Universität Rostock Traditio et Innovatio

Ensilage of Jack bean and cowpea grains sole or mixed with sorghum to improve their nutritional value as feedstuff for growing-finishing pigs

Dissertation

zur Erlangung des akademischen Grades

Doktor der Agrarwissenschaften (Dr. agr.)

an der Agrar- und Umweltwissenschaftlichen Fakultät
der Universität Rostock

Rostock, 2014

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urn:nbn:de:gbv:28-diss2015-0227-3

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Datum der Einreichung: 05. September 2014

Datum der Verteidigung: 12. Juni 2015

Contents

Abbreviations

AA	amino acid
AC	acetic acid
AD	apparent digestibility
ADF_{OM}	acid detergent fiber (exclusive residual ash)
ADL_{OM}	acid detergent lignin (exclusive residual ash)
ADT	additive
AEE	acid ether extract
ALA	alanine
ANF	anti-nutritional factors
ARG	arginine
ASP	asparagine
BA	butyric acid
BC	buffering capacity
BW	body weight
CG	cyanogenic glycosides
Con-A	concanavalin A
CF	crude fiber
cfu	colony forming units
CP	crude protein
CT	condensed tannins
CWP	cowpea
CYS	cystine
DM	dry matter
FM	fresh matter
GLU	glutamic acid
GLY	glycine
HIS	histidine
HU	hemagglutination unit
ILE	isoleucine
JBN	Jack bean
LA	lactic acid
LAB	lactic acid bacteria
LEG	legume grain (either Jack bean or cowpea)
LEU	leucine
LYS	lysine
m.a.s.l.	meters above sea level

MET	methionine
MOL	molasses
MOR	moisture re-constituted
ME	metabolizable energy
NA	not analyzed
ND	not detected
NDF_{OM}	neutral detergent fiber (exclusive residual ash)
n.s.	not significant
OM	organic matter
PA	propionic acid
PET	pre-ensiling treatment
PHE	phenylalanine
PRG	pre-germinated
PRO	proline
RFT	Rostock fermentation test
SER	serine
SID	standardized ileal digestibility
SOK	soaked
SOR	sorghum
STI	storage time
THR	threonine
TI	trypsin inhibitor
TIA	trypsin inhibitory activity
TIU	trypsin inhibitory unit
TTM	treatment
TYR	tyrosine
VAL	valine
VFA	volatile fatty acids
w/v	weight per volume
WSC	water soluble carbohydrates

1 Introduction

Due to the increasing world population, the resulting food demand is a challenge to be faced. In 2010 a total of 925 million people were estimated to be undernourished, whereas the developing countries account for 98 % (FAO, 2010b). In the tropical countries of the less-developed world, the majority of people depend directly or indirectly mainly on agriculture and livestock-based activities (Preston, 1995). Efforts have been made to alleviate poverty by supporting agriculture and animal production. Preston (1995) considers though, that the lack of understanding of the ecological, socio-economic and cultural limitations inherent in these countries constrains severely the application of conventional development models so that autochthonous models and technologies have to be focused stronger, considering as well the efficient use of the abundant biodiversity of the tropics.

In Cuba, to face the increasing demand for animal products, integrated sustainable farming systems appropriate for small producers have been implemented, achieving a high level of production and causing only marginal environmental problems. However, a serious problem when raising animals in the tropics is still an insufficient feedstuff production to supply livestock farming. In particular, there is a lack of high quality feedstuffs rich in protein, as most developing countries in the tropics cannot afford to import those cost-intensive feedstuffs, or they are simply not available. Thus, the use of local protein sources is a promising solution.

The most viable option appears to be the exploitation of neglected legumes, which abound in the tropics (Udedibie & Carlini, 1998b). Recently, the grains of under-utilized legumes like Jack bean (*Canavalia ensiformis*) and cowpea (*Vigna unguiculata*) receive attention, being more adapted to adverse environmental conditions, highly resistant to diseases and pests and of good nutritional quality (Siddhuraju, 1994). However, legumes show some drawbacks which hamper their use in animal nutrition, as they contain several anti-nutritional factors, which make them less palatable or affect the digestibility of nutrients. Furthermore, in tropical regions with increased temperatures and a high humidity, drying and storage of dried feedstuffs are particularly problematic. Silages of legume grains are seen as an option, although a high buffering capacity and low levels of water soluble carbohydrates indicate poor ensilability characteristics.

The present study shall contribute to broaden the knowledge about the suitability of Jack bean and cowpea grains as a possible feedstuff for pigs. Thereby, ensilage should be investigated as a method for conservation of those legume grains sole or mixed with sorghum, whereas the application of pre-ensiling treatments of the grains is studied with respect to an increase of ensilability, the reduction of anti-nutritional factors and the improvement of the nutritional value.

2 Literature review

2.1 Particularities of animal production in the tropics

The tropics frame the area between the Tropic of Cancer and Capricorn. They are commonly characterized by their predominant high temperatures throughout the year and seasons, determined by the precipitation regime. The tropics are known to shelter over half of the world's biodiversity (Green *et al.*, 1996). Furthermore, their sensitivity and effects on the global climate are well documented (Galvin & Jones, 2009). However, the tropical developing countries face a serious problem, namely the scarcity of food for the human population and feed for the dwindling livestock industry (Udedibie & Carlini, 1998b). Thereby, the claim to maintain the biodiversity of the tropics does not contradict with the establishment of new feed sources, as there is a wide range of nutrients in the tropical flora which is currently not optimally used.

There are inherent reasons for the poor productivity of the tropical agriculture compared to the temperate zones. Gallup & Sachs (2000) highlighted many disadvantages of the tropics, among others a low soil quality, low crop yields, an irregular rainfall regime, pests and diseases, difficulties in transferring technology generated mainly for highly developed countries as well as the fact that agricultural research is mainly focused on the temperate zones. Paterniani (1990) and Brewbaker *et al.* (1985) concluded the same when analyzing corn production. As a consequence, in developing tropical countries the food production does not increase as fast as the population grows.

Many technical and economical endeavors at national and international levels tried to increase animal production and animal productivity in the tropics, but results in general have been meager. The most repeated error is the attempt to import technologies that proved to work under temperate climes, but not for staple foods with low performance under tropical conditions. Protein sources like soybean and other oilseeds and legumes are most of the time imported, which represents a barrier against the sustainability of the animal production. Although those feedstuffs could be planted, the available infrastructure is insufficient for their optimal use (Figueroa, 2003).

Alternative feedstuffs are commonly used by pig smallholders in most of the developing countries in the tropics. Studies about new feeding strategies have been made not only in tropical but also in temperate countries with variable results (Ly, 1990) to identify new feed sources and to optimize their nutritive efficiency and

profitability. In order to substitute cereals as a source of energy in pig rations, roots and tubers are seen as a choice. Particularly the roots of cassava (Lindberg *et al.*, 2000b; Parra *et al.*, 2002; Vasupen *et al.*, 2008) and sweet potato (Manfredini *et al.*, 1993; Gonzalez *et al.*, 2003; Moron *et al.*, 2006; Pietrosemoli *et al.*, 2006; Gupta *et al.*, 2009) were studied, mainly due to their exceptional yield and contents of starch and water soluble carbohydrates. There are experiences in using taro (Buntha *et al.*, 2008) and yam, but their cultivation is relatively rare.

The use of fresh fruits or their industrial byproducts is also seen as an option, but they are feasible only when used in farms close to the plantations or their processing industry. Plantains and bananas for example, when fed ripe or properly processed (*e.g.* dried, cooked, ensiled), are an alternative source of starch and sucrose, but low in crude fiber and crude protein (Ly, 2004). Naturally grown palm tree fruits (*Roystonea regia*) and pig production are commonly combined in extensive raising systems in Cuba. The fresh ripe oily nuts contain about 25 % ether extract (rich in unsaturated fatty acids) and 26 % crude fiber and are suggested by Ly (2000) to be fed ground to improve the animal´s performance. Other species like the extracted or raw African oil palm (*Elaeis guineensis* [Jacq.]) has been tested with good results replacing cereals in rations for fattening pigs (Ocampo, 1994). Others, like *Jatropha curcas,* gained popularity due to the nutritive value of the detoxified kernel meal, which supplemented with additional lysine is comparable with soybean meal for feeding pigs (Wang *et al.*, 2011).

Citrus pulp, a byproduct of the citrus juice industry, with crude protein contents of 6 to 8 % of dry matter (DM) and crude fiber contents of 11 to 18 % of DM have been used in pig rations in proportions of 10 to 20 % of DM and up to 40 % of DM when it is ensiled (Dominguez, 1995; Sotto *et al.*, 2009). Even the inclusion of dried coffee pulp has been as well reported in pig feeding (Okai *et al.*, 1985).

Sugar cane and its byproducts are used as feedstuffs in those countries where the sugar cane industry prevails. The animal's performance can be improved when sugar cane juice or molasses is used instead of the freshly ground plant (Xande *et al.*, 2010). Due to the low N content of molasses, a good response in animals is obtained when it is combined with a protein source like soybean (Hidalgo *et al.*, 2006). The nutritional value of broiler offal (Lallo *et al.*, 1997), shrimp byproducts (Lindberg *et al.*, 2000a) and catfish (Nguyen *et al.*, 2010) can be improved by fermentation with molasses after inoculation with lactic acid bacteria. Growing "Torula" yeast (*Candida*

spp.) in molasses produces a biomass with 40 to 50 % crude protein in the DM when it is industrially produced, enhancing the animal´s performance (Diaz *et al.*, 1996; Savon *et al.*, 1999).

The microbial protein is an important source of essential amino acids, which makes distillery byproducts a valuable source of nutrients. Nevertheless, feedstock species, processing methods, the yeast used for fermentation and analytical methods (Liu, 2011) have influence on the final product. “Torula” yeast´s vinasse may supply up to 30 % of the crude protein in cereal based pig diets (Piloto *et al.*, 2009), a growth rate of 810 $g \cdot d^{-1}$ and a feed conversion rate of 3.6 was obtained when 20 % of fresh distillers solubles and soy bean were fed to pigs (Sarria & Preston, 1992). In contrast, condensed molasses residue (63 % DM) from the microbial production of citric acid can provide only a small percentage of the daily ration for pigs due to its low crude protein content and gross energy value (Weigand & Kirchgessner, 1980).

The omnivorous nature of pigs provides the opportunity to include roughages in their rations, where crop byproducts are produced in high amounts and with acceptable nutritional quality. Dried or ensiled leaves of cassava and sweet potato were identified to be a valuable source of protein (Lindberg *et al.*, 2000b; Ogle *et al.*, 2005; Ly *et al.*, 2010; 2011). The use of trees, shrub leaves (Ly *et al.*, 2001) and aquatic plants (Ly *et al.*, 2002) receives only little attention in the tropics, their tremendous yield and chemical composition could be of better use (Table 1).

Table 1: Chemical composition of the leaves of selected trees, shrubs and aquatic plants

		DM (%)	**OM**	**NDF** (% DM)	**N**
Multifunctional trees and shrubs	*Acacia auriculiformis*	40.8	93.4	68.0	2.73
	Flemingia macrophylla	41.8	94.1	73.0	3.19
	Gliricida sepium	27.1	93.0	59.1	3.27
	Leucaena leucocephala	43.5	93.1	66.0	3.09
	Morus alba	33.3	86.3	31.5	3.54
	Trichanthera gigantea	26.3	85.4	50.8	3.46
Aquatic plants	*Azolla pinnata*	6.8	82.9	42.8	4.50
	Pistia stratiotes	6.8	78.6	47.3	5.54
	Spirodella polyrhiza	4.7	90.5	58.2	4.57

adapted from Ly *et al.* (2001; 2002); DM, dry matter; OM, organic matter; N, nitrogen; NDF, neutral detergent fiber

Animal manures as feedstuffs are especially attractive, as feed costs are reduced and a partial solution for the manure management and the environmental pollution is provided. Cattle manure silages have been included in pig rations to cover part of the requirements in molasses and maize-based diets (Diaz & Elias, 1976; Diaz *et al.*, 1979). Cattle manure is rich in so called "growth promoting factors" including undigested food, vitamin B complex, essential amino acids and certain hormonal remains. However, its economic value as a feedstuff is much lower than its value as a fertilizer (Martin Jr *et al.*, 1983). The demand for biogas substrates reduces even more its chances to be used as animal feedstuff.

Alternative feedstuffs in general have lower nutrient concentrations than conventional ones. The protein is of low biological quality, which requires supplementation of high concentrated N sources and essential amino acids, which are cost-intensive. Furthermore, alternative feedstuffs often have a high moisture content, which makes them difficult to store for long periods, as drying causes extra costs. Grains of underutilized tropical legumes overcome those drawbacks. They have an acceptable nutritional composition and their dry matter content offers the possibility to conserve them for longer periods of time.

2.2 The production of Jack bean, cowpea and sorghum grains in the tropics

The import of feed ingredients for livestock production is not beneficial, as most of the developing countries in the tropics cannot afford the requisite foreign exchange. The tendency of the increasing food commodity prices (Figure 1) in the last 20 years is reported by FAO (2011).

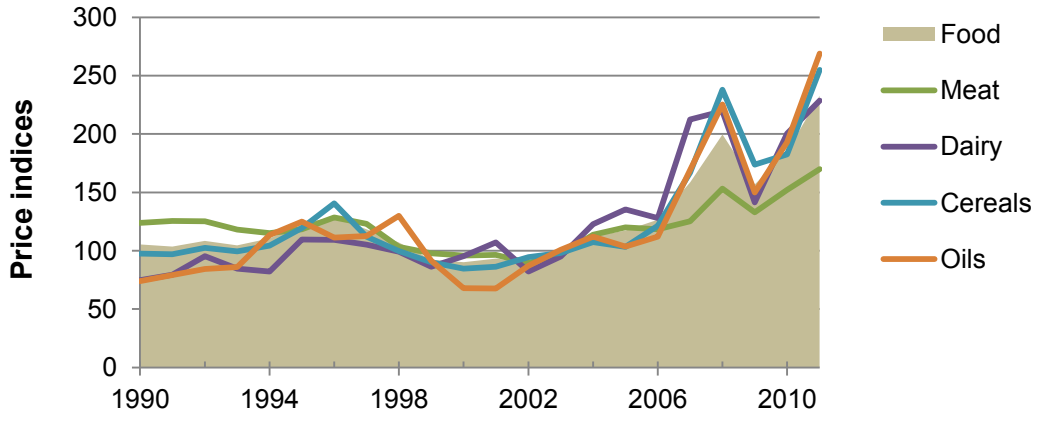


Figure 1: The food commodity price indices in the monthly international prices of major food commodities from January 1990 to April 2011 (FAO, 2011)

Mainly in developing countries, there is an interest in new food sources, especially those ones rich in protein. The prevailing attitudes to the environment´s protection and to a sustainable agriculture also favor the production of legumes (Deshpande & Damodaran, 1990; Lambert & Fenwick, 1991). Moreover, due to symbiotic abilities they play an important role in colonizing disturbed ecosystems (Arianoutsou & Thanos, 1996; Smil, 1999).

Legumes have been included in the diets of animals and humans since their cultivation (Graham & Vance, 2003). There are evidences that bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) were each domesticated more than 3000 years ago in America and Asia, respectively (Hymowitz & Singh, 1987; Kaplan & Lynch, 1999). Unfortunately, the improvement of legume crop yields has not kept pace with that of cereals. FAO (2010c) noted, that the world yield of cereals increased by 150 $kg \cdot ha^{-1}$ between 1999 and 2008 with the exception of the last recorded period (2008-2009), where no increase was observed. The world legume yield per hectare and year did not increase between 1999 and 2007 and only by 30 $kg \cdot ha^{-1}$ in the period from 2007 to 2009. Partially, this difference is due to the unfavorable environmental conditions under which many legume species are grown, as they are often cropped after corn or rice at the end of the growing season (Graham & Vance, 2003) without an adequate agro-technical support (fertilizers, irrigation, pesticides, herbicides, fuel or machinery). As a consequence, grains and their byproducts are limited and expensive as they are the main source of nitrogen in the diet of animals and humans.

The exploitation of neglected novel legumes abounding in the tropical region seems a viable option to ease the current pressure on the conventional legumes (Cáceres *et al.*, 1995; Udedibie & Carlini, 1998b). Unfortunately, they don´t receive the same attention by science and are not included in the pig feeding systems like the conventional ones (Figueroa, 2003).

Originated in the New World (Sauer, 1964), **Jack bean** (*Canavalia ensiformis* [L.] DC), as well known as Riesenbohne, Haba de Caballo or Pois sabre (Legel, 1984), has been identified as a legume adaptable to a wide range of agronomic and climatic conditions with a high potential for exploitation as a source of protein and energy for livestock (Arora, 1995; Cáceres *et al.*, 1995; Akpapunam & SefaDedeh, 1997a; Sridhar & Seena, 2006).

The plant is characterized by a vegetative cycle of 170 - 240 days, a fast germination (2 - 3 days), 60 - 100 cm height and taproots. It is able to grow up to 900 m.a.s.l. and it tolerates drought, shadow and moderate floods (Morris, 2007). Poor soil conditions (low phosphorus contents and a pH between 4.3 and 8.0) are accepted due to the colonization of mycorrhiza and rhizobia (Udedibie & Carlini, 1998b). Yields have been reported between 0.5 and 6.0 $t \cdot ha^{-1}$ (Bressani *et al.*, 1987; Kessler, 1990; PROSEA, 1992; Carlini & Udedibie, 1997) in close relation with the region and the experimental conditions. Sridhar & Seena (2006) compiled from data of several authors an overview of the chemical composition of Jack bean raw flour (Table 2).

Table 2: Chemical composition of raw Jack bean seeds (Canavalia ensiformis [L.] DC)

Component		Content
Dry matter	(%)	86.5 - 96.2
Crude protein		22.8 - 35.3
True protein		24.2 - 28.2
Albumins		7.8 - 8.6
Globulins		13.0 - 14.6
Prolamins		0.6 - 0.9
Glutelins		1.8 - 2.0
Ether extract	(% DM)	1.6 - 12.1
Crude fiber		4.7 - 11.4
Ash		2.3 - 5.8
Total starch		24.7 - 36.9
Digestible starch		26.1
Resistant starch[1]		10.8
Nitrogen free extract		45.8 - 65.4
Gross energy	(MJ $kg \cdot DM^{-1}$)	14.7 - 19.1

according to Sridhar & Seena (2006)
[1]Resistant starch content is calculated as total starch minus digestible starch.

Cowpea (*Vigna unguiculata* [L.] Walp), also known as blackeye bean and southern pea (Uzogara & Ofuya, 1992); caupí, caritas, fríjol in Venezuela; Augenbohne or Kuhbohne in Germany (Cook *et al.*, 2005), is one of the most important food legumes in the tropic and sub-tropic regions, where drought is a major production constraint due to low and erratic rainfall (Singh *et al.*, 1997). As much as 1 $t \cdot ha^{-1}$ of dry grain was produced in a Sahelian environment with only 181 mm of rainfall and a high evaporative demand (Ehlers & Hall, 1997). Cowpea is a warm-season crop well adapted to many areas of the humid tropics and temperate zones. It is tolerant to

shadow, heat and drought, but intolerant to frost (Ehlers & Hall, 1997; Singh *et al.*, 1997). Germination is rapid at temperatures above 18 °C and growing ranges between 0 and 1500 m.a.s.l.. Cowpea has the capacity to fix nitrogen in soils with adverse conditions like low dry matter contents, a pH range of 4.5 to 9.0, low contents of organic matter (< 0.2 %) and sand contents over 85 % (Uzogara & Ofuya, 1992; Singh *et al.*, 1997; Sprent *et al.*, 2010). Yields are reported between 0.3 and 4.0 $t \cdot ha^{-1}$ (Cook *et al.*, 2005). Sarmiento *et al*. (2011) refer to a range between 1.5 and 2.5 $t \cdot ha^{-1}$ and for the variety INIFAT 93 a yield of 1.05 to 1.25 $t \cdot ha^{-1}$ was reported in Cuba (Diaz *et al.*, 2000). Not only its resistance to extreme environments, but as well its nutrient composition makes cowpea a potential source for animal feeding (Makinde *et al.*, 1996a; Makinde *et al.*, 1996b; Erlwanger *et al.*, 1999; Castro *et al.*, 2002b; Ibrahim *et al.*, 2002; Diaz *et al.*, 2003; Sarmiento *et al.*, 2011). Selected parameters of the nutritional value of cowpea are given in Table 3.

Table 3: Chemical composition of raw cowpea seeds (Vigna unguiculata [L.] Walp)

Component		Content
Dry matter	(%)	87.7 - 94.3
Crude protein	(% DM)	22.0 - 26.7
True protein*		23.4 - 24.0
Ether extract		1.0 - 2.5
Crude fiber		5.6 - 7.8
Ash		2.6 - 4.3
Nitrogen free extract		33.1 - 60.0
Gross energy	(MJ $kg \cdot DM^{-1}$)	13.5 - 14.1

Source: Bressani (1985); PROSEA (1992); Uzogara & Ofuya (1992); León *et al.* (1993); Souci *et al.* (2000); Castro *et al.* (2002b); Diaz *et al.* (2003); Frota *et al.* (2008); USDA (2010)
*Values of true protein not necessarily correspond to the range of crude protein reported.

Despite its potential, the world production of cowpea is meager. In 2009, 5.7 million t were produced, which is considerably low compared to soybeans with 223.2 million t (FAO, 2010a).

Nowadays, high prices are not only associated to legumes but as well to cereals (see Figure 1). Substituting conventional cereals for native alternatives in rations for pigs is a topic being considered by sustainable feeding systems in developing countries.

Sorghum (*Sorghum bicolor* [L.] Moench), a major cereal in the world behind rice, wheat, corn and barley, is a staple food for humans in the semi-arid tropics of Africa, Asia and Latin America (ICRISAT, 2004). It has the ability to cope with many types of stresses, including heat, salinity and water logging (Ejeta & Knoll, 2007). Furthermore, it is considered as a model species for drought tolerance due to its inherent drought tolerant characteristics (Ali *et al.*, 2009; Pérez *et al.*, 2010; Rajarajan & Ganesamurthy, 2011). Primarily it is a crop of hot, semi-arid tropical environments with only 400 - 600 mm rainfall, which are too dry for corn.

Due to its favorable chemical composition (Table 4), sorghum grains have been evaluated with good results as a substitute for conventional cereals in diets for pigs (Myer *et al.*, 1985; Myer & Gorbet, 1994; Myer & Gorbet, 2004; Nyannor *et al.*, 2007; Benz *et al.*, 2011), broilers and laying hens (Jacob *et al.*, 1996; Castro, 1999; Ambula *et al.*, 2003; Barcellos *et al.*, 2006; Dominguez *et al.*, 2009), dairy and beef cattle (Mitzner *et al.*, 1994; Santos *et al.*, 1997; Chiou *et al.*, 1999; Theurer *et al.*, 1999; Menezes *et al.*, 2009) and horses (Al Jassim, 2006; Gobesso *et al.*, 2008; Gollcher *et al.*, 2010).

Table 4: Chemical composition of sorghum seeds (Sorghum bicolor [L.] Moench)

Component		Content
Dry matter	(%)	89.4
Crude protein	(% DM)	9.4
Ether extract		3.4
Crude fiber		2.1
Ash		1.6
NDF_{OM}		10.6
ADF_{OM}		4.9
ADL_{OM}		0.4
Starch		70.1
Gross energy	(MJ kg·DM^{-1})	16.2

Source: NRC (2012)

According to FAOSTAT (2010a), sorghum reached a world production quantity of 62.1 million t with an average yield of 1.4 t·ha^{-1} in 2009. Africa and America are the first world producer regions (Figure 2), whereas the United States is the first world producer country. Currently, the world production of sorghum is irrelevant compared to corn and wheat, with 682 and 817 million t in 2009, respectively.

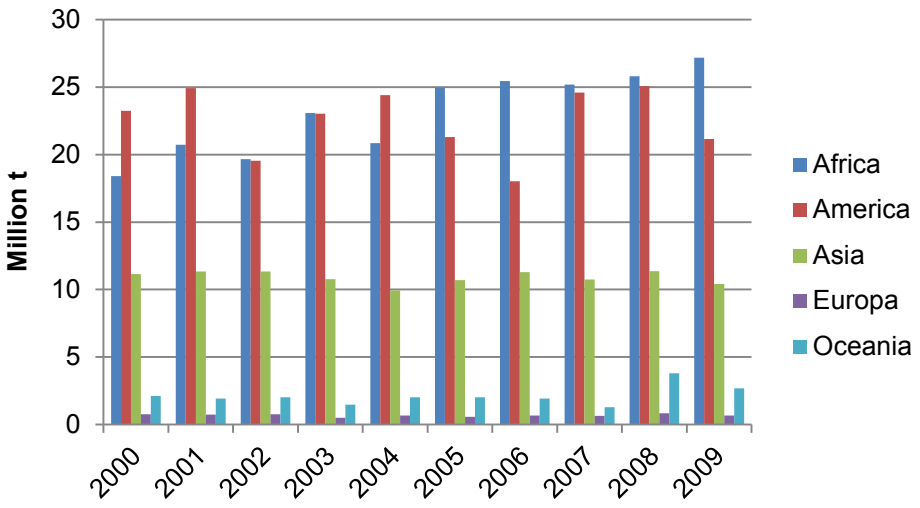


Figure 2: World production of sorghum grains between 2000 and 2009 (FAOSTAT, 2010a)

Despite their nutritional value and adaptability potential, Jack bean, cowpea and sorghum are not commonly used as a feed or cultivated in the same proportion than other seeds. Among various reasons, those grains are well known to contain anti-nutritional factors (ANF) and toxic compounds able to induce adverse effects in farm animals. Hence, their use in diets is limited, mainly for non-ruminants.

2.3 Anti-nutritional factors in Jack bean, cowpea and sorghum grains

Plants protect themselves against pests and predators, which is associated with the action of certain substances that have a proved harmful effect on animals and humans. These compounds are able to produce a violent and immediate reaction or, more commonly, a subtle effect is manifested after a persistent ingestion. The resulting low palatability of the plants and the reduction of the digestive efficiency have a negative impact on the animal´s performance, leading to death in the worst case. Since their discovery, these defensive mechanisms are known as anti-nutritional factors (ANF). However, as recently the effects of ANF have been described not only as negative, but as well positive or both (Champ, 2002; Jamroz & Kubizna, 2008), the terms ‘secondary plant metabolites’ or ‘bioactive substances’ considering their impact on health, are preferred (Champ, 2002; Makkar *et al.*, 2007; Jezierny *et al.*, 2010). Nevertheless, the term ‘ANF’ will be used in the present work.

2.3.1 Main anti-nutritional factors in Jack bean, cowpea and sorghum grains and their impact on animal physiology

The incorporation of alternative feedstuff sources in animal nutrition mandatory involves an increased occurrence of ANF, as these substances are found in a wide range of tropical plants. The main ANF with a proved negative effect on animal performance can be divided in the groups of protease inhibitors, lectins or phytohaemagglutinins, amylase inhibitors, non-protein amino acids, phenolic compounds, glycosides, alkaloids, non-starch polysaccharides and others like *e.g.* phytic acid or gossypol (Champ, 2002; Jezierny *et al.*, 2010). With an improvement of analytical methods it might be possible that more substances will be included.

Jack bean stands out by its content of mainly concanavalin A, canavanine, trypsin inhibitors, cyanogenic glycosides, polyphenols and saponins (Akpapunam & SefaDedeh, 1997a; b; Sridhar & Seena, 2006). In cowpea, the most prominent ANF are lectins, trypsin inhibitors, cyanogenic glycosides and tannins, although only moderate levels of these substances are present (Prinyawiwatkul *et al.*, 1996b; Olivera-Castillo *et al.*, 2007). Sorghum grains are well known to contain variable quantities of tannins (Brandon *et al.*, 1982; Saura-Calixto *et al.*, 2009; Ojeda *et al.*, 2010) as well as phytic acid (Ojeda *et al.*, 2010) and non-starch polysaccharides (Verbruggen *et al.*, 1993; Castro *et al.*, 2002a; Muralikrishna & Rao, 2007) depending on the cultivar. In the following, a description of the most common ANF in the evaluated grains will be given.

CONCANAVALIN-A (CON-A)

Concanavalin-A (Con-A) is the plant lectin most studied and was first isolated and crystallized by Summer & Howell (1936). Although lectins can be found in animals like jellyfish, *Nemopilema nomurai* (Imamichi & Yokoyama, 2010), in the coelomic fluid of sea cucumber, *Holothuria scabra* (Gowda *et al.*, 2008) and in the heart tissue of buffalo, *Bubalus bubalis* (Ashraf *et al.*, 2010), they occur in abundance mainly in the seeds of legumes, whereas Jack bean is the natural source of Con-A (Carlini *et al.*, 1988; Udedibie & Carlini, 1998b). Lectins are proteins respectively glycoproteins, which have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharides or oligosaccharides (Sze & Tzi, 2011). They can interact as well with proteins, membranes and other molecules to influence plant cell morphogenesis and nodule development (Brewin & Kardailsky, 1997) and play an

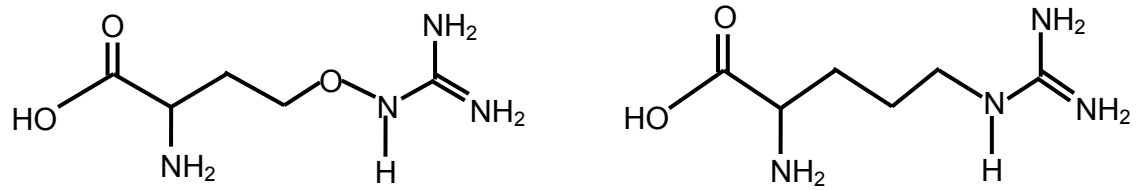
important role in the nitrogen fixation. Bezrukova *et al.* (2011) indicated a close relationship between lectins and the hormonal systems controlling cell division.

Like most of the ANF, lectins form a defense barrier against plagues and diseases. Con-A has been reported to increase mortality in insects like *e.g. Acyrthosiphon pisum* (Fitches *et al.*, 2008), *Aedes aegypti* (Sa *et al.*, 2009) and *Myzus persicae* (Jaber *et al.*, 2008). Its antifungal activity has been proved against *Aspergillus flavus* (Baker *et al.*, 2009), *Microsporum canis* (Pinheiro *et al.*, 2009), *Saccharomyces cerevisiae, Colletotrichum musae* and *Fusarium oxysporum* (Boleti *et al.*, 2007). Moreover, studies demonstrated the *in vivo* anti-tumor (Li *et al.*, 2008; Zhang *et al.*, 2010) and antiviral (Keyaerts *et al.*, 2007; Swanson *et al.*, 2010) effects of lectins. Due to their affinity to glycoproteins and carbohydrates they have been used to isolate glycoproteins (Alvarez-Manilla *et al.*, 2010), to obtain information about the carbohydrate composition of samples (Pilobello & Mahal, 2007) and in the diagnosis of parasite diseases (Yang *et al.*, 2010).

Con-A accounts for 15 to 35 $g \cdot kg^{-1}$ DM of the Jack bean seed (Sharon & Lis, 1972; Hague, 1975), representing about 20 % of the total protein in the grain (Dalkin & Bowles, 1983). However, lectins have undesirable effects on farm animals, mainly monogastrics, as they are mainly resistant to digestion (Pusztai *et al.*, 1990; Udedibie & Carlini, 1998b; Kelsall *et al.*, 2002). Con-A is able to agglutinate the red blood cells of chicken, guinea pigs, rabbits, sheep, rats and most of the human blood types (Liener, 1974a; Carlini *et al.*, 1988). It has also been reported to bind rat and human nerve cells (Gulati *et al.*, 1986). Presumably, the harmful effect is caused by the ability to bind the membrane receptors of the epithelial cells of the small intestine. As a result, changes may be induced in the structure of the absorptive epithelium (microvilli lining), changing the surface of the small intestine, leading to an interference with the absorption of nutrients (Liener, 1974b; 1997; Kunzelmann *et al.*, 2004). As well, Con-A is known to inhibit the activity of enzymes of the brush border of the enterocytes and to interfere with the adherence of enterobacteria and the intestinal wall (Carlini & Udedibie, 1997; Naughton *et al.*, 2000). Therefore, it is assumed that side effects on immune functions, the protein metabolism, enzyme activities and hormonal regulations can occur (Herzig *et al.*, 1997; Ovelgonne *et al.*, 2000; Kordas *et al.*, 2001).

CANAVANINE

The alkaline and toxic amino acid canavanine (2-amino-4-[guanidinooxid]-butyric acid), a naturally occurring structural analogue of L-arginine (Figure 3), was originally isolated from Jack bean (Kitagawa & Tomiyama, 1929), but has also been reported to be present in over 500 species of the *Leguminosae* (Turner & Harborne, 1967; Bell *et al.*, 1978; Lavin, 1986).



canavanine *arginine*

Figure 3: Chemical structure of canavanine and arginine

Canavanine is the principal non-protein amino acid present in Jack bean grains and one of the major nitrogen storage compounds, accounting for more than 95 % of the free amino nitrogen (Bell, 1958; Rosenthal, 1977a; b; Rosenthal & Rhodes, 1984; Rosenthal *et al.*, 1988). The canavanine content of *Canavalia* spp. (*Canavalia ensiformis*) ranges from 25 - 51 $g \cdot kg^{-1}$ DM (Rosenthal, 1972; Natelson, 1985; Sridhar & Seena, 2006). It is able to manifest antiviral effects (Bell, 1974) and it is thought to promote symbiotic rhizobium-legume interactions by inhibiting other competing bacteria (Cai *et al.*, 2009). By being incorporated in the cell nucleus and other proteins interfering with DNA and RNA synthesis of plants and animals, canavanine influences most the regulatory and catalytic reactions of the arginine metabolism, mimicking arginine uptake, formation of structural components and other cellular processes (Rosenthal, 2001). As canavanine-containing proteins are unable to form crucial ionic interactions, this results in an altered protein structure and a loss of functions, which can lead to cell death (Bence & Crooks, 2003). In consequence, canavanine is a highly effective protective allelochemical, providing a significant chemical barrier to predation and diseases (Rosenthal, 2001). Due to this mechanism, canavanine is used for anti-cancer treatment schemes based on an artificially created arginine starvation (Vynnytska *et al.*, 2011).

The first reaction to canavanine-containing diets when fed to pigs is an intake reduction (Enneking *et al.*, 1993; Belmar & Morris, 1994b). Animal feeding trials showed a number of deleterious effects following the consumption of legumes containing canavanine. In chicken, decreased feed intake and growth and a significant reduction in plasma concentration of basic amino acids was determined. The L-canavanine as well reduced the intestinal absorption of amino acids sharing similar transport systems throughout the brush border membrane vesicles (Herzberg *et al.*, 1971; Rueda *et al.*, 2003). Although evidence of its toxicity to mammals *in vivo* is limited, some canavanine fed animals showed abnormalities similar to those seen in the human systemic *lupus erythematosus*, an autoimmune disease that adversely affects the kidneys and the skin (Bell, 2003). This syndrome was provoked as well in monkeys fed alfalfa sprouts (Bardana *et al.*, 1982; Malinow *et al.*, 1982) and in humans after long ingestions of alfalfa tablets (Roberts & Hayashi, 1983).

TRYPSIN INHIBITORS

The existence of protease inhibitors in plants is known since 1938. They are widely spread throughout the plant kingdom, but especially occur in the *Leguminosae*, *Gramineae* and *Solanaceae* (Richardson, 1977). However, as protease inhibitors are abundant in legumes, they are the most studied species (Udedibie & Carlini, 1998b).

The trypsin inhibitor was first described in Jack bean by Orru & Demel (1941) and later confirmed by Borchers & Ackerson (1950), whereas in cowpea it was first reported by Borchers & Ackerson (1947) and later by Jaffe (1950). Sorghum was as well thought to contain trypsin inhibitors (Filho, 1974; Mulimani & Vadiraj, 1991). However, the influence on the protein digestion is supposed to be caused rather by the action of tannins (Loomis & Battaile, 1966; Anderson, 1968; Los & Podsédek, 2004). Studies verified the presence of two kinds of cowpea protease inhibitors, namely trypsin inhibitors and trypsin-chymotrypsin inhibitors (Gennis & Cantor, 1976; Gatehouse *et al.*, 1980), which later were renamed Kunitz and Bowman-Birk family, respectively.

In addition to their role of regulating proteolytic activities, protease inhibitors protect fluids or tissues from degradation by unwanted or foreign proteolytic activities (Neurath, 1984). In plants, they occur particularly in storage tissues such as seeds or tubers. Those parts might act as depot or safe storage forms of protein, which are immune to digestion until required during germination or sprouting (Richardson,

1981). However, the increase in endopeptidase in the storage tissues during seed germination cannot be accounted for by the inactivation of an "equivalent amount" of inhibitors (Chrispeels & Baumgartner, 1978).

The trypsin inhibitors themselves contribute to the exceptional nutritional value of legumes. In the total protein of Lima and Navy bean for example, it represents approximately 32 and 40 % of the total cystine, respectively (Kakade *et al.*, 1969), although the assumption that they are a source of protein used during germination has to be questioned. For instance, while the content of trypsin inhibitors decreased during germination of Jack bean (Akpapunam & SefaDedeh, 1997b), no decline was determined for 10 days of germination in *Phaseolus vulgaris* (Nielsen & Liener, 1988), or even trypsin inhibitors were found to increase (Richardson, 1981; Savelkoul *et al.*, 1992). Besides, there is consensus about their capacity to target multiple digestive enzymes of predators and pathogens (Richardson, 1981; Ryan, 1990).

The inhibitors of the serine proteases (trypsin and chymotrypsin) found in plant feedstuffs are the most studied (Belitz & Weder, 1990). They are polypeptides which form well-characterized stable complexes with trypsin and chymotrypsin on a one-to-one molar ratio, obstructing their binding sites and disrupting their enzymatic action (Udedibie & Carlini, 1998b). Inactivation of trypsin in the gut by trypsin inhibitors from soybeans induced the intestinal mucosa releasing cholecystokinin (CCK). This hormone stimulates the pancreatic acinar cells to produce more trypsin, chymotrypsin, elastase and amylase. When this negative feedback continues, an important loss of S-containing amino acids is created, leading to growth reduction, pancreatic hypertrophy/hyperplasia and carcinogenic effects (Liener, 1976; Savelkoul *et al.*, 1992). Grant *et al.* (1995) found that rats fed a soybean or cowpea based diet, showed an extensive increase in the relative and absolute weights of the pancreas and an increase in the incidence of macroscopic pancreatic nodules and possible pancreatic neoplasia. However, the negative feedback through CCK does not seem to be the only mechanism by which pancreatic proteases secretion is controlled. Pusztai *et al.* (1997) proved that soybean inhibitors remained effective in stimulating pancreatic secretion after elimination of their inhibitory activity by complex formation.

HYDROGEN CYANIDE

The cyanogenic glycosides (CG) are glycosides of α-hydroxy nitriles. All known compounds are β-linked, mostly with D-glucose (Poulton, 1990; Vetter, 2000). Most

CG derive from the five hydrophobic protein amino acids tyrosine, phenylalanine, valine, leucine and isoleucine (Poulton, 1990). The CG are not toxic themselves, but due to an enzymatic or acid hydrolysis hydrogen cyanide (HCN) is liberated (Makkar *et al.*, 2007), so that free HCN is not found in intact plant cells. The enzymatic reaction starts by splitting off the carbohydrate part by one or more β-glycosidases (pH 4.0 - 6.2), after which the corresponding cyanohydrins are formed. This intermediate may decompose either spontaneously or enzymatically in the presence of an α-hydroxy nitrile lyase (pH 5.0 - 6.5) to release HCN and an aldehyde or ketone (Figure 4). The non-enzymatical decomposition proceeds rapidly at alkaline pH, but is negligible below pH 5.5 (Poulton, 1990). Vetter (2000) found this to be possible in a wider range of pH (> 4) and temperatures (> 35 ºC).

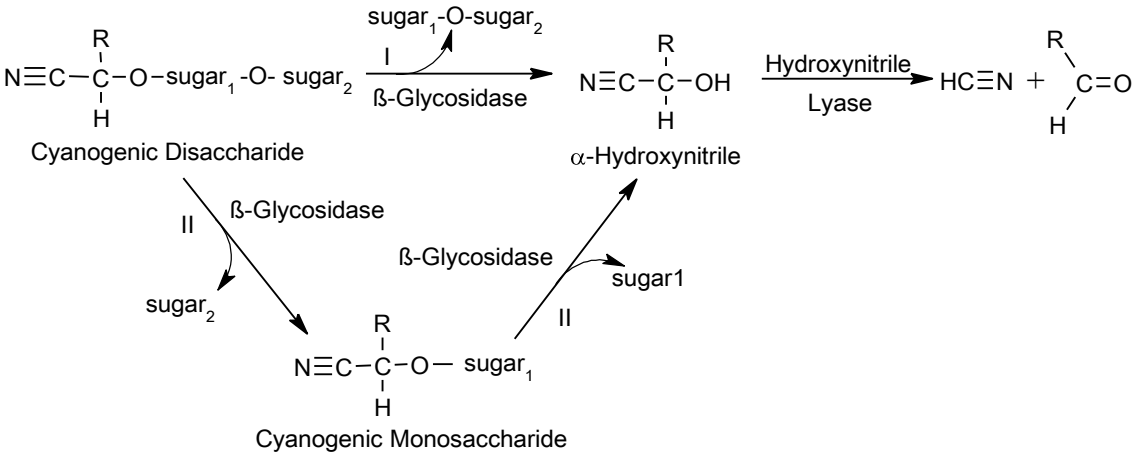


Figure 4: Simultaneous (I) and sequential (II) mechanisms for the catabolism of cyanogenic disaccharides (Poulton, 1990)

The hydrolysis of CG occurs at a significant rate only after tissues have been disrupted by animal mastication, fungal attack or mechanic destruction. It is postulated that CG represent a defense barrier against predators (Poulton, 1990; Vetter, 2000) or might serve as nitrogen storage compounds (Selmar *et al.*, 1988; Selmar *et al.*, 1990). As well, their role as a promoter for pre-germination has been confirmed by Maruyama *et al.* (1996).

Hydrogen cyanide primarily involves the inhibition of cytochrome-c-oxidase (Panda & Robinson, 1995), a vital enzyme in the tricarboxylic acid cycle, blockading the aerobic synthesis of ATP (Egekeze & Oehme, 1979; Younes & Strubelt, 1988; Laurena *et al.*, 1994; Makkar *et al.*, 2007). The toxicity symptoms of ingested HCN are peripheral

numbness and light-headedness followed by mental confusion and stupor, cyanosis, twitching and convulsion with terminal coma. The HCN can be converted into thiocyanate (Sorbo, 1953), which induces the formation of nitrosamine, a proven carcinogen (Makkar *et al.*, 2007).

Monogastric animals are less susceptible to HCN intoxication than ruminants, as a high rumen pH and microorganisms accelerate cyanogenesis. Because of a bitter taste, rejection by animals is the first reaction when fed legume grains rich in CG (Makkar *et al.*, 2007). Betancur-Ancona *et al.* (2008) evaluated HCN in seeds of *Canavalia ensiformis* and determined contents of 159 $mg \cdot kg^{-1}$ DM. They assumed that the different contents found by Bernal & Jiménez (1990) and Linder (1995) with 110 $mg \cdot kg^{-1}$ and 20 $g \cdot kg^{-1}$ DM, respectively, could be attributed to the cultivar. Sridhar & Seena (2006) stated a range of HCN between 0 and 112 $mg \cdot kg^{-1}$ DM. Cowpea seeds contain HCN from 4.8 to 6.0 $g \cdot kg^{-1}$ DM, reported by Umoren *et al.* (1997) after evaluation of raw grains of four varieties. Onwuka (2006) found lower contents (83.8 $mg \cdot kg^{-1}$) and Olivera-Castillo *et al.* (2007) did not detect any amount of HCN. Like in the case of Jack bean, this could be related to the cultivar, the crop's location or the environmental conditions. In dry sorghum grains undetectable or low amounts (1 to 29 $mg \cdot kg^{-1}$) of dhurrin were found, a cyonagenic glycoside which results in HCN. However, dhurrin can be drastically increased during sprouting (Panasiuk & Bills, 1984; Ahmed *et al.*, 1996).

TANNINS

Tannins are polyphenolic water soluble compounds with molecular weights between 500 and 3000 Da (Perezmaldonado *et al.*, 1995; Santos-Buelga & Scalbert, 2000; Li *et al.*, 2006; Saura-Calixto *et al.*, 2009). They can be divided into two major groups: hydrolysable and condensed tannins (Makkar *et al.*, 2007; Saura-Calixto *et al.*, 2009).

Hydrolysable tannins are esters of phenolic acids, either gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins), and a polyol, which is usually β-D-glucose or quinic acid as presented in Figure 5 (Salminen *et al.*, 1999; Clifford & Scalbert, 2000; Santos-Buelga & Scalbert, 2000). Gallotannins are easily degraded by bacteria, fungi and yeasts, while ellagitannins are more difficult to be degraded by microorganisms, due to their complex structures with additional C–C bonding (Li *et al.*, 2006).

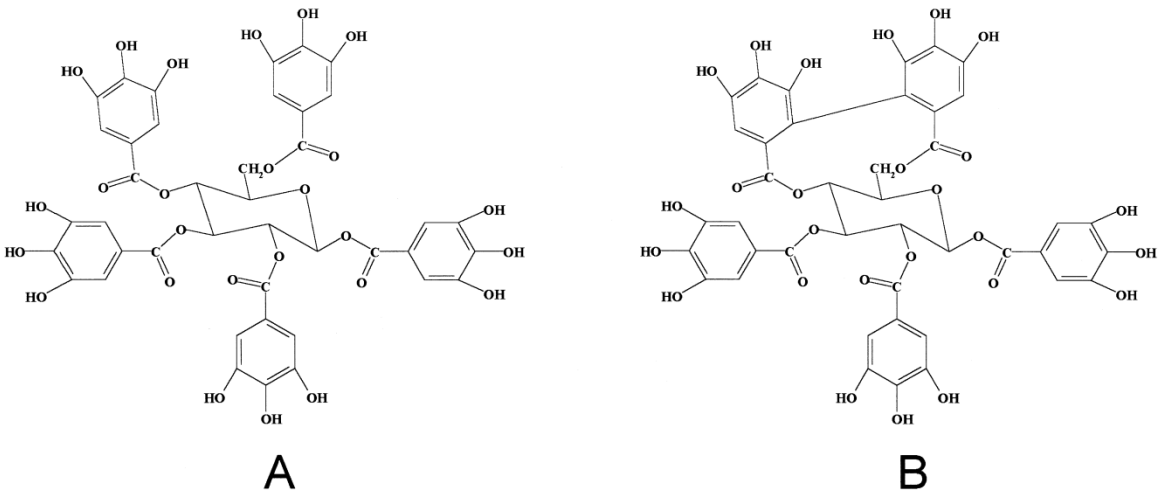


Figure 5: Structures of pentagalloylglucopyranose (A, gallotannin) and trigalloyl-HHDP-glucopyranose (B, ellagitannin) (Salminen *et al.*, 1999)

Condensed tannins (CT, Proanthocyanidins) are polymers made of elementary flavan-3-ol units that are linked by C-C and occasionally C-O-C bondings (Santos-Buelga & Scalbert, 2000). The flavan-3-ols are dominated mainly by the catechins and gallocatechins, which may also exist as gallate esters of the C-3-hydroxy group (Bors *et al.*, 2001). In Figure 6 the basic form of CT is presented. A key feature of proanthocyanidins is that they are transferred to anthocyanidins upon heating in acidic media (Santos-Buelga & Scalbert, 2000).

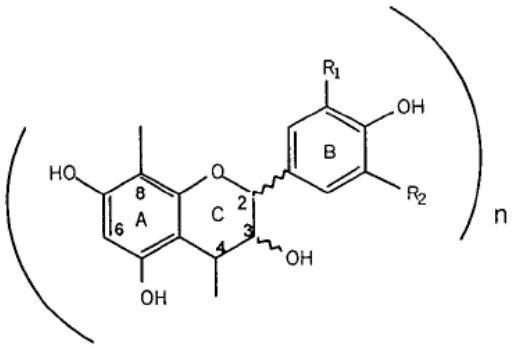


Figure 6: Basic structure of proanthocyanidins. R_1=H, R_2=H: propelargonidins; R_1=H, R_2=OH: procyanidins; R_1=OH, R_2=OH: prodelphinidins (Santos-Buelga & Scalbert (2000))

Tannins form complexes with proteins (*e.g.* enzymes, hormones, toxins) by covalent, hydrogen and ionic bonding (Mitaru *et al.*, 1984). This is an essentially dynamic surface phenomenon and generally reversible, which basically involves proteins that possess an open, random coil type conformation and whose principal forces are

hydrophobic effects (Santos-Buelga & Scalbert, 2000). These interactions are similar to antigen-antibody interactions in that a binding agent and ligand of comparable sizes associate multivalently to form soluble and insoluble complexes (Hagerman & Butler, 1981).

The affinity of proline-rich proteins (PRP) and histatins with tannins is considered as the first defensive animal reaction to minimize the adverse effects of tannins after ingestion. Furthermore, the tannin-PRP complex is assumed to resist endogenous or microbial enzyme attack in the digestive tract (Robbins *et al.*, 1987). Kamphues *et al.* (2010) described an adaptation of pigs to counteract the effects of tannins by increasing size, mass and proline content of parotid glands after 7 days of consuming diets with high tannin content. However, when the capacity of the defensive barrier is exceeded, digestibility of amino acids can be severely affected and the endogenous protein losses increase (Jansman, 1993; Steendam *et al.*, 2004; Myrie *et al.*, 2008). The intensity of interaction between tannins and proteins is determined by the nature of both, being the relative ratio of tannins and protein in solution and physical and chemical conditions such as type of medium, temperature, pH value, ionic strength and incubation time (Hagerman & Butler, 1989). As well, the higher the polymerization of tannins, the lower the affinity with proteins (Santos-Buelga & Scalbert, 2000).

Tannins can form complexes as well with starch, inhibiting α-amylase attack (Deshpande & Salunkhe, 1982), although they are known to bind directly α-amylase, too, reducing its activity (Yan & Bennick, 1995; Santos-Buelga & Scalbert, 2000). Having o-dihydroxyphenyl groups, CT are excellent chelators of Fe (III) (Santos-Buelga & Scalbert, 2000) and they also form complexes with Al (III) and Cu (II) (Kennedy & Powell, 1985; Kipton *et al.*, 1987).

According to Jansman (1993), the bitter or astringent taste produced by tannins is associated with the precipitation of mucoproteins or a direct binding of taste receptors, which reduces palatability and hence negatively affects the voluntary feed intake. Moreover, tannins provoke necrotic effects on the gastric mucosa and glandular atrophy. When absorbed, tannins cause kidney and liver failure, leading to the death of the animal (Jansman, 1993; Makkar *et al.*, 2007).

Most of the tannins are found in the seed's coat (Reddy *et al.*, 1985; Adebooye & Singh, 2007; Han *et al.*, 2009), which underlines their role as a first defense barrier against predators and microorganisms. In the grains of Jack bean, cowpea and

sorghum they are present in different amounts. Sridhar & Seena (2006) stated in Jack bean seeds contents of tannins from 0 to 900 mg·100 g^{-1}·DM and of total phenols from 730 to 1818 mg·100 g^{-1} DM, whereas condensed tannins were not found. Ibrahim *et al.* (2002) evaluated the effect of fermentation, soaking and pre-germination on cowpea and determined 210 mg·100 g^{-1} DM tannins in the raw grains using the Folin-Denis reagent method. Onwuka (2006) on the contrary reported higher quantities (3.42 g·100 g^{-1} DM) in cowpea using the same method.
Like in Jack bean and cowpea, the tannin content in sorghum grains depends on the cultivar (Reddy & Pierson, 1994). Elmaki *et al.* (1999) classified them in low (0.32 % of DM) and high (1.44 % of DM) tannin containing cultivars in order to test different methods for deactivation. Ahmed *et al.* (1996) showed a variation of 220 to 410 mg·100 g^{-1} DM in three cultivars. Castro *et al.* (2002a) found a similar variability, when determining tannins with a range of 0.04 to 1.99 % of DM in four Cuban sorghum varieties.

2.3.2 Deactivation of anti-nutritional factors

Numerous attempts have been made to decrease the negative effects of ANF in feedstuffs. Among them, soaking, peeling, boiling, germination, fermentation, expansion and toasting are the most recurrent methods described in literature. None of these techniques is new and some are used already for thousands of years. However, nowadays the knowledge is provided to understand how those methods work and how they can be optimized (Hill, 1998). Even when highly developed methods for the deactivation of ANF (*e.g.* the application of microwaves and irradiation) are used at present, none of them provide the definitive solution so far. For selecting an adequate deactivation method, the following points should be considered:

- Different chemical structures of ANF produce differences in their chemical and physical properties as consequence
- Different ANF may appear at the same time in one feedstuff
- Deactivation methods might reduce some ANF, but drastically increase others
- The final product after deactivation might be as harmful as the not treated one or even more

- Deactivation methods transform as well other nutrients in the feedstuff, which in some cases is not desired
- ANF play important roles in the seed´s physiology and its defense against predators
- The quality of the deactivated product (physical and chemical characteristics, palatability, etc.) has to be in accordance with the requirements of the animal species to be fed
- Most of the commonly used deactivation technologies are expensive and high energy consuming

Among the deactivation methods, those ones involving the use of high temperatures (cooking, roasting, autoclaving, extruding, etc.) have been widely applied. They deactivate most ANF by denaturation. Adebooye & Singh (2007) studied cooking of whole cowpea grains, which reduced the total phenol content. When the seeds were previously decorticated, the result was improved by 10 %.

Combining different deactivation methods achieves the best results. Onwuka (2006) reported about the advantage combining soaking and boiling. Soaking for 12 h followed by boiling for 80 min reduced trypsin inhibitor activity from 25.6 to 3.2 TIU·mg^{-1} DM in vegetable cowpea. Further reductions were observed for haemagglutinin (from 49.5 to 9.5 HU·mg^{-1} DM), cyanogenic glycosides (from 83.8 to 5.1 mg·kg^{-1} DM), alkaloids (from 9.6 to 0.5 % DM) and tannins (from 3.4 to 1.3 % DM). Ibrahim *et al.* (2002) proved that the combination of either pressure or pre-germination with cooking was more effective than cooking alone in cowpea.

Pizzani *et al.* (2006) toasted Jack bean meal at 220 and 230 ºC (3 min) and at 240 ºC (2 min) and reduced the original canavanine content by more than 90 %. Nevertheless, the true metabolizable energy (TME) content of raw Jack bean was not improved by toasting. No significant differences were found between TME of raw Jack bean and toasted at 200, 220 and 230 ºC (3 min) or at 240 ºC (1 or 2 min), whereas toasting Jack bean meal at 180 ºC (3 min) or 230 ºC (1 or 2 min) significantly reduced TME. No haemagglutinating activity was detected in toasted Jack beans, but Con-A binding to duodenal mucosa ranged from moderate to weak. Reactive lysine and protein solubility was reduced as both, temperature and toasting time, increased. The effect of extrusion in Jack bean was assessed by Zamora (2003), who found increased digestibility values for protein (from 58 to 90 %) and starch (38 to 53 %). Protease inhibitor activities (trypsin and chymotrypsin) and α-

amylase inhibitor activity were reduced by 95 % and no haemagglutinating activity was detected. However, the canavanine content remained on the same level. By cracking Jack bean grains before cooking, Udedibie & Carlini (1998a) completely eliminated the haemagglutinating activity of the beans within 1 h of normal and 15 min of pressure cooking. When whole grains were used, 45 min of pressure cooking inactivated Con-A, whereas it took at least 3 h with normal cooking (96 ºC).

Rackis *et al.* (1986) indicated that it is necessary to apply a minimum of 1200 $J \cdot g^{-1}$ DM to inactivate urease and 1670 $J \cdot g^{-1}$ DM to destroy 95 % of the trypsin inhibitors in soybean. Nevertheless, during heating the availability of amino acids, especially lysine and cystine, is affected due to their susceptibility to the Maillard reaction (Parsons, 1996; Bruce *et al.*, 2006), which also reduces the efficacy of pancreatic and intestinal enzymes (Stein *et al.*, 2008). The same author proved that the loss of reactive lysine increased by the 25-fold when the samples were heated at 130 °C.

Due to its thermostability (melting point at 184 ºC), poor results have been obtained using heat for deactivation of canavanine in Jack bean (Belmar *et al.*, 1999; Sridhar & Seena, 2006). According to Tepal *et al.* (1994) and Zamora (2003), extrusion at 110 resp. 155 ºC had no influence on the reduction of canavanine. Later studies concluded that temperature has to be increased to reduce canavanine, but expected that nutrients will be affected at the same time, which was proven by Michelangeli *et al.* (2004b), who evaluated the ileal and fecal digestibility of various dietary components of toasted Jack bean (194 ± 2 °C, 18 min) in pigs. The original canavanine content of raw Jack bean was reduced by 95 % after toasting. However, toasting of Jack bean grains as well reduced total amino acids and their digestibility by approximately 50 %. Activity of haemagglutinins was not detected in toasted grains, but NDF_{OM} and ADF_{OM} contents were markedly increased due to the toasting procedure. The authors concluded, that even when ANF were reduced, the resulting nutritional value of the toasted grains for growing pigs was rather low. In a second experiment, the effect of feeding growing pigs with diets containing 20 % of Jack bean, toasted under various conditions of temperature and time, was evaluated. Pig performance varied depending on the toasting conditions, but only the diet with 20 % Jack bean toasted at 194 ºC (18 min) showed no significant differences in feed intake and weight gain compared with the control diet (Michelangeli *et al.*, 2004a). The

effect of heating on other ANF like *e.g.* tannins still has to be questioned (Jansman, 1993; Agbede & Aletor, 2005).

Other deactivation methods like germination, soaking and fermentation are alternatives to be considered when the cost-intensive use of heat cannot be afforded. Furthermore, those methods are assumed to improve the nutritional value of the feedstuffs. Pre-germination of cowpea seeds for 24, 48, 72 and 96 h resulted in a gradual decrease of the oligosaccharide content with pre-germination time while the level of monosaccharides increased. Protein and energy content increased slightly, while calcium and iron decreased with pre-germination and both ascorbic acid and niacin increased significantly, while thiamine decreased significantly according to Akinlosotu & Akinyele (1991). Ghavidel & Prakash (2007) observed that pre-germination significantly increased protein, thiamin, *in vitro* iron and calcium bioavailability and *in vitro* starch and protein digestibility of cowpea. Tannins were reduced by 28 % in pre-germinated grains and by 47 % after dehulling. Soaking cowpea seeds (24 h, 25 ºC) before milling resulted in a 20 % decrease of TIA (Prinyawiwatkul *et al.*, 1996a). Soaking (16 h) in bicarbonate solution remarkably deactivated ANF, as TIA was reduced from 24 % to 16 % when soaked only in water. The same effect was observed with oligosaccharides and phytic acid, but not with tannins. Pre-germination is assumed to be effective in reducing all ANF including tannins and for soaking was stated that the longer the soaking time, the higher the reduction (Ibrahim *et al.*, 2002). Depending on the soaking time (10, 20, 30 h) tannins were reduced in a following pre-germination (72 h) in low and high tannin-containing cultivars of sorghum (Elmaki *et al.*, 1999).

Several species and genera of yeasts, molds or bacteria have been evaluated in fermentation processes with legume seeds (Reddy & Pierson, 1994). Khattab & Arntfield (2009) studied the effect of *Saccharomyces cerevisiae* when they fermented two cowpea varieties for 24 h and found reduced contents of total phenols and tannins. They assumed that this effect was attributed either to polyphenol oxidase or to the microflora fermentation. Furthermore, fermentation resulted in a reduction of TIA (38 - 47 %) and oligosaccharides (71 - 72 %), which they assumed was caused by the secretion of hydrolytic enzymes by the yeast or the action of the naturally present α-galactosidase stimulated by the pH acidification.

During a spontaneously appearing fermentation by the native microflora of cowpea grains a decrease of verbascose (80 %) and stachyose (6 %) was observed,

whereas raffinose increased by 13 % as Akinyele & Akinlosotu (1991) reported. Moreover, increases of sucrose (8 %), fructose (105 %), glucose and galactose (56 %), as well as of thiamin, niacin, phosphorus, gross energy and protein were determined. Prinyawiwatkul *et al.* (1996a) inoculated cowpea with a commercial *Rhizopus microsporus* var*. oligosporus* starter culture and demonstrated an increased nutritional quality of cowpea flour, including the absence of raffinose and stachyose and increased B vitamin contents. However, a slight increase in TIA occurred after 18 h of fermentation and TIA at 24 h was higher (514 $mg \cdot g^{-1}$ flour) than that at 0 h (382 $mg \cdot g^{-1}$ flour). Ibrahim *et al.* (2002) used *Rhizopus oligosporus* and *Lactobacillus plantarum* (DSM 20205) as inoculants and eliminated completely TIA and oligosaccharides and reduced remarkably phytic acid, whereas tannins noticeably increased. Egounlety & Aworh (2003) reported similar results for the same ANF, except that tannins were not evaluated.

Belmar *et al.* (1999) reported in their review about the use of fermentation respectively ensiling methods and their impact on the deactivation of ANF in Jack bean. The “fermentation” with urea, dry or in solution (Carlini & Udedibie, 1997), has been applied with good results in poultry feeding (Udedibie & Carlini, 1998b; Belmar *et al.*, 1999). It is considered that urea has a denaturing effect on thermostable ANF (Udedibie *et al.*, 1994), but so far it could not be clarified how urea improves the nutritive value of Jack bean. Risso & Montilla (1992) did not find the same positive effects using a similar proceeding with Jack bean for pig feeding.

2.4 Particularities of grain silages

Although the use of microorganisms to deactivate ANF in the fermentation process is not a novelty, the effect of ensiling grains on ANF is not well documented.

The term “fermentation” refers to processes that obtain energy from the oxidation of organic compounds such as carbohydrates, which use an endogenous electron acceptor, which is usually an organic compound, but not oxygen. On the contrary, in “respiration” electrons are donated to an exogenous electron acceptor, such as oxygen, via an electron transport chain (Li, 2004; Prescott *et al.*, 2004). However, the generally accepted view is established in literature, that fermentation is any process of chemical change in organic substrates through the action of microbial enzymes (Li,

2004; Singleton & Sainsbury, 2006). Nevertheless, the strict biochemical definition of fermentation is frequently overlooked.

Ensilage is a fermentation process itself, but with certain particularities that make it unique compared to other forms of fermentations. McDonald *et al.* (1991) enounced the principal once:

1. Anaerobic conditions have to be achieved.
2. Undesirable microorganisms (*e.g.* clostridia) have to be suppressed, promoting those ones performing lactic acid fermentation.

The basic theory of ensilage is comprehensively described in literature (Woolford, 1984; McDonald *et al.*, 1991; Buxton *et al.*, 2003). Therefore, no details will be given at this point.

Legumes are regarded as rather unsuitable for ensiling due to three main factors: they have a high buffering capacity (BC), they tend to have low contents of water soluble carbohydrates (WSC) and they are low in dry matter (DM) when used solely as forages (McDonald *et al.*, 1991). Although the DM content in grains of Jack bean and cowpea is favorable, the high BC and low WSC content remain as limiting factors for ensiling.

In the tropics, conservation of grains affronts difficulties due to the influence of a high temperature and humidity, which promotes the development of fungus and undesired bacteria, affecting the nutritional quality. Ensiling represents an alternative to preserve the nutritional value of legume grains for animal feeding.

Ensiled grains can show significant contents of lactic acid as the result of microbial activity, but lactic acid level is lower and pH is higher than in ensiled forages (Pieper *et al.*, 2010). The quality of fermentation is thereby determined by the level of moisture, previous processing and ensiling of fresh or dried grains followed by reconstitution (Buchanan-Smith *et al.*, 2003). Corn silages with a moisture content of 275 $g \cdot kg^{-1}$ or more contained more lactic acid than the drier corn (Goodrich *et al.*, 1975). Similar results were obtained in silages of reconstituted sorghum (moisture contents of 250, 300 or 350 $g \cdot kg^{-1}$), where lactic and acetic acid increased and pH and ethanol decreased as moisture level increased. However, ammonia-N was lower in a moisture level of 250 $g \cdot kg^{-1}$ (Huck *et al.*, 1999). In practice, grains with moisture content < 250 $g \cdot kg^{-1}$ are usually more difficult to compact to produce the desired anaerobic environment than moister grains. However, when grains contain a

moisture $> 300\ g \cdot kg^{-1}$ their feeding value is affected. Under field conditions this optimal moisture content corresponds to fully mature grains even before the technological ripeness (Buchanan-Smith *et al.*, 2003). In the case of Jack bean and cowpea seeds the adequate moisture content to produce high quality silages still has to be evaluated.

As a fermentative process, ensiling might offer several advantages over other methods of feed processing and preservation. It is particularly useful for the processing of hard legume seeds, improving digestibility through reducing bean flatulence and the elimination of ANF, in particular trypsin inhibitors (Deshpande & Salunkhe, 2000).

Fermentation of a cereal-legume mix is especially beneficial with respect to the complementation of their amino acid content. The sulfur-containing amino acids methionine and cysteine are often limiting in legumes, while cereal proteins are generally deficient in lysine (Deshpande & Salunkhe, 2000). Therefore, combined ensiling of cereal and legume grains can be seen as a possibility for harvesting and preserving mature high moisture grains as an integral diet.

The main carbohydrate in cereals is starch, a polysaccharide not available for the majority of lactic acid bacteria. To use cereals as an effective source of water soluble carbohydrates, amylase or amylase-rich materials should be added to the mixed silages (McDonald *et al.*, 1991) or an alternative carbohydrate source like molasses should be used. The latter has been proven to increase lactic acid and to reduce pH and ammonia-N levels (Migwi *et al.*, 2000; Van Man & Wiktorsson, 2002; Huisden *et al.*, 2009; Nkosi *et al.*, 2009; Lima *et al.*, 2010; Lima *et al.*, 2011). On the other hand, the role of plant enzymes involved in the hydrolysis of starch and hemicelluloses releasing WSC (Muck, 1988) and the direct fermentation of amylolytic lactic acid bacteria (LAB), among them *L. plantarum*, indicates that WSC addition is not always mandatory to produce good quality silages. Reddy *et al.* (2008) showed the steps how LAB can use starch (Figure 7).

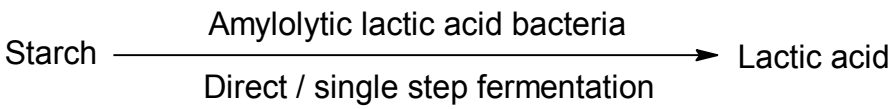


Figure 7: Scheme of lactic acid production from starch as substrate (Reddy *et al.*, 2008)

Homolactic bacteria are part of the epiphytic microflora of legumes. After a natural fermentation of cowpea for 4 days at 25 ºC, *Lactobacillus casei, Lactobacillus leichmanii, Lactobacillus plantarum* and *Pediococcus pentosaceus* were isolated (Zamora & Fields, 1979). These bacteria are homofermentative, producing an entire lactic acid fermentation, which would improve conservation of DM, reduce solubilization of N and give greater aerobic stability at feed-out (Buchanan-Smith *et al.*, 2003). Contrary to classical believe, well fermented silages containing high proportions of lactic acid in the total fermentation acids are prone to aerobic instability. Indeed, silages with high concentrations of acetic and butyric acids are associated with silages which are quite aerobically stable (Wilkinson & Davies, 2013).

The high DM content of grains seems to play a role in depressing epiphytic lactic acid bacteria when low moisture fermentations take place. Thus, using inoculants in silage is seen positive. Pieper *et al.* (2010) compared the effect of inoculants in silages of reconstituted (250 g·moisture kg^{-1}) wheat, barley and triticale grains and obtained higher lactic acid contents and a lower pH than in not inoculated ones. Sebastian *et al.* (1996) reported the same results with corn at a moisture content of 220 g kg^{-1}.

3 Task and aim

Although the potential of feed resources for pigs in the tropics is superior to that of the temperate zone (Ly, 1993), animal feeding systems established by most of the countries in this region, especially Cuba, are mainly based on soybean and cereals like wheat or corn. Paradoxically, their availability is not in accordance with the animal production demands. Therefore, they have to be imported with extra costs and the risk of subordination to the world market in consequence.

Non-conventional feedstuffs like Jack bean and cowpea represent an alternative due to their positive chemical composition, adaptability, resistance and low input demands. However, their content of anti-nutritional factors still limits their inclusion in pig rations. To overcome this problem, different deactivation methods have been tested with variable results. Most of them involve high implementation costs and energy consumes, which is difficult to afford for low-input farmers and furthermore is environmentally questionable.

For a long time, fermentation has been used to process feedstuffs. Numerous evidences demonstrate its influence on the improvement of the nutritional value of feedstuffs (Belmar *et al.*, 1999; Granito *et al.*, 2002; Azeke *et al.*, 2005; Nout & Kiers, 2005; Baik & Han, 2012; Muzquiz *et al.*, 2012; Torino *et al.*, 2013). Therefore, the question arises, if silages of Jack bean and cowpea sole or mixed with sorghum would not only conserve well, but produce the same positive effects on the nutritional value of these feedstuffs. To answer this question the following hypothesis was enunciated:

“It is possible to produce good quality silages of Jack bean and cowpea grains sole or mixed with sorghum grains with their main anti-nutritional factors being reduced, which increases their nutritional value as a feedstuff for growing-finishing pigs.”

The main objectives of the present study were formulated as follows:

1. Preparation of silages of Jack bean and cowpea grains sole or mixed with sorghum grains, which have a high fermentation quality.
2. Reduction of the main anti-nutritional factors in grains of Jack bean and cowpea sole or mixed with sorghum through ensiling.
3. Evaluation of the effect of ensiling on the animal performance through the determination of standardized ileal digestibility of essential amino acids and

apparent digestibility of selected nutrients using pigs under experimental conditions.

In order to implement the main objectives, the following work plan was developed:

1. The ensilability of Jack bean and cowpea grains in two different mixtures with sorghum should be evaluated through an *in vitro* rapid fermentation test (RFT) using a lactic acid bacteria inoculant and molasses as additives.
2. The influence of soaking and germination as pre-ensiling treatments on the reduction of buffering capacity and the increase of water soluble carbohydrates of the legume grains should be tested.
3. The effect of an additive (lactic acid bacteria and/or molasses) and mixing with sorghum on the pH value and the main fermentation products (lactic acid, volatile fatty acids, alcohols, ammonia) in Jack bean and cowpea lab scale silages (ROMOS) after different pre-ensiling treatments (soaking, pre-germination or remoistening to 65 % dry matter) should be evaluated.
4. The effect of storage time on the reduction of selected anti-nutritional factors in silages of Jack bean and cowpea grains sole or mixed with sorghum grains should be determined.
5. The fermentation quality and the effect on selected anti-nutritional factors of ensiled Jack bean and cowpea mixed with sorghum using additives and a selected pre-ensiling treatment should be evaluated as well on a large-scale (ton silages).
6. The effect of ensiled Jack bean-sorghum and cowpea-sorghum mixtures on the standardized ileal digestibility of amino acids compared with their equivalent raw mixtures should be demonstrated through a digestibility trial with minipigs with an ileal-rectal anastomosis.
7. The influence of ensiled Jack bean-sorghum and cowpea-sorghum mixtures on the fecal digestibility of selected nutrients compared with their equivalent raw mixtures should be analyzed through a digestibility trial with growing-finishing pigs.

4 Materials and methods

4.1 Experimental procedure

Harvested seeds of Jack bean (JBN), cowpea (CWP) and sorghum (SOR) at their ripe stage were thoroughly sun dried, winnowed and stored. A chemical evaluation of selected nutrients (DM, OM, CP, CF, AEE, NDF_{OM}, ADF_{OM}, ADL_{OM}, starch and ash) in the grains was made. Furthermore, selected ANF were determined for JBN (trypsin inhibitory activity, canavanine, hydrogen cyanide and tannins), CWP (trypsin inhibitory activity, hydrogen cyanide and tannins) and SOR (hydrogen cyanide and tannins). Buffering capacity and water soluble carbohydrates (WSC) were determined in all grains to estimate ensilability.

An *in vitro* fermentation test (Rostock Fermentation Test, RFT) was run to estimate ensilability before ensiling on a lab scale. Hereby, additives (molasses and lactic acid bacteria inoculant) were applied in different mixtures of JBN and CWP with SOR. Changes in pH during incubation (0, 14, 18, 22, 26 and 38 h) and chemical analysis of filtrates (lactic acid, volatile fatty acids, ammonia and ethanol) at the end of the test were used as selecting criteria for treatments of silages.

After the evaluation of results in RFT, Rostock model silages (ROMOS) were prepared. Three different experiments were conducted depending on the used pre-ensiling treatment. Beans were remoistened to approximately 65 % of DM, soaked or pre-germinated (each pre-ensiling treatment sole and mixed with SOR). A change in the chemical composition of the grains caused by the pre-ensiling treatments was expected to improve ensilability. The 65 % DM silages were thought to act as a control variant. Silages were disposed in a factorial design, where the effects of the type of bean, the pre-ensiling treatment used, the mixture with SOR, the use of additives and the storage time were the main factors to be evaluated. Fermentation products in silages after 60 days of storage and ANF at four storage times (0, 5, 20 and 60 days) were the used variables.

Silages of JBN+SOR and CWP+SOR with selected pre-ensiling treatment and additive were used in pig feeding trials. The selection criteria for the combination of pre-ensiling treatment and additive did not only consider results of model silages, but also practical reasons for future on-farm use. Therefore, grain-sorghum mixtures were ensiled in plastic tons for 60 days. Selected nutrients, fermentation products and ANF were determined to evaluate the nutritional quality of the silages.

Silages and their respective raw mixtures as control were fed to minipigs with an ileal-rectal anastomosis and the standardized ileal amino acid digestibility was calculated using the regression method. Fecal digestibility of selected nutrients (DM, OM, ash, starch, CF, AEE, NDF_{OM}, ADF_{OM} and ADL_{OM}) was determined in fattening-finishing pigs using the same feedstuffs. The calculation was made by difference.

4.2 Selection and processing of the plant material

Fully mature Jack bean (JBN, *Canavalia ensiformis* [L.] DC) grains were hand harvested and sun dried at the Agricultural Experimental Station "Álvaro Barba" (22º43'N, 79º90'W), Central University of Las Villas, Santa Clara, Cuba. Seeds were planted on brown carbonated soils and neither fertilizer nor irrigation was applied. Cowpea (CWP, *Vigna unguiculata* [L.] Walp.) var. INIFAT-93 seeds were purchased from a local market (State´s Seeds Distributer, Villa Clara, Cuba). Sorghum (SOR, *Sorghum bicolor* [L.] Moench) was purchased at Scharnebecker Mühle Di Ha GmbH, Scharnebeck, Germany. All grains were stored in cellular nylon bags prior to use.

4.3 Ensiling experiments with grains of Jack bean and cowpea sole or mixed with sorghum

4.3.1 Rostock Fermentation Test (RFT)

In order to conduct the *in vitro* method of RFT (Pieper *et al.*, 1989; Zierenberg, 2000), grains of JBN and CWP were milled to 4 mm mesh size. From the coarsely ground material 50 g was mixed with 200 ml of deionized water in glass beakers of 600 ml capacity. The following treatments were applied (n=3): control without additive (LEG), molasses (MOL, 4 %), *Lactobacillus plantarum* (LAB, $3x10^5$ $cfu \cdot g^{-1}$, DSM 8862 and 8866), MOL+LAB. Furthermore, sorghum (SOR) grains (4 mm mesh size) were mixed with legume grains to reach either 20 or 24 % crude protein of the dry matter in the mix and as well combined with LAB (SOR+LAB) (Table 5), whereas 20 and 24 % of crude protein would meet the requirements between weaning and growing-fattening phase.

Table 5: Treatments used in the Rostock Fermentation Test

Treatment	MOL (4 %)	LAB ($3x10^5$ cfu·g^{-1})	SOR (20% CP in the DM)	SOR (24% CP in the DM)
LEG				
LEG+MOL	+			
LEG+LAB		+		
LEG+MOL+LAB	+	+		
LEG+SOR			+	
LEG+SOR+LAB		+	+	
LEG+SOR				+
LEG+SOR+LAB		+		+

cfu, colony forming units; CP, crude protein; DM, dry matter; LAB, lactic acid bacteria; LEG, legume grains (Jack bean or cowpea); MOL, molasses; SOR, sorghum grains

The mixtures were stirred with a glass stick, which remained in the beaker throughout the whole RFT procedure and beakers were covered with aluminium foil and incubated at 30 °C. The pH value was measured after 0, 14, 18, 22, 26 and 38 h potentiometrically using a calibrated pH analyzer with glass and reference electrodes (precision 0.01, temperature compensation 0 - 70 ºC). Before measurements, each sample was well stirred with the glass stick. The pH electrode was disinfected with ethanol (70 %) between measurements to avoid microbial contamination among treatments. After the 38 h measurement time, extracts were filtrated and stored at -20 °C before analysis of fermentation products.

4.3.2 Preparation of model silages

Air-dry grains of JBN, CWP and SOR were coarsely ground (4 mm mesh size) and mixed with deionized water to reach a DM content of 65 % in the remoistened grains. Treatments (n=3) for silages were performed according to Table 6. Unlike treatments including SOR in RFT, the treatments LEG+SOR+MOL and LEG+SOR+MOL+LAB were included, whereas in all treatments mixed with SOR only 18 % of crude protein in the dry matter of the mix was adjusted in accordance to the diet that would be given to the animals in the feeding experiment.

Table 6: Treatments used for silages of remoistened grains of Jack bean and cowpea

Treatments	**MOL** (4%)	**LAB** ($3x10^5$ cfu·g^{-1})	**SOR** (18 % CP in the DM)
LEG			
LEG+MOL	+		
LEG+LAB		+	
LEG+MOL+LAB	+	+	
LEG+SOR			+
LEG+SOR+MOL	+		+
LEG+SOR+LAB		+	+
LEG+SOR+MOL+LAB	+	+	+

Laboratory-scale silages were prepared following the vacuum-packed Rostock model silage (ROMOS) method (Hoedtke & Zeyner, 2011). For the vacuum sealing of the plastic bag silages, a vacuum sealer (V.300, LAVA vacuum-package, Bad Saulgau, Germany) was used. Six-hundred grams of samples was packed into polyethylene bags (PA-PE 20/70, 200×300 mm, LAVA vacuum package, Bad Saulgau, Germany). Silage material was pre-compressed by hand before the bags were air-evacuated and heat-sealed. In order to prevent deformation of ROMOS bags, adhesive tape was wrapped around the sealed polyethylene bags. To avoid bloating, the wrapped bags were perforated with a needle (2×50 mm, Rometsch GmbH, Heilbronn, Germany), which was disinfected after each bag with ethanol (70%) and put immediately into a second bag (PA-PE 20/70, 200×300 mm, LAVA vacuum package, Bad Saulgau, Germany) which was air-evacuated with the same proceeding and sealed at once to maintain anaerobic conditions. ROMOS were stored at 30 ºC for 5, 20 and 60 days.

At each opening day, silages were homogenized and a representative sample was taken to determine DM, pH value, selected ANF and losses. The latter were calculated as percentage of the initial weight before ensiling. Selected nutrients and fermentation products of filtrates were determined only in 60-day silages. For pH measurement and analysis of filtrates, a portion of 50 g fresh silage was used. Samples were weighed into 600 ml beakers, mixed with 200 ml deionized water, stirred with a glass stick, covered with aluminum foil and kept overnight (4 ºC). The

pH was measured after extracts reached room temperature. Extracts were filtrated through filter paper (Whatman, grade Nº 4) and kept at -20 °C prior to analysis.

4.3.3 Pre-ensiling treatments

4.3.3.1 Soaking

For evaluation of a suitable soaking pre-ensiling treatment to be used for lab scale silages, three soaking times and four proportions of grain to water were used to assess the effect on the buffering capacity (BC) and selected parameters (DM, crude protein and ash). For both legumes a thorough selection of grains was made by hand. Quantities of 100 g were weighed into 600 ml beakers and deionized water was added as shown in Table 7.

Table 7: Experimental design of the soaking trial with Jack bean and cowpea grains (n=4)

Soaking time	18 h	24 h	30 h
Grain:water ratio (w/v)	1:2	1:2	1:2
	1:3	1:3	1:3
	1:4	1:4	1:4
	1:5	1:5	1:5

Beakers were covered with aluminum foil and incubated at 30 ºC. After soaking water was drained, grains were rinsed with deionized water, oven-dried at 60 ºC for 72 h and milled (1 mm mesh size) before being analyzed.

4.3.3.2 Pre-germination

The pre-germination trial with the legume grains was conducted to evaluate the influence of pre-germination on selected chemical parameters (WSC, DM and CP), which were thought to show an impact on ensilability. Furthermore, selected ANF were determined (TIA, HCN and canavanine) according to chapter 4.5.5, as through germination a reduction of those ANF was expected.

Following the method of Oboh *et al.* (2002), grains were soaked for 30 min in a sodium hypochlorite (1 %) solution and thoroughly rinsed afterwards (5 times) with deionized water. Grains were soaked (9 h) in deionized water at the ratio of 1:3 (w/v).

Sterile Petri dishes were lined with filter paper on which cotton wool was spread and sprayed with deionized water. Grains were hand selected and 30 JBN and 200 CWP seeds were selected per dish (n=3). The pre-germination trial was conducted under a light and darkness regime in a temperature regulated chamber (25 ºC). The treatment under light regime was given 12 $h \cdot d^{-1}$ natural light and 12 $h \cdot d^{-1}$ in total darkness. As the experiment was done in a room with only one window, additional artificial light (150 W bulb) was used to homogenize light in the whole room. Seeds under total darkness regime remained in the same room but were covered by a carton box. Grains were sprayed with deionized water two times per day to maintain humid conditions. Samples were collected at 12, 24, 48, 72 and 96 h and at 12, 24, 48 and 72 h for JBN and CWP, respectively. Pre-germinated grains were oven dried at 60 ºC, milled (1 mm mesh size) and stored for chemical analysis.

4.3.4 Lab scale silages with pre-treated seeds

4.3.4.1 Model silages with soaked seeds

In accordance to the results of the soaking trial, the appropriate treatment was selected to prepare ROMOS. The necessary quantity of JBN and CWP was hand selected and soaked in tap water for 24 h at a grain to water ratio of 1:4 (w/v) in plastic boxes (80 l volume) in a tempered room (20 ºC). Afterwards, grains were rinsed with tap water and drained for 20 min in stainless steel sieves.

Soaked grains were crushed (universal feed masher, Bad Liebenwerda, Germany) to approx 4 mm mesh size. Each treatment (Table 8) was prepared in a dough mixer (25 kg capacity). The mixer was disinfected between treatments with ethanol (70 %) to avoid carry-over of the inoculant. The ensiling procedure followed the method of ROMOS described in chapter 4.3.2.

Table 8: Treatments used for silages of soaked grains of Jack bean and cowpea

Treatments	MOL (4%)	LAB ($3x10^5$ cfu·g^{-1})	SOR (18 % CP in the DM)
LEG			
LEG+MOL	+		
LEG+LAB		+	
LEG+MOL+LAB	+	+	
LEG+SOR			+
LEG+SOR+LAB		+	+
LEG+SOR+MOL+LAB	+	+	+

cfu, colony forming units; CP, crude protein; DM, dry matter; LAB, lactic acid bacteria; LEG, legume grains (Jack bean or cowpea); MOL, molasses; SOR, sorghum

4.3.4.2 Model silages with pre-germinated seeds

After calculating the required amount of seeds for ROMOS, grains were pre-germinated in total darkness 72 h and 48 h for JBN and CWP, respectively. The same methodology as described in chapter 4.3.3.2 was followed. Times were selected according to the highest content of WSC after pre-germination. Treatments (n=3) for ROMOS (see 4.3.2) were made in accordance to Table 6.

4.4 Standardized ileal digestibility of amino acids and apparent fecal digestibility of selected nutrients

4.4.1 Constitution of diets used for digestibility trials

In both feeding trials, namely the determination of ileal and fecal digestibility, the same feedstuffs were tested. For silages, soaking (grain:water ratio 1:4 and 24 h soaking time) as pre-ensiling treatment was chosen for JBN and CWP grains in accordance to chapter 4.3.3. After soaking, beans were drained for 12 h and crimped with a meat mincer to a particle size of approx. 4 mm. Legume grains were later mixed with SOR (ground to 4 mm mesh size) to achieve 18 % of crude protein in the mix. Molasses and lactic acid bacteria were added according to chapter 4.3.1. The material was mixed thoroughly in a dough mixer and subsequently ensiled anaerobically in plastic tons (120 l capacity) with a sealed lid over a period of 60 days

at room temperature (approx. 20 ºC). After opening, silages were well homogenized. Samples were taken for chemical analysis and the determination of aerobic stability. To maintain silage quality during the feeding trials, silages were aliquoted and stored at -20 °C until being used. Aliquots were thawed at 4 °C the day before feeding.
In a simple palatability test, JBN silage was refused by the animals in all possible proportions proposed to be tested. Therefore, JBN containing silage had to be excluded from the feeding trail.
Besides the silages containing CWP, the corresponding raw (not ensiled) mixture of CWP and SOR was offered as feed. Both grains were milled (4 mm mesh size) and mixed in the same proportion like in silages to reach 18 % crude protein in the mix. However, neither molasses nor lactic acid bacteria were applied.

4.4.2 Determination of the standardized ileal digestibility of amino acids

4.4.2.1 Animals and housing

The experimental protocol was approved by the Ethical Committee of the Ministry of Agriculture, Food Safety and Fishery Mecklenburg-Western Pomerania, Germany (Permission No.: LALLF M-V/TSD/7221.3-2.1-026/09). The experimental design, collection and treatment of chyme and analyses of feed and ileal effluent were conducted according to the recommendations of GfE (2005b).
Eight adult castrated minipigs (strain Minilewe) were used, that were fitted with an end-to-end ileo-rectal anastomosis (IRA), conserving the ileo-ceco-colic valve and isolating the colon completely (Hennig *et al.*, 1986; Laplace *et al.*, 1994). Pigs were housed individually in floor pens (3 m^2 per pig) for the first 9 days of an adaptation period, during which the proportion of the feed to be tested was gradually increased. During the following 7 days, which comprised 2 days for adaptation and 5 days for collection of ileal effluents, animals were kept in metabolic cages (0.8 m^2 per pig).
In the subsequent cycles with duration of 10 days each, pigs were housed in the floor pens for 3 days adaptation and the following 7 days in accordance to the first cycle. The rooms were air conditioned and temperature and light was controlled (18 to 20 °C; 06.00 to 18.00 lighting). All animals were declared healthy at the beginning of the trial. The perianal region was washed daily with warm water and protected against feces irritation with a zinc oxide containing cream.

4.4.2.2 Experimental design and diets

The trial was disposed in a Latin square design and standardized ileal digestibility was calculated by the regression method. As there are restrictions in feeding raw CWP grains due to its content of ANF, the CWP was limited to 10, 20 and 30 % of the DM in the tested diets. Therefore, wheat (milled to 4 mm mesh size) was included as basal feed ingredient.

Two experiments were undertaken. In experiment 1 with four animals with a mean body weight (BW) of 64.0 ±2.1 kg, grain silage was fed and in experiment 2 (four animals with a mean BW of 58.6 ±1.9 kg) the raw mixture of grains was used. The composition of the diets is presented in Table 9.

During both experiments the feed supply was restricted to a daily level of 35 g DM·kg^{-1} BW$^{0.75}$. The daily rations were divided into two equal meals mixed with water (1:2.5, w/w) and fed at 07.00 and 14.00. In addition to drinking water, pigs were offered twice a day 200 ml of an electrolyte solution (composition, g·l^{-1}: 5.38 NaCl, 0.680 $CH_3COONa \times 3\ H_2O$, 0.372 KCl, 0.548 $CaCl_2 \times 6\ H_2O$, 0.304 $MgCl_2 \times 6\ H_2O$; concentrations, g·l^{-1}: 3.218 Na, 0.195 K, 0.036 Mg, 0.100 Ca, 3.651 Cl) with little modifications to prevent mineral depletion due to IRA (GfE, 2002). Due to animals' adverse reaction, $CH_3COONa \times 3\ H_2O$ was reduced tenfold. The pigs consumed solution volumes of 20 to 24 ml·kg^{-1} BW$^{0.75}$ and day, and the daily Na supply was adjusted to the 2.5-fold of the requirements of intact pigs.

To minimize the effect of endogenous amino acids (AA) on apparent digestibility (AD) values and in accordance to the results of Furuya & Kaji (1989) and Fan *et al.* (1994), the German Society of Nutrition Physiology (GfE, 2002) introduced the following threshold levels in assay diets (g·kg^{-1} DM): 9.0 lysine, 7.0 threonine, 3.0 methionine and 12.0 leucine. The assay diets were supplemented with crystalline AA up to the abovementioned levels. Crystalline AA are considered to be 100 % digestible (Chung & Baker, 1992), so that they were not considered in the intake.

Table 9: Composition of diets (g·kg^{-1} DM) used in the experiments for determination of ileal digestibility

	Experiment 1			
	1	2	3	4
Ingredients	Wheat	Silage + wheat (10% CWP of DM)[1]	Silage + wheat (20% CWP of DM)	Silage + wheat (30% CWP of DM)
Wheat	947	755	563	371
Silage[2]	-	195	391	586
Mineral mix[3]	35	35	35	35
L-leucine	4.2	2.8	1.4	0
L-lysine*HCl	8.2	6.9	5.6	4.4
L-threonine	4.1	3.5	3.0	2.4
DL-methionine	1.7	1.5	1.4	1.3
	Experiment 2			
	1	2	3	4
Ingredients	Wheat	Mix + wheat (10% CWP of DM)[1]	Mix + wheat (20% CWP of DM)	Mix + wheat (30% CWP of DM)
Wheat	947	755	564	372
Raw mix[2]	-	195	391	586
Mineral mix[3]	35	35	35	35
L-leucine	4.2	2.7	1.3	0
L-lysine*HCl	8.2	6.6	5.0	3.4
L-threonine	4.1	3.5	3.0	2.4
DL-methionine	1.7	1.5	1.3	1.2

CWP, cowpea; DM, dry matter

[1] The percentage of CWP was calculated depending on the daily DM intake per animal.

[2] Mixture cowpea and sorghum to reach 18 % crude protein in the DM

[3] Mineral and vitamin premix (Spezialfutter Neuruppin GmbH, Neuruppin, Germany); content of minerals and vitamins per kg diet: 5.87 g Ca, 0.25 g Mg, 1.63 g P, 1.25 g Na, 125 mg Co, 0.75 mg I, 125 mg Fe, 75 mg Mn, 0.25 mg Se, 178.75 mg Zn, 0.50 mg Co, 12500 I.U. vitamin A-palmitate, 1250 I.U. cholecalciferole, 37.5 mg tocopherole, 1.88 mg methylnaphthochinon, 2.5 mg thiamin, 6.25 mg riboflavin, 3.75 mg pyridoxine, 25 µg cobalamine, 15.63 mg Ca-pantothenate, 31.25 mg nicotinic acid, 125 mg choline chloride, 0.31 mg folic acid, 75 µg biotin

4.4.2.3 Chyme collection and analysis

The ileal effluents were quantitatively collected in metal containers adapted to the metabolic cages with a solution of methanol and formaldehyde (95:5, v/v) to prevent microbial fermentation and N losses (Laplace *et al.*, 1994). Total amounts were sampled once a day, pooled and frozen at -20 °C. At the end of the collecting period, the total effluent was weighed, homogenized and approximately 500 g fresh sample was taken for chemical analysis. Prior to freeze-drying, methanol and formaldehyde solution was removed by air-drying for 24 h. Dried digesta samples as well as feed samples were ground to 1 mm mesh size.

Feedstuffs and digesta were analyzed for AA composition (except tryptophan) according to Hackl *et al.* (2010). Samples of 2 g of each diet and dried effluents of all individual animals were hydrolyzed with 60 ml of 6 N HCl for 22 h at 110 ºC and then filtrated. For analysis of sulphur-containing AA, samples of 2 g each were oxidized using 10 ml of an oxidative mixture containing formic acid and H_2O_2 (9:1, v/v) and kept at 4 ºC overnight. Consecutively, 1 N $KMnO_4$ was added until no more discoloration took place and then samples were hydrolyzed with 60 ml of 6 N HCl for 22 h at 110 ºC. After cooling, the samples were transferred to flasks, filled up to 500 ml and filtrated. The AA in the filtrates were quantified by HPLC (Shimadzu, Kyoto, Japan) using a cation column (LC K06; Alltech-Grom GmbH, Rottenburg-Hailfingen, Germany). The temperature of the column was programmed to oscillate in a range between 57 and 74 ºC and a pH gradient from 3.45 to 10.85. The buffer flow rate was 0.45 ml·min^{-1}. The AA solutions were mixed with ninhydrin at a flow rate of 0.25 ml·min^{-1} for color development at 128 ºC and determined by use of an UV-detector at 570 nm (proline at 440 nm).

4.4.3 Determination of fecal digestibility of selected nutrients

4.4.3.1 Animals and housing

The experimental protocol was approved by the Ethical Committee of the Ministry of Agriculture, Food Safety and Fishery Mecklenburg-Western Pomerania, Germany (Permission No.: LALLF M-V/TSD/7221.3-2.1-010/10). The experimental design, collection and handling of excreta and chemical analyses of diets and excreta were conducted according to the recommendations of GfE (2005a).

Six castrated pigs of the breed ‘German Landrace‘ were selected with an initial mean BW of 49.0 ±2.1 kg. Pigs were housed individually in fattening pens (1.5 m² per pig) for the first 4 days of an adaptation period. After that time, animals were kept in metabolic cages (0.8 m² per pig) for 3 more days of adaptation and another 5 days for collecting feces. At the end of the cycle, pigs were weighed to recalculate the daily DM intake and housing started again in the fattening pens for the first days of adaptation like described before. The rooms were air conditioned and temperature and light was controlled (18 to 20 °C; 06.00 to 18.00 lighting). Animals finished the trial with a mean BW of 66.6 ±4.4 kg.

4.4.3.2 Experimental design and diets

The experiment was conducted using a Latin square design, whereas 6 pigs were allotted in three groups (2 pigs per group). In the assay diet wheat acted as basal feed ingredient and silage and its corresponding raw mixture as assay feed ingredient. The assay diet was adjusted so that CWP will not exceed 30 % in the DM, which corresponded to more than 50 % of the assay ingredient (CWP+SOR silage) in the assay diet as recommended (GfE, 2005a). The DM intake was adjusted at the beginning of each cycle in correspondence to the BW of the animals.

The daily rations were divided into two equal meals mixed with water (1:2.5, w/w) and fed at 07.00 and 14.00. Minerals and vitamins were supplied according to the requirement. As the content of lysine in the diet was insufficient, the necessary amount was provided. Free access to drinking water was given after each meal. The composition of the diets in each cycle is presented in Table 10.

Table 10: Composition of diets (g·kg^{-1} DM) used in the experiment for determination of fecal digestibility

Ingredients	**Diets**		
	1	2	3
Wheat	952	366	366
Silage (CWP+SOR)	-	586	-
Mixture (CWP+SOR)	-	-	586
Mineral mix[1]	35	35	35
L-lysine*HCl	13	13	13

[1]Composition according to Table 9.

4.4.3.3 Feces collection and analysis

The feces were quantitatively collected in metal containers adapted to the metabolic cages. Total amounts were sampled once a day, pooled and frozen at -20 °C. At the end of the collecting period, feces were thawed and homogenized and a representative sample was taken and freeze-dried for further chemical analysis. For the determination of crude protein (N x 6.25) sub-samples of the fresh feces were used. Dry matter of the fresh feces was determined by oven drying (105 °C, 17.5 h) to a constant weight. Dried feces and feedstuffs were ground (1 mm mesh size) and stored until being analyzed. The methods for determination of proximate nutrients (DM, ash, CP, AEE, NDF_{OM}, ADF_{OM}, $ADL_{OM,}$ CF and starch) are described in chapter 4.5.1.

4.5 Chemical analysis

4.5.1 Chemical analysis of the plant material, silages, chyme and feces

The dry matter (DM) was determined according to the guidelines of the VDLUFA (1993). A representative sample of air-dried seeds, silages, chyme or feces was milled to 1 mm mesh size (Brabender, Duisburg, Germany). Milled samples were stored in sealed glass flasks at room temperature for further analyses. Approximately 2 g (accurate weighing) of the milled samples were oven dried (105 ºC) for 3 h until constant weight.

In silages, at every opening day, bags were opened and the whole content mixed in a disinfected tray. After organoleptic evaluation of the quality, a representative portion per bag was divided in a couple of trays and protected with aluminum foil. Trays were stored at -20 °C before being freeze-dried (4 to 5 days). After freeze-drying the trays were weighed and milled (1 mm mesh size). Then the same proceeding described before for DM determination was followed. A dry matter correction was not made for volatile silage products as currently there still remain uncertainties, when the freeze drying method is used instead the classical oven drying (Eriksson & Ericson, 2012). Furthermore, equations for correcting silage dry matter of grain silages should be developed in the future.

Ash was determined in all materials by ashing at 600 ºC for 6 h in a muffle furnace. The mineral composition of soaked JBN and CWP of selected soaking treatments was analyzed at the Zentrum für Lebensmittel und Tierernährung, Oberschleißheim, Germany. According to Stürmer (2005), a hydrolysis of the samples was made with HNO_3 for Ca, Na, K, P and Mg using a microwave (EHTOS 1600, MLS, Leutkirch, Germany). For Cl, samples were washed with water and the slurry was used for analysis. Ca, Na and K were measured using a flame spectrophotometer (EFOX 5053, Eppendorf, Hamburg, Germany). For Mg an Atomic Absorption Spectrophotometer (AAnalyst 800, Perkin Elmer, Shelton, USA) was used. P was determined spectrophotometrically (Genesys 10 UV, Thermoscientific, Waltham, USA). The determination method of Cl was based on the principle of colometric impulse titration by the use of a silver electrode pairing (Chloroidmeter 50cl, SLAMED, Frankfurt, Germany).

Crude protein (N × 6.25) was analyzed with Kjeldatherm and Vapodest (Gerhardt, Königswinter, Germany). Neutral detergent fiber (NDF_{OM}, exclusive residual ash) and

acid detergent fiber (ADF_{OM}, exclusive residual ash) were determined by wet chemical analyses (Goering & Van Soest, 1970) with prevail enzymatic hydrolysis and crude fiber (VDLUFA, 1993) using a FOSS analyzer (Fibertec 2010, Rellingen, Germany). Applying the Soxhlet principle, acid ether extract was determined after acid hydrolysis and petroleum ether extraction (Soxtec 1047 and 2050, FOSS Tecator, Hoganas, Sweden).

Water soluble carbohydrates (WSC) were analyzed as monomeric and dimeric sugars in water extracts (1 h at 25 ºC) by HPLC (HPX-87C, Biorad, Hercules, CA, USA) according to Menge-Hartmann *et al*. (2009) with a flow rate of 0.65 $ml \cdot min^{-1}$ at refractive index detector (column temperature 80 ºC). For the determination of starch, an enzymatic procedure using amylase (Thermamyl 120, Novo Nordisk A/S, Denmark) was chosen (Schmidt *et al.*, 2005). Concentration of glucose was measured by HPLC after enzymatic hydrolyses and the starch content was calculated by considering the increase of glucose.

For buffering capacity (BC), 0.5 g of plant material was mixed with 50 ml deionized water, shaken for 2 min using a magnetic stirrer and left for 30 min at room temperature. The BC was determined by titration with lactic acid (0.1 $mol \cdot l^{-1}$) to a pH of 4.0 (Weissbach, 1967).

4.5.2 Determination of fermentation products

Filtrates of RFT and silage extracts were analyzed for fermentation products. Lactic acid was determined by HPLC (Aminex HPX-87H, Biorad) with a flow rate of 0.60 $ml \cdot min^{-1}$ at the UV detector. Volatile fatty acids (acetic, propionic, butyric, valeric and iso-valeric acids) and alcohols (ethanol, propanol and 2,3-butanediol) were quantitatively separated by gas chromatography (GC-14A, CLASS-VP, Shimadzu, Kyoto, Japan). Nitrogen was used as carrier gas at a pressure of 1 $kg \cdot cm^{-2}$. The temperature of the injector and flame ionization detector was kept constant at 190 ºC each. The temperature of the column oven was programmed at 110 ºC during the first 1.5 min and increased to 170 ºC at a rate of 12 $ºC \cdot min^{-1}$ thereafter. Ammonia was determined in the filtrates using a modified CONWAY (Conway & Byrne, 1933) micro-diffusion technique (Voigt & Steger, 1967).

4.5.3 Determination of the aerobic stability of silages

Aerobic stability was determined in the silages prepared for the feeding experiments after 60 days of storage by means of temperature rise (Honig, 1990). Samples (n=4) representing 100 g of DM were filled into plastic containers (1.25 l capacity), of which the base as well as the lid were provided with a hole of 1 cm diameter to guarantee air circulation. A thermocouple was fixed through the hole in the lid in the geometrical centre of the sample and the closed test container was put into a cylinder made of styrofoam. Temperature was measured at intervals of 6 h for 7 days and recorded by data logger software (Version 4.2, PS ES Electronics Services, Nieuwendijk, Netherlands). Silages were considered as aerobically unstable if the temperature of the silage sample and the room temperature (set at 20 °C) differed by more than 3 °C. Moreover, pH and DM were determined at the beginning and at the end of the test.

4.5.4 Determination of deoxynivalenol

The plant material was analyzed for deoxynivalenol (DON) in accordance to Oldenburg *et al.* (2007) at the Bundesforschungsinstitut für Tiergesundheit, Friedrich-Loeffler-Institut, Braunschweig, Germany. Samples were analyzed by HPLC equipped with a diode array detector (SPD-M10AVP, Shimadzu, Duisburg, Germany). Separations were done on an Aqua C18 column, 5 µm, 250x4.6 mm (Phenomenex, Aschaffenburg, Germany). Quantification was made at 218 nm. Previously, a clean-up with an immuno-affinity column (IAC, DONprep^{TM}, R-Biopharm AG, Darmstadt, Germany) was made. A mechanical shaker was used to extract 10 g sample (1 mm mesh size) in 80 ml deionized water for 1 h. Afterwards, 20 ml extract was filtered through a fluted filter and 3 ml of the filtrate was afterwards filtered through a polivinylidene fluoride membrane syringe filter (0.45 µm, Amchro, Hattersheim, Germany). Through an IAC, 2 ml of the extract was passed under gravity. The column was washed once with 2 ml and twice with 1.5 ml of deionized water using a slight vacuum. Four successive portions of 1 ml methanol were passed slowly. The solvent was separated from the eluted DON using a rotator evaporator. The last traces were blown out under a gentle stream of nitrogen. The authors

recommend, that dilutions of the extract should be made before passing through IAC when DON surpasses 3 mg·kg^{-1} DM due to column limited capacity.

4.5.5 Determination of anti-nutritional factors

Sample preparation

Prior to analysis, samples were finely milled in a ball mill (Retsch, MM 200, Germany), whereas overheating had to be prevented. The oscillation frequency was set to 30 Hz and the grinding time to 5 min to reach a particle size of ≤ 5 µm. Samples were conserved in glass flasks and protected from light.

Trypsin inhibitory activity (TIA)

The determination of the trypsin inhibitory activity followed the method of Kakade *et al.* (1974) with modifications made by Smith *et al. (*1980*)*. Finely ground samples (0.6 g) were mixed with 30 ml NaOH (0.01 M). The pH of the resulting slurry was adjusted to the range of 9.4 - 9.6 with either NaOH (0.1 M) or HCl (0.1 M). Samples were extracted overnight (4 ºC) and the unfiltered and not centrifuged supernatant was used for analysis. Dilutions were made with deionized water, so that 2 ml of sample extract produced an inhibition of 40 to 60 % to reduce the relative standard deviation. Tubes of 10 ml volume were prepared in duplicate as follows:

(a) *Reagent blank*: 2.0 ml deionized water
(b) *Standard trypsin*: 2.0 ml standard trypsin solution (from bovine pancreas, Sigma, T8802, 13000 units·mg protein^{-1}; 4 mg·200 ml^{-1} 0.001 M HCl) + 2.0 ml deionized water
(c) *Sample blank*(s): 2.0 ml diluted sample extract
(d) *Samples*: 2.0 ml diluted sample extract + 2.0 ml standard trypsin solution

A solution of Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA; Sigma, B4875) was prepared dissolving 40 mg of BAPNA in 1 ml of dimethyl sulphoxide and diluting it to 100 ml with Tris buffer (Sigma 93389) previously warmed to 37 ºC. After mixing (Vortex, Genie 2, Scientific Industries, USA) and pre-heating to 37 °C for 10 min, 5 ml BAPNA solution was added to each tube, mixed and incubated for exactly 10 min at 37 ºC. The reaction was stopped by acetic acid (1.0 ml, 30 % v/v).

Standard trypsin (2.0 ml) was then added to tubes (a) and (c). After centrifugation (5000 rpm, 10 min, 20 ºC), the released p-nitroaniline was measured photometrically at 410 nm (Spectronic, Genesys 5, Milton Roy, USA). The residual trypsin activity was measured in $mg \cdot g^{-1}$ DM of the sample, whereas TIA was calculated as follows:

A_I=	(Ab-Aa)-(Ad-Ac)	A= absorbance of tubes (a) to (d)
% inhibition=	$100 * A_I/(Ab-Aa)$	($>$ 40 % and $<$ 60 %)
TIA=	$factor * D * A_I/S$	(mg pure trypsin inhibited per g sample) factor = [ml (0.01 M NaOH) + ml (0.1M NaOH or 0.1M HCl)]/19

A_I = change in absorbance due to trypsin inhibition per ml diluted sample extract
D = dilution
S = sample weight

Hydrogen cyanide (HCN)

Following the acid hydrolysis method developed by Bradbury *et al.* (1991), 100 mg of samples were weighed into screw-capped plastic tubes of 10 ml volume in duplicate. To each tube, 10 ml phosphoric acid (0.1 M) was pipetted in two steps. At first, 3 ml were added and shaken for 30 seconds. Afterwards, the remaining 7 ml were pipetted and the extract was placed in an ultrasonic bath for two intervals of 5 min each with a break of 5 min in between to avoid heating. The supernatant was then separated by centrifugation (4800 rpm, 15 min, 20 ºC) and conserved in the fridge if necessary.

Into 10 ml plastic test tubes, 2 ml of the extract and 2 ml H_2SO_4 (4 M) were pipetted and cooked in a water bath (100 ºC, 50 min). Depending on necessities, the tubes were either cooled in an ice bath or overnight in the fridge (4 ºC). After that, 5 ml NaOH (3.6 M) were pipetted and the solution was left for 5 min. From the mixture, 1 ml was transferred into clean screw-capped test tubes with 7 ml phosphate buffer (0.2 M, pH 6). Then, 0.4 ml of chloramine-T ($5\ g \cdot l^{-1}$) was added. The tubes were cooled in ice for 5 min and 1.6 ml of pyridine/barbituric acid solution was pipetted, which was prepared by dissolving 1 g barbituric acid in 40 ml pyridine and finally brought to 200 ml with deionized water. For preparing the blank solution, chloramine-T and the pyridine/barbituric acid solution were substituted by 2 ml deionized water. After 1 h to let the color develop, the absorbance was measured at 583 nm.

For calculating HCN concentration (mg·100 g^{-1}), a calibration curve was prepared. From the HCN solution (75 mg brought to 100 ml with 0.2 M NaOH), aliquots (0.2, 0.4, 0.6, 0.8 and 1 ml) were taken and brought to 10 ml with a pre-mixture of solutions made to contain the same proportions as those ones used in the analyses (20 ml 0.1 M H_3PO_4 + 20 ml 4 M H_2SO_4 + 50 ml 3.6 M NaOH). From each, 1 ml was taken and the proceeding followed the same steps likewise the samples.

Canavanine

The determination of canavanine followed the method of Makkar *et al.* (2007) with modifications of the preparation of the extract suggested in the original protocol (Cacho *et al.*, 1989). Finely ground samples (0.5 g) were weighed into 50 ml-test tubes and 15 ml HCl (0.1 M) were added. The mixture was stirred for 2 min with a homogenizer (Polytron, PT 1600 E, Switzerland) at room temperature and centrifuged (4000 rpm, 20 min, 20 ºC). The supernatant was saved and a second extraction was performed with 10 ml HCl (0.1 M). Both the supernatants were combined and the pH was adjusted to 7.0 with NaOH (0.2 M). The solution was diluted to 100 ml final volume using phosphate buffer (0.2 M, pH 7.0).

Pentacyanoaminoferate (PCAF) was prepared by dissolving 10 g sodium nitroprusside in 55 ml of concentrated ammonia solution (32 % purity). To store the solution at 0 ºC in the darkness as recommended, it was kept in a styrofoam box filled with crushed ice inside a fridge. After 24 h a yellow-green precipitate (PCAF II and III) was obtained by filtration through a Büchner funnel using a vacuum pump to separate ammonia. Ethanol was added to the filtrate and the formed precipitate re-filtrated and combined with the first one. The pooled precipitate was washed until all ammonia was eliminated. The precipitate was dehydrated in pure H_2SO_4 containing desiccators for one day and afterwards passed into silica-gel pearls containing desiccators until it was completely dry. Finally, it was passed into an amber-colored flask covered by aluminum foil and conserved in desiccators in complete darkness until photoactivated PCAF was needed.

Photoactivated PCAF solution (1 %, w/v) was freshly made every time it was needed and used within one day. PCFA (1 g) was dissolved in 50 ml deionized water and then filled to 100 ml. The solution was transferred completely to a 200 ml-beaker and

illuminated by a 60 W table lamp for 1 h. The photoactivated PCFA was stored in a capped Erlenmeyer flask covered with aluminum foil in dark conditions.

The calibration curve was prepared pipetting 1 ml from five canavanine solutions with different concentrations (2, 4, 6, 8, 10 $\mu g \cdot ml^{-1}$) of canavanine (Sigma C1625, L-canavanine from *Canavalia ensiformis*, ≥ 98 % TLC) in 0.2 M potassium phosphate buffer of pH 7.0) in 10 ml-test tubes. To these solutions, 6.5 ml of the pH 7.0 phosphate buffer, 1 ml of potassium persulfate (1 %) and 0.5 ml of the aqueous PCAF (1 %) were added and completed to 10 ml with deionized water. A reagent blank was prepared with phosphate buffer in place of the canavanine solution. Solutions were thoroughly mixed and after 15 min absorbance measured at 520 nm against the reagent blank.

For the determination of canavanine in the samples, 1 ml of plant extract was taken and the same procedure was run as mentioned previously for the canavanine solutions. The concentration of canavanine was determined using the calibration curve and results were expressed as g canavanine·100 g^{-1} DM.

Polyphenols (*condensed tannins, total phenols and non-tannin phenols*)

For the extract preparation, 100 mg of the finely ground sample was weighed into screw-capped test tubes in duplicates and shaken for 30 sec with 3 ml previously cooled acetone (70 %). In a second step, additional 7 ml acetone was added and the tubes were placed in an ultrasonic bath for two times 5 min with 5 min break in between. The bath was cooled with crushed ice (the quantity was restricted to avoid ultrasonic waves´ interference). The supernatant was collected after centrifugation (4800 rpm, 15 min, 4 ºC) and protected from light inside a fridge. The extract had to be used within two days.

Condensed tannins

The method for the determination of condensed tannins is based on Porter *et al*. (1986). A butanol-HCl solution was prepared mixing 95 ml n-butanol and 5 ml HCl (37 % of purity), from which 1.5 ml were pipetted into screw-topped plastic tubes. Immediately after that, 250 µl from the extract was added, whereas for the blanks 250 µl acetone (70 %) was taken. After 10 sec of shaking, 50 µl of a $(NH_4)Fe(SO_4)_2$ solution (2 g brought to 100 ml 2 N HCl) were added and shaken for 10 more

seconds. The tubes were closed and cooked in a water bath (100 ºC, 1 h), the blanks remained at room temperature. After cooking, the tubes were cooled in ice for 10 min. The absorbance was measured at 550 nm using the blank as auto zero. The calculation was made as follows:

Condensed tannins (% DM) = absorbance*156.5*dilution factor/DM (%)

Total phenols

Using the same extract , 250 µl was pipetted into 10 ml-test tubes in addition to 750 µl cold deionized water and 500 µl Folin-Ciocalteus (1 N). After shaking, the solution was left for 3 min before 2500 µl Na_2CO_3 solution (88.5 g Na_2CO_3 x 10 H_2O brought to 500 ml with deionized water) was added, followed by shaking. The samples were left in darkness (1 h) for the color development and the absorbance was measured at 725 nm.

For the calculation a calibration curve was produced every time new solutions were made. Aliquots (10, 20, 40, 60, 80 and 90 µl) were taken from a tannin solution (12.5 mg tannic acid brought to 25 ml with deionized water) and brought to 1 ml with cold deionized water. The Folin-Ciocalteus and Na_2CO_3 solutions were added following the same procedure described before, whereas for the blanks, deionized water was used instead of the sample extract or tannin solution.

Non-tannin phenols

In 10 ml-test tubes 200 mg polivinyl-polipirrolidone (PVPP) were weighed in duplicate for every sample and shaken with 2 ml cold deionized water for 10 sec. From the same extracts, 2 ml was pipetted into each tube containing PVPP and after shaking the solutions were left to react for 15 min in an ice bath before shaking once again followed by centrifugation (4800 rpm, 15 min, 4 ºC). The supernatant (500 µl) was mixed with 500 µl cooled deionized water, whereas for the blank 1000 µl deionized water was used. The solution was mixed with 500 µl Folin-Ciocalteus (1 N) and left for reaction (3 min) after shaking. Afterwards, 2500 µl Na_2CO_3 solution was pipetted and the tube was shaken once again. The absorbance was measured at 725 nm after 1 h of color development at room temperature.

4.6 Calculations

The estimation of the metabolizable energy of the digestible nutrients in the grains and diets was made in accordance to the recommendations of the German Society of Nutrition Physiology (GfE, 2008). The used equation was as follows:

ME (MJ·kg^{-1}DM) =	**CP** x 0.021503	(g kg^{-1} DM)
	+ **AEE** x 0.032497	(g kg^{-1} DM)
	- **CF** x 0.021071	(g kg^{-1} DM)
	+ **Starch** x 0.016309	(g kg^{-1} DM)
	+ **Organic rest*** x 0.014701	(g kg^{-1} DM)

AEE, acid ether extract; CF, crude fiber; CP, crude protein

*Organic rest calculated as the difference between the OM and the sum of CP, AEE, CF and starch (each in g kg^{-1}).

No interaction between the feed ingredients wheat and silages or raw mixture was expected. Therefore, the regression method was selected for the determination of the standardized ileal digestibility of amino acids (GfE, 2005b). The equations used for the calculation are described in chapter 4.7.

As the tested ingredient (T_{Ingr}), silages or raw mixture, were only part of the tested diet (T_{Diet}), the digestibility coefficient (DC) of the nutrients supplied by T_{Ingr} (DC_{Ingr}) was calculated by difference, whereas wheat was used as basal diet (BD):

$$DC_{Ingr}\,(\%) = \frac{DC_{Diet}\,(\%) - DC_{BD}\,(\%)*(1-a)}{a}$$

DC_{Diet} and DC_{BD} = Digestibility of nutrient from T_{Diet} and T_{BD} calculated as follow:

$$DC\,(\%) = \frac{\text{Input-Output}}{\text{Input}}\,100$$

$$a = \frac{\text{Analyzed nutrient content in } T_{Ingr}\,(g\cdot kg^{-1}\,DM)*\text{Inclusion rate of } T_{Ingr} \text{ in } T_{Diet}\,(kg\cdot kg^{-1}\,DM)}{\text{Analyzed nutrient content in } T_{Diet}\,(g\cdot kg^{-1}\,DM)}$$

Hereby, energy intake of the animals was calculated as the 2.5-fold of the maintenance requirement (ME_m) as:

ME_m ($MJ \cdot d^{-1}$) = 0.72 kg $BW^{0.63}$ (GfE, 1987).

4.7 Statistical analysis

A General Linear Model was run to find significant interactions among soaking time, grain:water ratio and the BC, DM and CP in JBN and CWP, as well as among the time and light regime on the WSC, DM and CP and selected ANF during pre-germination of JBN and CWP, generated by IBM SPSS version 19 (SPSS software for Windows Inc., Chicago, IL, USA). P-values less than 0.05 were considered to be significant.

Differences between treatments in RFT fermentation parameters (pH value, lactic acid, acetic acid, butyric acid, ethanol and ammonia), ROMOS fermentation parameters (pH value, fermentation losses, lactic acid, acetic acid, butyric acid, ethanol, propanol, 2,3-butanediol and NH_3-N of total N) and minerals in selected treatments after soaking were analyzed using One-way ANOVA followed by Duncan's multiple range test or Dunnett-T3 in case of no variance homogeneity. $P<0.05$ was considered to be statistically significant. IBM SPSS version 19 (SPSS software for Windows Inc., Chicago, IL, USA) was used. Differences in pH among incubation times within treatments and between treatments for each incubation time of RFT are presented in the appendix.

The silage data (DM, fermentation losses, pH value, lactic acid, acetic acid, butyric acid, ethanol, 2,3-butanediol as well as ANF in the selected treatments) were analyzed by analysis of variance using the GLIMMIX procedure of SAS/STAT and a model containing the fixed effects pre-ensiling treatment (levels: moisture reconstitution, soaking, pre-germination), legume (levels: JBN, CWP), additive (levels: control, molasses, lactobacillus, molasses plus lactobacillus) and sorghum (levels: mixed, not mixed).

In addition, LS-means (LSM) corresponding to each fixed effect in the model and their standard errors (SE) were calculated and all LS-means were pair wise tested using the Tukey-Kramer procedure for pair wise multiple comparisons. The data analysis for multiple interactions was generated using SAS/STAT software,

Version 9.2 (SAS/STAT, 2009) of the SAS System for Windows. Information was gathered in graphics made using the same software and every single significant difference was summarized in tables presented in the appendix.

For comparing standardized ileal digestibility of amino acids, Proc GLIMMIX and a model with the fixed effect Group (levels: raw mixture and silage), the covariate x and the interaction Group*x was used to estimate, test and compare the intercepts and slopes of the regression lines:

$$\mathbf{y}_{mixture} = \mathbf{a}_{mixture} + \mathbf{b}_{mixture} * \mathbf{x}$$

$$\mathbf{y}_{silage} = \mathbf{a}_{silage} + \mathbf{b}_{silage} * \mathbf{x}$$

y: the standardized precaecal digestibility of the selected AA in the silage or raw mixture
x: the contribution level of selected AA from wheat to the assay diet
a: the standardized precaecal digestibility of selected AA in fed silage or raw mixture
b: the standardized precaecal digestibility of selected AA in fed wheat

Test results with P-values less than 0.05 are considered to be significant.

A General Linear Model was used to evaluate the effect of ensiling on the apparent digestibility of selected nutrients. P-values less than 0.05 were considered to be significant.

5 Results

5.1 Silages of remoistened Jack bean and cowpea grains sole or mixed with sorghum

5.1.1 Chemical analysis of the plant material

The chemical composition of raw grains is shown in Table 11. As expected, JBN and CWP are characterized by a high CP and ash content compared to SOR. The CF in JBN was found to be remarkably higher compared to CWP. This has to be taken into consideration when used in rations for pigs.

Table 11: Selected chemical parameters of Jack bean, cowpea and sorghum grains

Grain	DM	CP	AEE	CF	NDF_{OM}	ADF_{OM}	ADL_{OM}	Ash
	($g\ kg^{-1}$)	($g\ kg^{-1}$DM)						
JBN	888	287	24.3	106	225	138	3.30	31.7
CWP	902	261	21.8	60.6	170	89.5	5.90	45.1
SOR	866	95.4	35.9	23.7	93.8	36.6	3.20	15.0

ADF_{OM}, acid detergent fiber; ADL_{OM}, acid detergent lignin; AEE, acid ether extract; CF, crude fiber; CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; NDF_{OM}, neutral detergent fiber; SOR, sorghum

Water soluble carbohydrates (WSC) were found in not noteworthy contents in the studied plant material (Table 12). The minimum of 2 % WSC in the fresh matter to expect a good ensilability (Honig & Pahlow, 1986) was only achieved in CWP. Furthermore, it can be assumed that the low WSC content in JBN (< 20 $g\ kg^{-1}$ DM) suggests the use of an additional source of WSC for ensiling. The use of SOR will not contribute to increase WSC content in mixed silages as believed. In SOR, the lowest WSC quantities (2.0 $g\ kg^{-1}$ DM) were found. Although its buffering capacity (BC) was lowest compared to the legumes, SOR had a WSC/BC ratio of only 0.1.

Table 12: Content of water soluble carbohydrates and buffering capacity, their ratio and the content of starch of Jack bean, cowpea and sorghum grains

Grain	WSC ($g\ kg^{-1}$ DM)	BC ($g\ LA \cdot kg^{-1}$ DM)	WSC/BC	Starch ($g\ kg^{-1}$ DM)
JBN	18.0	90.0	0.2	359
CWP	23.0	63.0	0.4	387
SOR	2.0	31.0	0.1	739

BC, buffering capacity; CWP, cowpea; DM, dry matter; JBN, Jack bean; LA, lactic acid; SOR, sorghum; WSC, water soluble carbohydrates

In the legume grains notable quantities of starch were determined. In SOR, like in most of the cereals, this nutrient was found approximately the two-fold time (739 g kg^{-1} DM) compared to JBN and CWP.

5.1.2 Rostock Fermentation Test using Jack bean and cowpea sole or mixed with sorghum

In RFT of cowpea, inoculation with LAB had a distinct impact on the decrease of pH (Figure 8 and Table A1 in the appendix). After 14 h, inoculated treatments were different ($P<0.05$) to treatments without inoculant, but not among them ($P>0.05$) until the last measurement (38 h). By addition of molasses a fast decrease of pH was not achieved. In inoculated treatments, the proportion in which SOR was mixed did not have any influence. After 38 h the control showed the highest pH (5.50) and no differences were observed among the not inoculated treatments ($P>0.05$).

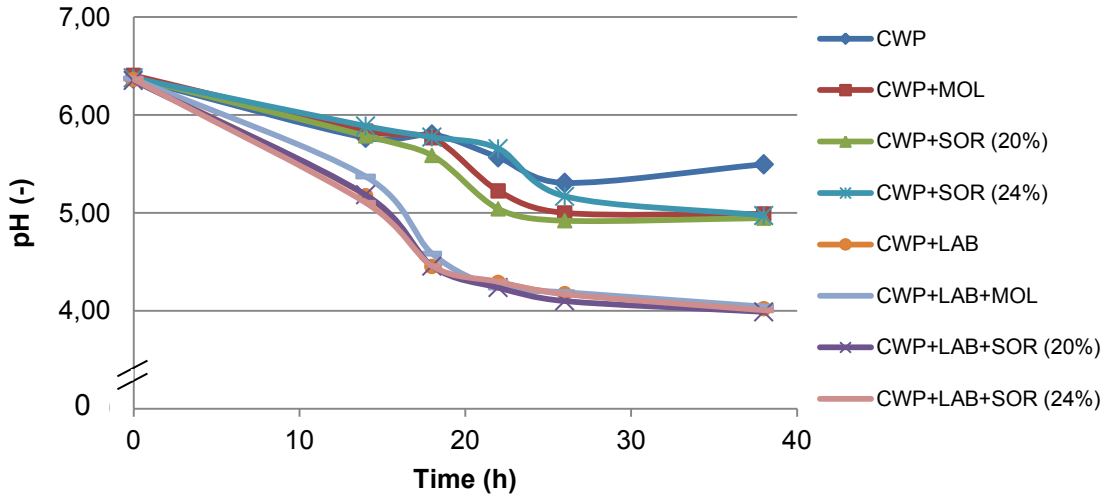


Figure 8: Change of pH during Rostock Fermentation Test for cowpea grains sole or mixed with sorghum (n=3)

CWP, cowpea; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

The analysis of RFT filtrates after 38 h revealed the inoculated treatments as those with the highest ($P<0.05$) lactic acid and lowest volatile fatty acids production compared to the not inoculated variants (Table 13). By contrast, production of ethanol and NH_3 was more pronounced in treatments without inoculation. An additional source of fermentable carbohydrates did not seem to be compulsory to initiate lactic acid fermentation, though was the addition of LAB.

Table 13: Fermentation parameters of different treatments with cowpea in filtrates of RFT after 38 h incubation (n=3)

	Lactic acid	Acetic acid	Butyric acid	Ethanol	NH_3
			(% DM)		
1	0.00^{a} ±0.00	1.76^{f} ±0.00	1.64^{c} ±0.16	1.53^{f} ±0.19	0.30^{f} ±0.03
2	2.40^{c} ±0.41	1.41^{e} ±0.11	1.20^{b} ±0.30	0.99^{e} ±0.00	0.24^{e} ±0.01
3	0.22^{a} ±0.12	1.04^{c} ±0.01	1.85^{c} ±0.16	0.52^{c} ±0.06	0.14^{c} ±0.01
4	1.95^{b} ±0.22	1.21^{d} ±0.03	0.93^{b} ±0.26	0.74^{d} ±0.04	0.18^{d} ±0.00
5	6.83^{f} ±0.04	0.67^{a} ±0.00	0.00^{a} ±0.00	0.16^{a} ±0.00	0.07^{a} ±0.00
6	6.85^{f} ±0.14	0.64^{a} ±0.05	0.00^{a} ±0.00	0.15^{a} ±0.00	0.08^{a} ±0.01
7	4.34^{d} ±0.04	0.62^{a} ±0.01	0.00^{a} ±0.00	0.30^{b} ±0.01	0.11^{b} ±0.00
8	4.75^{e} ±0.13	0.81^{b} ±0.05	0.00^{a} ±0.00	0.34^{b} ±0.03	0.14^{c} ±0.00

^{a,b} Mean values with different superscripts in the same column differ significantly ($P<0.05$).
Treatments: 1, CWP; 2, CWP+MOL; 3, CWP+SOR (20% CP); 4, CWP+SOR (24% CP); 5, CWP+LAB; 6, CWP+MOL+LAB; 7, CWP+SOR+LAB (20% CP); 8, CWP+SOR+LAB (24% CP)
CP, crude protein; CWP, cowpea; LAB, lactic acid bacteria ($3 \cdot 10^5$ cfu·g^{-1}); MOL, molasses (4 %); SOR, sorghum

Contrary to cowpea, the effect of an inoculant on the reduction of pH was not remarkable in Jack bean (Figure 9 and Table A2 in the appendix). Only in the case of JBN+LAB+SOR a faster ($P<0.05$) pH drop at 14 and 18 h was determined in contrast to the rest. At the other measurement times no differences ($P>0.05$) were observed among treatments until the end of RFT (38 h). Molasses had no effect on the reduction of pH. Why the expected influence of LAB and molasses on pH reduction was not observed for JBN deserves further discussion.

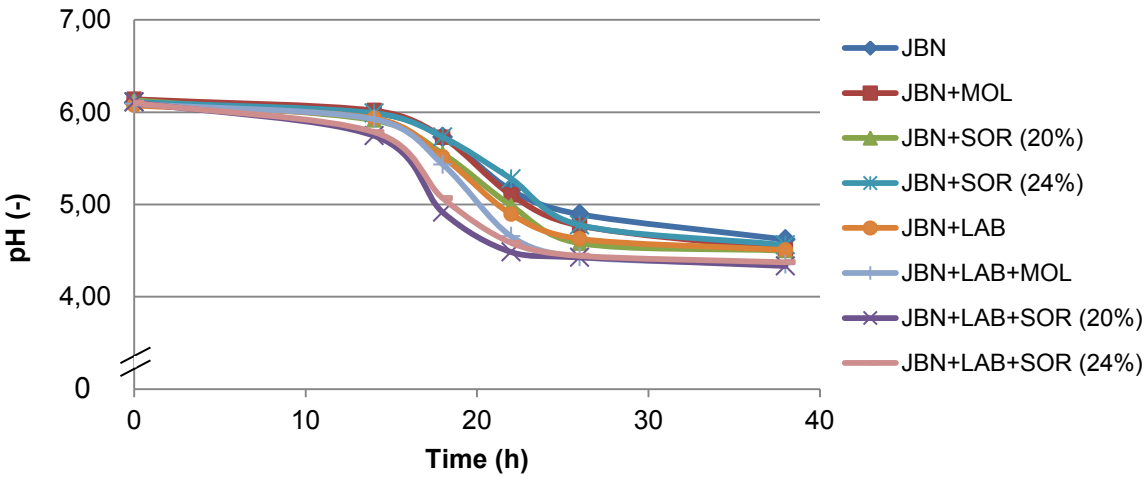


Figure 9: Change of pH during Rostock Fermentation Test for Jack bean grains sole or mixed with sorghum (n=3)

JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

The lactic acid content in the analyzed filtrates corresponded to the pH curves, as no outstanding differences were observed among treatments. However, the lactic acid production was significantly high when LAB was used. The highest content was measured in JBN+LAB and JBN+MOL+LAB with 3.71 and 4.63 % DM, respectively (Table 14). Although no LAB was added in JBN+MOL, this treatment produced as much lactic acid as JBN+LAB+SOR (24 %) with 2.80 and 2.86 % DM, respectively. The production of unfavorable fermentation parameters like acetic acid, butyric acid, ethanol and NH_3 was generally marginal.

Table 14: Fermentation parameters of different treatments with Jack bean in filtrates of RFT after 38 h incubation (n=3)

	Lactic acid	Acetic acid	Butyric acid	Ethanol	NH_3
			(% DM)		
1	2.26^{a} ±0.24	1.14^{bc} ±0.26	0.05 ±0.04	0.34^{b} ±0.04	0.11^{e} ±0.01
2	2.80^{c} ±0.00	1.28^{c} ±0.06	0.01 ±0.03	0.36^{b} ±0.04	0.18^{f} ±0.08
3	2.23^{a} ±0.02	0.83^{b} ±0.03	0.05 ±0.01	0.19^{a} ±0.03	0.07^{b} ±0.01
4	2.36^{ab} ±0.09	0.90^{b} ±0.05	0.03 ±0.01	0.25^{ab} ±0.01	0.08^{c} ±0.00
5	3.71^{d} ±0.06	0.93^{b} ±0.09	ND	0.18^{a} ±0.07	0.09^{d} ±0.00
6	4.63^{e} ±0.07	0.45^{a} ±0.36	0.01 ±0.00	0.15^{a} ±0.00	0.09^{d} ±0.00
7	2.52^{b} ±0.01	0.84^{b} ±0.00	ND	0.19^{a} ±0.02	0.06^{a} ±0.00
8	2.86^{c} ±0.01	1.01^{bc} ±0.00	ND	0.22^{a} ±0.00	0.07^{b} ±0.00

[a,b] Mean values with different superscripts in the same column differ significantly ($P<0.05$).
Treatments: 1, JBN; 2, JBN+MOL; 3, JBN+SOR (20% CP); 4, JBN+SOR (24% CP); 5, JBN+LAB; 6, JBN+MOL+LAB; 7, JBN+SOR+LAB (20% CP); 8, JBN+SOR+LAB (24% CP);
CP, crude protein; JBN, Jack bean; LAB, lactic acid bacteria ($3 \cdot 10^5$ cfu$\cdot$g^{-1}); MOL, molasses (4 %), ND, not detected; SOR, sorghum

Results of RFT suggested that LAB should be used for an adequate fermentation in silages using JBN and CWP. The high DM of the seeds surely limits the growth of the epiphytic flora that commonly grows in other fresh plant materials with higher moisture. Mixing with SOR reduced lactic acid production but did not interfere with pH reduction, which could be attributed to the lower BC of SOR (Table 12). No appreciable differences were observed due to the different SOR proportions.

5.1.3 Model silages with remoistened grains

During the storage period, DM content in JBN silages was slightly reduced with increasing storage time (P<0.05) but the differences were only marginal among treatments (Table 15). The pH value was remarkably reduced (P<0.05) in treatments inoculated with LAB. In general, JBN silages did not show considerable fermentation losses during storage. However, losses increased (P<0.05) with storage time (0.8, 1.3 and 2.4 % at days 5, 20 and 60, respectively). In general, silages without additive (JBN and JBN+SOR) showed the highest pH values and fermentation losses. Outstanding was the case of JBN+SOR with 3.5 % of losses compared to other treatments.

Table 15: Influence of storage time and treatment on dry matter, pH and fermentation losses of ensiled remoistened Jack bean grains sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	**pH** (-)	**Losses**[1] (%)
Storage time (d)			
5	652^{a}	4.99^{a}	0.8^{a}
20	647^{b}	4.68^{b}	1.3^{b}
60	644^{c}	4.56^{c}	2.4^{c}
Ensiling treatment			
JBN	649^{c}	5.54^{a}	1.5^{c}
JBN+MOL	653^{b}	5.29^{b}	0.9^{a}
JBN+LAB	641^{f}	4.46^{d}	1.3^{b}
JBN+MOL+LAB	644^{e}	4.38^{e}	1.2^{b}
JBN+SOR	642^{ef}	5.29^{b}	3.5^{d}
JBN+SOR+MOL	657^{a}	4.61^{c}	1.3^{b}
JBN+SOR+LAB	647^{d}	4.20^{f}	1.3^{b}
JBN+SOR+MOL+LAB	651^{bc}	4.19^{f}	1.0^{a}
pooled SD	±2.51	±0.032	±0.19
P-values			
Storage time (ST)	<0.001	<0.001	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	<0.001	<0.001	0.009

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] Weight losses in respect to the initial weight.
DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

There was a similar tendency in the results observed in silages of remoistened CWP (Table 16). Like in JBN, the addition of LAB resulted in lower pH values after 5, 20

and 60 days of storage compared to treatments without inoculant (P<0.05). However, the lowest pH was achieved when SOR was mixed with CWP (CWP+SOR+LAB and CWP+SOR+MOL+LAB with 4.18 each, P<0.05). The use of additives reduced fermentation losses, so that CWP and CWP+SOR showed the highest losses with 2.2 and 3.4 %, respectively (P<0.05).

Table 16: Influence of storage time and treatment on dry matter, pH and fermentation losses of ensiled remoistened cowpea grains sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	**pH** (-)	**Losses**[1] (%)
Storage time (d)			
5	647^{a}	5.1^{a}	0.9^{a}
20	643^{b}	4.8^{b}	1.4^{b}
60	647^{a}	4.7^{c}	2.5^{c}
Ensiling treatment			
CWP	642^{b}	5.7^{b}	2.4^{b}
CWP+MOL	652^{e}	5.8^{a}	1.0^{de}
CWP+LAB	645^{c}	4.3^{e}	1.0^{de}
CWP+MOL+LAB	644^{c}	4.3^{e}	1.0^{de}
CWP+SOR	638^{a}	5.6^{c}	3.4^{a}
CWP+SOR+MOL	652^{e}	4.9^{d}	1.9^{c}
CWP+SOR+LAB	645^{c}	4.2^{f}	1.1^{d}
CWP+SOR+MOL+LAB	648^{d}	4.2^{f}	0.9^{e}
pooled SD	±2.14	±0.032	±0.17
P-values			
Storage time (ST)	<0.001	<0.001	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	0.002	<0.001	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] Weight losses in respect to the initial weight.
CWP, cowpea; DM, dry matter; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

As shown in Table 17, the content of fermentation products in the filtrates of JBN silages after 60 days of storage confirm the necessity of LAB to achieve a good fermentation quality. The highest levels of lactic acid were found in JBN+MOL+LAB, JBN+LAB and JBN+SOR+MOL+LAB with 5.2, 4.6 and 4.1 % DM, respectively. Butyric, propionic, valeric and iso-valeric acids were found only in traces (< 1 % DM).

Table 17: Fermentation products in model silages of remoistened Jack bean grains after 60 days of storage (n=3)

	DM	pH	LA	AC	Ethanol	Butanediol	NH_3-N/N_{total}
	(%)	(-)	(% DM)				(%)
1	64.2 ±0.08	5.02^{ab} ±0.08	2.1^{a} ±0.05	0.5^{ab} ±0.02	0.8^{a} ±0.07	ND	1.5^{b} ±0.09
2	65.0 ±0.10	4.81^{a} ±0.02	2.5^{ab} ±0.17	0.4^{b} ±0.01	0.2^{c} ±0.02	ND	1.4^{ab} ±0.04
3	64.3 ±0.06	4.44^{c} ±0.03	4.6^{c} ±0.12	0.8^{c} ±0.04	0.2^{c} ±0.01	ND	1.2^{a} ±0.21
4	64.3 ±0.07	4.36^{cd} ±0.05	5.2^{c} ±0.22	0.7^{ac} ±0.05	0.2^{c} ±0.03	ND	1.2^{a} ±0.15
5	63.2 ±0.40	4.96^{b} ±0.02	1.0^{d} ±0.05	0.3^{b} ±0.04	1.1^{a} ±0.02	2.7^{b} ±0.50	3.7^{d} ±0.10
6	64.9 ±0.07	4.49^{c} ±0.00	3.1^{b} ±0.01	0.6^{acd} ±0.01	0.4^{b} ±0.02	0.3^{ab} ±0.02	4.2^{e} ±0.24
7	64.2 ±0.25	4.21^{d} ±0.02	3.6^{e} ±0.01	0.5^{b} ±0.02	0.3^{bc} ±0.06	0.2^{a} ±0.02	1.8^{c} ±0.22
8	64.8 ±0.16	4.19^{d} ±0.02	4.1^{c} ±0.06	0.5^{bd} ±0.03	0.2^{c} ±0.01	0.0^{a} ±0.01	1.5^{b} ±0.01

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, JBN; 2, JBN+MOL; 3, JBN+LAB; 4, JBN+MOL+LAB; 5, JBN+SOR; 6, JBN+SOR+MOL; 7, JBN+SOR+LAB; 8, JBN+SOR+MOL+LAB;
AC, acetic acid; DM, dry matter; JBN, Jack bean; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses; ND, not detected

In JBN+SOR considerable amounts of ethanol (1.1 % DM) and butanediol (2.7 % DM) were analyzed, whereas in other treatments contents were much lower (< 1% DM). The production of N-NH_3/N_{total} was only marginal, except in JBN+SOR and JBN+SOR+MOL with 3.7 and 4.2 % N-NH_3/N_{total}, respectively (P<0.05, Table 17).

In CWP silages the use of LAB increased (P<0.05) lactic acid as expected (Table 18), whereas in CWP+LAB and CWP+MOL+LAB the highest amounts were produced with 4.9 and 5.8 % DM, respectively. The content of acetic acid was generally low (< 0.5 % DM) and butyric acid was detected only in traces in CWP+MOL, CWP+SOR+MOL and CWP. Propionic, valeric and iso-valeric acids were not detected.

The production of alcohols was negligible throughout. However, the lowest contents were found in treatments with addition of LAB. Like JBN+SOR, CWP+SOR showed the highest production of ethanol and butanediol (1.3 and 2.5 % DM, respectively). The use of LAB reduced the formation of N-NH_3/N_{total}, whereas there was no impact (P>0.05) if MOL or SOR were included (Table 18). The treatment CWP+SOR showed the highest proportion of NH_3-N of the total N (4.5 %).

Table 18: Fermentation products in model silages of remoistened cowpea grains after 60 days of storage (n=3)

	DM	pH	LA	AC	Ethanol	Butanediol	NH_3-N/N_{total}
	(%)	(-)	(% DM)				(%)
1	64.0 ±0.40	5.28^{a} ±0.05	1.8^{abe} ±0.02	0.2^{a} ±0.04	0.6^{ab} ±0.18	0.7^{a} ±0.08	3.2^{a} ±0.07
2	65.2 ±0.32	5.27^{a} ±0.03	1.7^{b} ±0.09	0.3^{a} ±0.03	0.5^{a} ±0.15	0.1^{b} ±0.03	2.3^{b} ±0.06
3	64.9 ±0.03	4.28^{bd} ±0.02	4.9^{c} ±0.08	0.4^{b} ±0.03	0.2^{a} ±0.00	0.0^{b} ±0.00	1.0^{cd} ±0.03
4	64.6 ±0.30	4.31^{b} ±0.01	5.8^{d} ±0.17	0.5^{ab} ±0.07	0.2^{a} ±0.03	0.0^{b} ±0.00	1.0^{d} ±0.07
5	63.8 ±0.25	5.26^{a} ±0.05	1.4^{ab} ±0.11	0.2^{a} ±0.04	1.3^{b} ±0.14	2.5^{c} ±0.27	4.5^{e} ±0.20
6	65.1±0.39	4.67^{c} ±0.02	2.8^{e} ±0.19	0.3^{a} ±0.03	0.4^{a} ±0.10	0.9^{a} ±0.12	3.9^{ae} ±0.19
7	64.8 ±0.44	4.19^{d} ±0.00	3.6^{f} ±0.16	0.4^{ab} ±0.04	0.3^{a} ±0.03	0.1^{b} ±0.02	1.7^{c} ±0.13
8	65.2 ±0.04	4.18^{d} ±0.01	3.7^{f} ±0.19	0.3^{ab} ±0.03	0.2^{a} ±0.02	0.0^{b} ±0.01	1.4^{cd} ±0.12

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, CWP; 2, CWP+MOL; 3, CWP+LAB; 4, CWP+MOL+LAB; 5, CWP+SOR; 6, CWP+SOR+MOL; 7, CWP+SOR+LAB; 8, CWP+SOR+MOL+LAB
AC, acetic acid; CWP, cowpea; DM, dry matter; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses

5.1.4 Anti-nutritional factors in silages of remoistened grains

For the determination of selected ANF only the four treatments with the best fermentation results were selected (LEG+LAB, LEG+MOL+LAB, LEG+SOR+LAB and LEG+SOR+MOL+LAB), as in those treatments a reduction of ANF was expected.

5.1.4.1 Trypsin inhibitory activity (TIA)

After 60 days of storage, TIA in JBN silages was reduced (P<0.05) compared to the raw (not ensiled) plant material (Table 19). However, a slight increase was observed from day 5 to 60 (except JBN+MOL+LAB), suggesting that the highest reduction takes place during the first 5 days of ensiling. In JBN+LAB and JBN+MOL+LAB after 5 days a reduction of 44 and 29 %, respectively, was observed. Between day 5 and 60 TIA increased by 4 % in JBN+LAB but not in JBN+MOL+LAB, where a subsequent reduction of 17 % was observed. In silages mixed with SOR, the highest reduction of TIA likewise took place in the first 5 storage days (25 % for JBN+SOR+LAB and 29 % for JBN+SOR+MOL+LAB). An increase of approximately 11 % was detected between day 5 and 60 in both treatments (Table 19).

Table 19: Trypsin inhibitory activity (mg TI·g^{-1} DM) in silages of remoistened Jack bean and cowpea sole or mixed with sorghum (n=3)

Grain	Storage Time*	Treatments			
	(d)	3	4	7	8
JBN	0[1]	59.73^{a} ±0.52	59.73^{a} ±0.52	25.98^{a} ±0.39	25.98^{a} ±0.39
	5	33.51^{b} ±0.38	42.65^{b} ±0.55	19.53^{b} ±0.38	18.55^{b} ±0.96
	20	35.95^{c} ±0.08	34.32^{c} ±1.21	22.49^{c} ±0.58	21.88^{c} ±1.07
	60	35.06^{c} ±0.99	35.27^{c} ±0.60	22.03^{c} ±0.49	20.78^{c} ±1.37
CWP	0[1]	140.55^{a} ±0.30	140.55^{a} ±0.30	71.98^{a} ±0.16	71.98^{a} ±0.16
	5	79.06^{b} ±0.66	75.19^{b} ±0.71	36.61^{b} ±1.16	36.05^{b} ±0.74
	20	47.76^{c} ±0.39	47.43^{c} ±0.76	18.17^{c} ±0.63	17.21^{c} ±0.20
	60	32.35^{d} ±0.19	35.79^{d} ±1.39	15.36^{d} ±0.67	14.16^{d} ±0.15

^{a,b} Mean values with different superscripts in the same column differ significantly for each grain (P<0.05).
[1] Raw legume (JBN or CWP) for treatments 3 and 4, raw legume and sorghum mixture to reach 18 % CP in the DM for treatments 7 and 8.
Treatments: 3, LEG+LAB; 4, LEG+MOL+LAB; 7, LEG+SOR+LAB; 8, LEG+SOR+MOL+LAB
CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum; TI, trypsin inhibited
*Storage times set in correspondence to silage phases.

A marked reduction (P<0.05) in TIA was observed in silages with CWP (Table 19), whereas in CWP+LAB and CWP+MOL+LAB the reduction reached 77 and 75 %, respectively. A reduction of 79 and 80 % was detected in CWP+SOR+LAB and CWP+SOR+MOL+LAB, respectively. Contrary to JBN silages, the progressive increase of the storage time corresponds to a decreasing TIA.

5.1.4.2 Hydrogen cyanide (HCN)

In contrast to the expected effect of ensilage, HCN increased from day 0 to day 60 of storage in all treatments under evaluation in both legumes (Table 20) except in JBN+LAB and JBN+MOL+LAB, where a reduction of 17 and 25 %, respectively, was achieved (P<0.05).

Table 20: Hydrogen cyanide (mg·100 g^{-1} DM) in silages of remoistened Jack bean or cowpea sole or mixed with sorghum (n=3)

Grain	Storage time (d)	Treatments			
		3	4	7	8
JBN	0[1]	80.48^{a} ±0.28	80.48^{a} ±0.28	39.58^{a} ±0.20	39.58^{a} ±0.20
	60	66.85^{b} ±0.76	60.51^{b} ±0.56	48.95^{b} ±0.65	47.74^{b} ±0.54
CWP	0[1]	8.57^{a} ±0.18	8.57^{a} ±0.18	7.47^{a} ±0.02	7.47^{a} ±0.02
	60	25.46^{b} ±0.37	26.87^{b} ±0.94	33.03^{b} ±0.60	32.16^{b} ±0.58

^{a,b} Mean values with different superscripts in the same column differ significantly for each grain (P<0.05).
[1] Raw legume (JBN or CWP) for treatments 3 and 4, raw legume and SOR mixture to reach 18 % crude protein in DM for treatments 7 and 8.
Treatments: 3, LEG+LAB; 4, LEG+MOL+LAB; 7, LEG+SOR+LAB; 8, LEG+SOR+MOL+LAB
CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

5.1.4.3 Canavanine

Increasing the storage time contributed to a reduction of canavanine in silages compared to the not ensiled plant material (P<0.05). The lower content of canavanine at day 0 in JBN+SOR+LAB and JBN+SOR+MOL+LAB compared to the other two treatments was due to the absence of this ANF in SOR. After 60 days of ensilage, a reduction of 9 and 22 % was achieved in JBN+LAB and JBN+MOL+LAB, respectively. In the treatments JBN+SOR+LAB and JBN+SOR+MOL+LAB canavanine was reduced by 23 and 25 %, respectively (Table 21).

Table 21: Canavanine (g·100 g^{-1} DM) in silages of remoistened Jack bean sole or mixed with sorghum (n=3)

Storage time	Treatments			
(d)	3	4	7	8
0[1]	3.91^{a} ±0.04	3.91^{a} ±0.04	1.87^{a} ±0.02	1.87^{a} ±0.02
5	3.97^{a} ±0.04	3.65^{b} ±0.04	1.55^{b} ±0.04	1.49^{c} ±0.02
20	3.83^{b} ±0.04	3.48^{c} ±0.03	1.52^{b} ±0.02	1.54^{b} ±0.00
60	3.55^{c} ±0.01	3.07^{d} ±0.04	1.45^{c} ±0.01	1.41^{d} ±0.02

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
[1] Raw JBN for treatments 3 and 4, raw JBN and sorghum mixture to reach 18 % CP in DM for treatments 7 and 8.
Treatments: 3, JBN+LAB; 4, JBN+MOL+LAB; 7, JBN+SOR+LAB; 8, JBN+SOR+MOL+LAB
CP, crude protein; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

5.2 Silages of soaked Jack bean and cowpea grains sole or mixed with sorghum

Although it was proved that selected silage treatments of remoistened grains of JBN and CWP have the potential for a good fermentation quality, it was assumed that those results could still be improved. For that reason, soaking was tested as a pre-ensiling treatment. The main focus was on the possibility to reduce the buffering capacity or to increase water soluble carbohydrates, which would have a direct influence on the ensilability and hence on the fermentation quality. Furthermore, an effect on the reduction of the assessed ANF was expected.

5.2.1 Soaking

In JBN (Table 22), soaking time influenced ($P<0.001$) the BC of the grains, whereas BC decreased with increasing soaking time.

Table 22: Effect of soaking time and grain:water ratio (w/v) on buffering capacity, dry matter, crude protein and ash in Jack bean grains (n=3)

	BC (g LA·kg^{-1} DM)	**DM** (g kg^{-1})	**CP** (g kg^{-1} DM)	**Ash** (g kg^{-1} DM)
Soaking time (h)				
0	84.9^{a}	872^{a}	274	33.0^{a}
18	59.0^{b}	386^{b}	294	31.2^{b}
24	58.2^{c}	379^{c}	293	33.1^{a}
30	57.7^{d}	376^{d}	294	31.0^{b}
Ratio (grain:water [w/v])				
0[1]	84.9^{a}	872^{a}	274^{a}	33.1^{a}
1:2	61.6^{b}	385^{b}	290^{b}	32.2^{b}
1:3	60.3^{c}	380^{c}	297^{b}	31.7^{bc}
1:4	54.8^{d}	378^{cd}	297^{b}	31.2^{c}
1:5	56.4^{e}	377^{d}	291^{b}	33.3^{a}
pooled SD	±0.316	±2.28	±0.623	±0.84
P-values				
Soaking time	<0.001	<0.001	0.819	<0.001
Ratio	<0.001	<0.001	0.023	<0.001
Soaking time*Ratio	<0.001	0.814	0.335	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor ($P<0.05$).
[1] Not soaked Jack bean grains.
BC, buffering capacity; CP, crude protein; DM, dry matter; LA, lactic acid

There was as well an effect of the grain:water ratio (P<0.001) on the BC, as BC was reduced gradually from 0 to 1:4, although it slightly increased again for the ratio 1:5. At all soaking times DM was drastically reduced. Presumably, the water passing through the testa was trapped in the interstitial tissue and cells increased moisture, as there was an obvious increase of the seeds' volume.

The CP was increased after soaking (P<0.05), independent of the soaking time and grain:water ratio. In the case of ash there was a contrary effect. Likewise BC, the longer the time of soaking, the more the content of total ash was reduced with the exception that after 24 h a small increase (P<0.05) was observed compared to 18 h. The highest reduction (32 %) occurred after 30 h. The grain:water ratio as well caused changes in the content of ash. The more water was added the lower was the ash content in the seeds, although this was observed only until a grain:water ratio of 1:4. At the ratio of 1:5 ash increased (33.3 g kg^{-1} DM) to the same values determined in the raw material (33.0 g kg^{-1} DM).

The influence of soaking in CWP followed a similar tendency like in JBN (Table 23).

Table 23: Effect of soaking time and grain:water ratio (w/v) on buffering capacity, dry matter, crude protein and ash in cowpea (n=3)

	BC (g LA·kg^{-1} DM)	**DM** (g kg^{-1})	**CP** (g kg^{-1} DM)	**Ash** (% DM)
Soaking time (h)				
0	61.2[a]	890[a]	243	38.2[a]
18	34.7[b]	411[b]	255	27.1[b]
24	33.2[c]	415[c]	256	25.2[c]
30	32.7[d]	416[c]	255	23.4[d]
Ratio (grain:water [w/v])				
0[1]	61.2[a]	890[a]	243[a]	38.2[a]
1:2	38.9[b]	416[b]	257[b]	29.1[b]
1:3	32.8[c]	417[b]	254[c]	24.2[c]
1:4	31.7[d]	411[c]	254[c]	24.4[c]
1:5	30.5[e]	411[c]	255[c]	22.0[d]
pooled SD	±0.316	±2.98	±1.55	±0.84
P-values				
Soaking time	<0.001	<0.001	0.164	<0.001
Ratio	<0.001	<0.001	<0.001	<0.001
Soaking time*Ratio	<0.001	0.492	0.444	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] Not soaked cowpea grains.
BC, buffering capacity; DM, dry matter; CP, crude protein; LA, lactic acid

With increasing soaking time of CWP the DM content decreased (P<0.001), whereas CP increased (P>0.05). Likewise, BC and the content of ash were reduced (P<0.001) with increasing soaking time and grain:water ratio. From 0 to 30 h soaking, BC and ash were reduced from 61.2 to 32.7 g LA·kg^{-1} DM and 38.2 to 23.4 g kg^{-1} DM, respectively. With increasing grain:water ratio (from 0 to 1:5), BC and ash were decreased (P<0.001) from 61.2 to 30.5 g LA·kg^{-1} DM and 38.2 to 22.0 g kg^{-1} DM, respectively.

It was feared that soaking would have a negative effect on the content of key minerals. Indeed, this pre-ensiling treatment affected most of the selected minerals in soaked grains compared to the unprocessed plant material (Table 24).

Table 24: Content of selected minerals in raw and soaked beans after different soaking treatments (n=4)

	Ca	P	Na	K	Cl	Mg
			(g·kg^{-1} DM)			
JBN (raw, n=1)	1.70	4.65	0.93	12.26	0.64	1.51
JBN (1:4/24h)[1]	1.63 ±0.01	4.27 ±0.07	1.01 ±0.01	11.04[a] ±0.09	0.76[a] ±0.06	1.44[a] ±0.09
JBN (1:4/30h)[1]	1.67 ±0.07	4.25 ±0.29	1.00 ±0.06	10.43[b] ±0.25	0.65[b] ±0.05	1.41[b] ±0.03
CWP (raw, n=1)	0.93	5.76	1.00	13.90	0.32	2.26
CWP (1:4/24h)[1]	0.76 ±0.01	4.06 ±0.18	0.98[a] ±0.02	7.14[a] ±0.06	0.12 ±0.00	1.40[a] ±0.02
CWP (1:5/30h)[1]	0.75 ±0.02	3.88 ±0.29	0.92[b] ±0.05	5.73[b] ±0.15	0.12 ±0.00	1.31[b] ±0.06

[a,b] Mean values with different superscripts in the same column differ significantly within each grain (P<0.05).
[1]grain:water ratio (w/v) and soaking time (h)
CWP, cowpea; JBN, Jack bean
Soaking treatments were selected in accordance to their impact on the buffering capacity reduction.

5.2.2 Model silages with soaked legume grains

In accordance with the results obtained in the soaking trial (see 5.2.1), JBN and CWP grains were soaked for 24 h at a grain:water ratio of 1:4, although a higher reduction of the BC was achieved at 30 h and a ratio of 1:5. However, the conditions were selected following practical reasons and with respect to a future on-farm application, mainly in order to save time and water.

Selected chemical characteristics of JBN and CWP after soaking in tap water and their mixtures with SOR are presented in Table 25.

Table 25: Selected chemical characteristics of soaked (24 h at 1:4 [w/v]) Jack bean and cowpea grains sole or mixed with sorghum used for model silages

Grains	DM (g kg^{-1})	CP (g kg^{-1} DM)	BC (g LA·kg^{-1} DM)
JBN	426.0	280.5	53.8
CWP	445.6	253.5	41.5
JBN+SOR[1]	592.8	178.3	34.0
CWP+SOR[1]	585.3	175.7	28.0

[1] Mixtures were prepared from soaked beans and not soaked SOR to reach 180 g kg^{-1} CP in DM.
BC, buffering capacity; CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; LA, lactic acid; SOR, sorghum

The contamination of the grains with harvest residues, which could not be removed completely due to the high amounts needed for the experiment and the quality of the water (tap water) used compared to the deionized water used in the soaking trials could be among the reasons why BC differs from the soaking experiment (see 5.2.1).

The DM of soaked JBN silages was not affected by the storage time and the differences observed among treatments were mainly associated to the use of SOR in mixed silages, which increased DM (Table 26).

Table 26: Influence of storage time and treatment on dry matter, pH and fermentation losses of ensiled soaked Jack bean grains sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	pH (-)	Losses[1] (%)
Storage time (d)			
5	480	4.40^{a}	0.6^{a}
20	480	4.41^{a}	1.3^{b}
60	481	4.61^{b}	2.7^{c}
Ensiling treatment			
JBN	403^{b}	5.04^{a}	1.8^{b}
JBN+MOL	415^{c}	4.37^{d}	1.6^{c}
JBN+LAB	398^{a}	4.95^{b}	2.0^{a}
JBN+MOL+LAB	413^{c}	4.22^{e}	1.6^{c}
JBN+SOR	575^{d}	4.54^{c}	1.4^{d}
JBN+SOR+LAB	575^{d}	4.09^{f}	1.3^{e}
JBN+SOR+MOL+LAB	583^{e}	4.11^{f}	1.1^{f}
pooled SD	±5.64	±0.032	±0.19
P-values			
Storage time (ST)	0.873	<0.001	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	0.014	<0.001	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
1 Weight losses in respect to the initial weight.
DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

There was a slight tendency to increase pH in the last 40 days of storage, whereas the treatments with the lowest pH corresponded to JBN+MOL, JBN+MOL+LAB, JBN+SOR+LAB, JBN+SOR+MOL+LAB. Losses increased with storage time and were lowest in mixed silages. Together with the control (JBN), the treatment JBN+LAB produced the silages with the lowest quality in all the evaluated variables.

There were only subtle variations of the DM during the storage time of soaked CWP silages, but the inclusion of SOR in mixed silages contributed to an increase of the DM content compared to those silages, where it was not included (Table 27). The pH value decreased gradually with the storage time ($P<0.001$) in all treatments, whereas on the contrary fermentation losses increased to 2.8 % FM at day 60 ($P<0.001$). Treatments inoculated with LAB showed the lowest pH values. However, CWP+LAB made an exception, which together with the control (CWP) was the treatment with the lowest silage quality (highest pH values and fermentation losses). Silages mixed with SOR, independent of the treatment, had the lowest fermentation losses.

Table 27: Influence of storage time and treatment on dry matter, pH and fermentation losses of ensiled soaked cowpea grains sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	**pH** (-)	**Losses**[1] (%)
Storage time (d)			
5	494^{a}	4.26^{a}	0.4^{a}
20	494^{a}	4.21^{b}	1.1^{b}
60	497^{b}	4.16^{c}	2.8^{c}
Ensiling treatment			
CWP	447^{b}	4.48^{a}	2.1^{a}
CWP+MOL	448^{b}	4.20^{d}	1.7^{b}
CWP+LAB	438^{a}	4.36^{b}	1.8^{ab}
CWP+MOL+LAB	446^{b}	4.05^{e}	1.7^{b}
CWP+SOR	561^{cd}	4.26^{c}	1.0^{c}
CWP+SOR+LAB	559^{c}	4.07^{e}	0.9^{c}
CWP+SOR+MOL+LAB	564^{d}	4.06^{e}	0.9^{c}
pooled SD	±3.62	±0.071	±0.42
P-values			
Storage time (ST)	<0.001	<0.001	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	<0.001	<0.001	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor ($P<0.05$).
[1] Weight losses in respect to the initial weight.
CWP, cowpea; DM, dry matter; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

Analysis of the fermentation quality of soaked JBN silages (Table 28) showed, that the lowest pH values for silages without SOR in the mix were achieved in the treatments JBN+MOL (4.36) and JBN+MOL+LAB (4.23). A pH value above 5 was found in JBN+LAB (5.40), which was similar to the control (JBN, 5.51). In silages mixed with SOR, the lowest pH values were determined in JBN+SOR+LAB (4.08) and JBN+SOR+MOL+LAB (4.10).

Table 28: Fermentation products determined in model silages of soaked jack bean grains after 60 days of storage (n=3)

	DM	pH	LA	AC	BA	Ethanol	Propanol
	(%)	(-)			(% DM)		
1	40.1^{a} ±0.57	5.51^{c} ±0.39	0.7^{a} ±1.46	1.1^{b} ±0.62	2.2^{b} ±1.50	0.7^{d} ±0.02	0.04^{d} ±0.01
2	41.8^{b} ±0.14	4.36^{b} ±0.03	5.7^{d} ±0.14	0.7^{a} ±0.05	0.0^{a} ±0.02	0.7^{d} ±0.08	0.00^{a} ±0.00
3	39.4^{a} ±0.09	5.40^{c} ±0.09	0.1^{a} ±0.09	0.9^{ab} ±0.26	2.9^{b} ±0.31	0.7^{cd} ±0.05	0.07^{e} ±0.01
4	42.1^{b} ±0.11	4.23^{ab} ±0.01	6.6^{e} ±0.13	0.8^{ab} ±0.04	0.0^{a} ±0.01	0.6^{bc} ±0.03	0.03^{c} ±0.00
5	57.2^{c} ±0.42	4.45^{b} ±0.05	2.6^{b} ±0.25	0.9^{ab} ±0.07	0.0^{a} ±0.00	0.8^{e} ±0.02	0.03^{c} ±0.00
6	57.6^{cd} ±0.33	4.08^{a} ±0.01	3.9^{c} ±0.15	0.5^{a} ±0.03	0.0^{a} ±0.00	0.6^{b} ±0.03	0.02^{c} ±0.00
7	58.0^{d} ±1.00	4.10^{a} ±0.00	4.2^{c} ±0.06	0.6^{a} ±0.07	0.0^{a} ±0.00	0.3^{a} ±0.03	0.01^{b} ±0.00

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, JBN; 2, JBN+MOL; 3, JBN+LAB; 4, JBN+MOL+LAB; 5, JBN+SOR; 6, JBN+SOR+LAB; 7, JBN+SOR+MOL+LAB
AC, acetic acid; BA, butyric acid; DM, dry matter; JBN, Jack bean; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

The highest lactic acid production was generally associated with the treatments with the lowest pH values. Interestingly, JBN+MOL and JBN+MOL+LAB showed the highest lactic acid production among all treatments with 5.7 and 6.6 % DM, respectively, although in JBN+SOR+LAB and JBN+SOR+MOL+LAB with only 3.91 and 4.2 % lactic acid in the DM, respectively, the lowest pH values were determined among all treatments (4.08 and 4.10, respectively). This finding could be attributed to the differences in the increase of DM and BC reduction by the addition of SOR.

The production of volatile fatty acids (VFA) and alcohols was meager for most of the treatments. However, apart from the control treatment (JBN), JBN+LAB was characterized by a low fermentation quality due to its high content of butyric acid (2.9 % DM). Butanediol, propionic, valeric and iso-valeric acids were not detected in any treatment.

Fermentative parameters of soaked CWP silages are presented in Table 29. Like in the case of JBN, the combination of CWP with MOL or MOL+LAB showed the best fermentation characteristics among the treatments not mixed with SOR. Special emphasize has to be given for their high lactic acid production of 6.3 % DM (CWP+MOL) and 6.2 % DM (CWP+MOL+LAB) and low pH (4.06 and 4.04, respectively). In both cases, the production of alcohol and VFA was negligible. Among the silages mixed with SOR, CWP+SOR+LAB and CWP+SOR+MOL+LAB produced the highest contents of lactic acid (4.0 and 4.4 % DM, respectively) and achieved the lowest pH values (4.06 and 4.05, respectively). Alcohols and VFA were generally low. Butanediol, butyric, propionic, iso-valeric and valeric acids were not detected in any of the treatments.

Table 29: Fermentation products determined in model silages of soaked cowpea grains after 60 days of storage (n=3)

	DM (%)	pH (-)	LA (% DM)	AC (% DM)	Ethanol (% DM)	Propanol (% DM)
1	45.3^{c} ±0.40	4.48^{d} ±0.04	4.5^{c} ±0.21	0.6^{d} ±0.08	0.3^{b} ±0.02	0.02 ±0.00
2	45.0^{bc} ±0.50	4.06^{a} ±0.01	6.3^{d} ±0.27	0.5^{c} ±0.04	0.4^{d} ±0.03	0.02 ±0.00
3	44.2^{a} ±0.51	4.34^{c} ±0.04	4.1^{b} ±0.07	0.5^{c} ±0.04	0.3^{b} ±0.02	0.02 ±0.00
4	44.7^{ab} ±0.25	4.04^{a} ±0.01	6.2^{d} ±0.19	0.5^{c} ±0.02	0.4^{d} ±0.02	0.02 ±0.00
5	56.2^{d} ±0.13	4.09^{b} ±0.01	3.9^{a} ±0.12	0.4^{ab} ±0.02	0.3^{c} ±0.02	0.02 ±0.00
6	56.0^{d} ±0.08	4.06^{a} ±0.01	4.0^{ab} ±0.10	0.3^{a} ±0.01	0.2^{a} ±0.01	0.02 ±0.00
7	56.5^{d} ±0.09	4.05^{a} ±0.00	4.4^{c} ±0.06	0.4^{b} ±0.02	0.2^{a} ±0.01	0.02 ±0.00

[a,b] Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, CWP; 2, CWP+MOL; 3, CWP+LAB; 4, CWP+MOL+LAB; 5, CWP+SOR; 6, CWP+SOR+LAB; 7, CWP+SOR+MOL+LAB
AC, acetic acid; CWP, cowpea; DM, dry matter; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

As shown in Table 30, the proportions of NH_3-N/N_{total} (%) of JBN+MOL (2.8 %), JBN+MOL+LAB (2.1 %), JBN+SOR+LAB (2.1 %), JBN+SOR+MOL+LAB (2.1 %), CWP+MOL (4.6 %), CWP+MOL+LAB (3.4 %), CWP+SOR+LAB (4.4 %) and CWP+SOR+MOL+LAB (3.8 %) confirm them as the treatments with the best fermentation quality in accordance to the results presented in Table 28 and Table 29.

Table 30: Ammonia production (% NH_3-N/N_{total}) in model silages with soaked grains after 60 days of storage (n=3)

Grain	Treatments						
	1	2	3	4	5	6	7
JBN	7.7^{e} ±0.14	2.8^{b} ±0.19	7.1^{d} ±0.20	2.1^{a} ±0.11	4.0^{c} ±0.40	2.1^{a} ±0.10	2.1^{a} ±0.03
CWP	6.7^{e} ±0.36	4.6^{c} ±0.13	5.8^{d} ±0.13	3.4^{a} ±0.08	5.8^{d} ±0.24	4.4^{c} ±0.10	3.8^{b} ±0.06

[a,b] Mean values with different superscripts in the same row differ significantly (P<0.05).
Treatments: 1, LEG; 2, LEG+MOL; 3, LEG+LAB; 4, LEG+MOL+LAB; 5, LEG+SOR; 6, LEG+SOR+LAB; 7, LEG+SOR+MOL+LAB
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum

5.2.3 Anti-nutritional factors in silages of soaked legume grains

5.2.3.1 Trypsin inhibitory activity (TIA)

The combination of soaking and ensiling did not produce the expected effect on TIA in silage treatments of JBN. In the case of JBN+MOL and JBN+MOL+LAB the TIA was significantly reduced during soaking by 22 %. However, an increase was found afterwards during ensiling. At day 60, in both treatments TIA was increased by approximately 30 % compared to the raw grains. Soaking followed by ensiling increased as well TIA in JBN+SOR+LAB and JBN+SOR+MOL+LAB (Table 31).

Table 31: Trypsin inhibitory activity (mg TI·g^{-1} DM) in four silage treatments of soaked Jack bean and cowpea sole or mixed with sorghum during three storage periods (n=3)

Grain	Storage time	Treatments			
	(d)	2	4	6	7
JBN	Raw[1]	35.35^{a} ±0.28	35.35^{a} ±0.28	19.93^{a} ±0.00	19.93^{a} ±0.00
	Soaked[2]	27.44^{b} ±0.08	27.44^{b} ±0.08	23.16^{b} ±0.77	23.16^{b} ±0.77
	5	31.09^{c} ±0.59	39.01^{c} ±0.69	21.35^{c} ±0.50	22.94^{b} ±0.53
	20	32.97^{d} ±0.94	32.61^{d} ±1.23	25.48^{d} ±0.43	25.07^{c} ±0.10
	60	45.81^{e} ±0.57	41.58^{e} ±0.78	28.08^{e} ±0.47	23.08^{b} ±0.47
CWP	Raw[1]	58.16^{a} ±0.28	58.16^{a} ±0.28	35.89^{a} ±0.00	35.89^{a} ±0.00
	Soaked[2]	42.27^{b} ±0.53	42.27^{b} ±0.53	31.90^{b} ±0.19	31.90^{b} ±0.19
	5	42.55^{b} ±1.74	60.77^{c} ±0.30	33.03^{c} ±0.48	29.68^{c} ±1.10
	20	37.41^{c} ±1.30	39.99^{b} ±0.80	21.71^{d} ±0.34	22.85^{d} ±1.10
	60	28.63^{d} ±1.26	29.91^{d} ±1.38	23.14^{e} ±0.22	23.16^{d} ±0.31

[a,b] Mean values with different superscripts in the same column differ significantly within each grain (P<0.05).
[1] Raw LEG for treatments 2 and 4, raw LEG and sorghum mixture to reach 18 % CP in the DM for treatments 6 and 7.
[2] Soaked LEG for 24 h and 1:4 grain:water ratio for treatments 2 and 4, soaked LEG for 24 h and 1:4 grain:water ratio mixed with SOR to reach 18 % CP in DM for treatments 6 and 7.
Treatments: 2, LEG+MOL; 4, LEG +MOL+LAB; 6, LEG+SOR+LAB; 7, LEG+SOR+MOL+LAB
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum; TI, trypsin inhibited

The effect of soaking in combination with ensiling produced a reduction of TIA by approximately 50 % in the treatments CWP+MOL and CWP+MOL+LAB after 60 days of storage time. However, the increase of TIA (approx. 5 %) in CWP+MOL+LAB after 5 days of ensilage compared to raw CWP was unexpected. In CWP+SOR+LAB and CWP+SOR+MOL+LAB TIA was reduced by 35 % after 60 days of storage. Thereby, there was no significant TIA reduction in CWP+SOR+MOL+LAB between days 20 and 60 (Table 31).

5.2.3.2 Hydrogen cyanide (HCN)

Ensiling of soaked JBN had a significant ($P<0.05$) effect on the HCN content (Table 32). In every studied treatment soaking had a negative effect on this parameter. Although the content of HCN increased during soaking ensiling produced a reduction after the first 5 days of storage with the highest reduction in JBN+MOL (23 %) and JBN+MOL+LAB (27 %). Between day 5 and 60 of ensilage differences were only small.

Table 32: Hydrogen cyanide (mg·100 g^{-1} DM) in four silage treatments of soaked Jack bean and cowpea sole or mixed with sorghum during three storage periods (n=3)

Grain	Storage time	Treatments			
	(d)	2	4	6	7
JBN	Raw[1]	104.24^{a} ±0.80	104.24^{a} ±0.80	56.82^{a} ±0.00	56.82^{a} ±0.00
	Soaked[2]	109.07^{b} ±2.78	109.07^{b} ±2.78	77.95^{b} ±0.11	77.95^{b} ±0.11
	5	80.21^{c} ±1.84	76.65^{c} ±1.13	64.94^{c} ±2.47	76.34^{b} ±2.06
	20	75.06^{d} ±1.46	87.86^{d} ±1.22	63.16^{c} ±1.13	83.48^{c} ±1.24
	60	76.76^{d} ±1.59	77.13^{c} ±0.89	62.29^{c} ±2.30	60.94^{d} ±3.22
CWP	Raw[1]	34.02^{a} ±1.29	34.02^{a} ±1.29	24.72^{a} ±0.00	24.72^{a} ±0.00
	Soaked[2]	41.31^{b} ±1.05	41.31^{b} ±1.05	36.08^{b} ±0.13	36.08^{b} ±0.13
	5	37.63^{c} ±2.57	38.11^{c} ±0.39	32.66^{c} ±0.69	25.55^{a} ±0.64
	20	38.63^{c} ±0.73	34.53^{d} ±1.63	32.33^{cd} ±2.03	23.98^{a} ±1.68
	60	37.08^{c} ±0.96	30.49^{e} ±1.25	30.55^{d} ±0.72	25.03^{a} ±0.82

^{a,b} Mean values with different superscripts in the same column differ significantly within each grain ($P<0.05$).
[1] Raw LEG for treatments 2 and 4, raw LEG and sorghum mixture to reach 18 % CP in the DM for treatments 6 and 7.
[2] Soaked LEG for 24 h and 1:4 grain:water ratio for treatments 2 and 4, soaked LEG for 24 h and 1:4 grain:water ratio mixed with SOR to reach 18 % CP in DM for treatments 6 and 7.
Treatments: 2, LEG+MOL; 4, LEG +MOL+LAB; 6, LEG+SOR+LAB; 7, LEG+SOR+MOL+LAB
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum

During 60 days of storage HCN in JBN+SOR+LAB and JBN+SOR+MOL+LAB was not reduced to lower levels than those found in the not ensiled material. Instead, an increase of approx. 10 % was produced after 60 days of storage, which could be attributed to the inclusion of SOR.

Like in the case of JBN, the combination of soaking and ensiling had a variable effect on HCN in CWP. Soaking increased HCN in every treatment and ensiling did not have any remarkable effect. Only in CWP+MOL+LAB a small but significant ($P<0.05$) reduction of approx. 10 % was produced after day 60 of storage (Table 32).

5.2.3.3 Canavanine

As presented in Table 33, a reduction of canavanine was observed after soaking and contents further decreased during ensiling in all treatments. After 60 days of storage, a significant reduction ($P<0.05$) of 17 % was produced in JBN+MOL and JBN+MOL+LAB and of 37 % in JBN+SOR+LAB, whereas the highest reduction was found in JBN+SOR+MOL+LAB (55 %).

Table 33: Canavanine (g·100 g^{-1} DM) in four silage treatments of soaked jack bean sole or mixed with sorghum during three storage periods (n=3)

Storage time	Treatments			
(d)	2	4	6	7
Raw[1]	3.38[a] ±0.03	3.38[a] ±0.03	1.66[a] ±0.01	1.66[a] ±0.01
Soaked[2]	3.24[b] ±0.01	3.24[b] ±0.01	1.54[b] ±0.04	1.54[b] ±0.04
5	2.88[c] ±0.05	3.01[c] ±0.01	1.36[c] ±0.06	1.21[c] ±0.04
20	2.92[c] ±0.06	2.90[d] ±0.03	1.21[d] ±0.05	1.05[d] ±0.06
60	2.82[c] ±0.10	2.82[d] ±0.05	1.04[e] ±0.04	0.75[e] ±0.08

[a,b] Mean values with different superscripts in the same column differ significantly ($P<0.05$).
[1] Raw JBN for treatments 2 and 4, raw JBN and sorghum mixture to reach 18 % CP in the DM for treatments 6 and 7.
[2] Soaked JBN for 24 h and 1:4 grain:water ratio for treatments 2 and 4, soaked JBN for 24 h and 1:4 grain:water ratio mixed with SOR to reach 18 % CP in DM for treatments 6 and 7.
Treatments: 2, JBN+MOL; 4, JBN +MOL+LAB; 6, JBN+SOR+LAB; 7, JBN+SOR+MOL+LAB
DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

Interestingly, in silages mixed with SOR a positive effect on the reduction of canavanine was observed, contrary to what was observed for HCN.

5.3 Silages of pre-germinated Jack bean and cowpea grains sole or mixed with sorghum

Considering that the content of water soluble carbohydrates (WSC) was found to be meager in both legume grains under evaluation, pre-germination was thought to improve ensilability by increasing WSC.

5.3.1 Pre-germination

The water application during the experiment and the moisture increase as a consequence of the germination itself caused significant changes in the DM in both legume grains. In general, DM was lower ($P<0.05$) by 1 % in JBN (Table 34) and 5 % in CWP (Table 35) when pre-germination took place in the darkness compared to the light regime. Furthermore, the DM was reduced ($P<0.001$) as the germination time was extended.

Table 34: Effect of illumination regime and germination time on dry matter, water soluble carbohydrates and crude protein in Jack bean (n=3)

	DM (g kg^{-1})	**WSC** (g kg^{-1} DM)	**CP** (g kg^{-1} DM)
Illumination regime			
Light	394^{b}	38.3^{b}	293
Darkness	390^{a}	37.0^{a}	292
Germination time (h)			
Raw	888	38.0	287
Soaked[1]	460	NA	290
12	454^{a}	16.4^{a}	289^{a}
24	406^{b}	42.2^{c}	289^{a}
48	380^{c}	38.1^{b}	290^{a}
72	367^{d}	43.4^{c}	295^{ab}
96	352^{e}	47.6^{d}	300^{b}
pooled SD	±5.56	±1.90	±3.94
P-values			
Illumination regime (IR)	0.029	0.022	0.581
Germination time (GT)	<0.001	<0.001	<0.001
IR*GT	0.080	0.529	0.349

^{a,b} Mean values with different superscripts in the same column differ significantly within each factor ($P<0.05$).
[1] According to the germination trial: after disinfection seeds were soaked for 9 h (see 4.3.3.2).
CP, crude protein; DM, dry matter; NA, not analyzed; WSC, water soluble carbohydrates

In JBN (Table 34), the WSC content was reduced by 57 % ($P<0.05$) after 12 h of germination compared to the raw (not treated) grain. In the following, after 96 h a gradual increase by up to 25 % (compared to the raw grain) was observed. Under light regime of germination the WSC production was slightly higher ($P<0.05$) than under darkness, whereas the illumination regime did not affect the CP content ($P>0.05$). Only a slight increase was detected comparing the CP content of the raw grain (287 g kg^{-1} DM) with the contents after 72 h (295 g kg^{-1} DM) and 96 h (300 g kg^{-1} DM).

After 12 h of germination, no decrease of WSC was found in CWP as was in JBN (Table 35). On the contrary, a gradual increase was produced reaching the highest value after 72 h (54.4 g kg^{-1} DM). Unlike JBN, the content of WSC was higher ($P<0.05$) during germination under darkness (40.4 g kg^{-1} DM) than under light (38.3 g kg^{-1} DM).

The CP content was not affected by the illumination regime ($P>0.05$) and only a marginal increase ($P<0.05$) was observed after 48 h.

Table 35: Effect of illumination regime and germination time on dry matter, water soluble carbohydrates and crude protein in cowpea (n=3)

	DM (g kg^{-1})	**WSC** (g kg^{-1} DM)	**CP** (g kg^{-1} DM)
Illumination regime			
Light	389[b]	38.3[a]	270
Darkness	372[a]	40.4[b]	271
Germination time (h)			
Raw	902	12.8	261
Soaked[1]	423	NA	262
12	419[a]	13.3[a]	262[a]
24	392[b]	44.4[b]	267[b]
48	353[c]	44.7[b]	274[c]
72	358[c]	54.4[c]	278[d]
pooled SD	±16.2	±1.73	±1.26
P-values			
Illumination regime (IR)	0.018	0.044	0.887
Germination time (GT)	<0.001	<0.001	<0.001
IR*GT	0.498	0.062	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor ($P<0.05$).
[1] According to the germination trial: after disinfection seeds were soaked for 9 h (see 4.3.4.2).
CP, crude protein; CWP, cowpea; DM, dry matter; NA, not analyzed; WSC, water soluble carbohydrates

5.3.2 Model silages with pre-germinated legume grains

As shown in silages with soaked legume grains (see 5.2.2), the inclusion of SOR increased DM of pre-germinated legume grain silages compared to those ones, where only legume grains were ensiled (Table 36). The addition of LAB contributed to a reduction of pH, especially in JBN+SOR+LAB (4.11) and JBN+SOR+MOL+LAB (4.08), where the lowest pH values were found. On the contrary, the control treatment (JBN, 4.85) and JBN+LAB (4.71) showed the highest pH value. All treatments had losses of 2 % of FM and below, whereas mixing with SOR contributed to a reduction of losses. Furthermore, losses were correlated with the storage time (P<0.001).

Table 36: Influence of storage time and treatment on dry matter, pH and losses of ensiled pre-germinated Jack bean sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	**pH** (-)	**Losses**[1] (%)
Storage time (d)			
5	519^{a}	4.38^{a}	0.7^{a}
20	515^{b}	4.44^{b}	1.4^{b}
60	515^{b}	4.36^{a}	2.8^{c}
Ensiling treatment			
JBN	432^{a}	4.85^{g}	2.0^{e}
JBN+MOL	449^{c}	4.32^{d}	2.0^{e}
JBN+LAB	433^{a}	4.71^{f}	2.1^{e}
JBN+MOL+LAB	443^{b}	4.18^{c}	1.8^{d}
JBN+SOR	592^{e}	4.44^{e}	1.5^{c}
JBN+SOR+MOL	598^{f}	4.46^{e}	1.3^{b}
JBN+SOR+LAB	586^{d}	4.11^{b}	1.4^{c}
JBN+SOR+MOL+LAB	597^{f}	4.08^{a}	1.0^{a}
pooled SD	±2.21	±0.032	±0.15
P-values			
Storage time (ST)	<0.001	<0.001	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	<0.001	<0.001	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] Weight losses in respect to the initial weight.
DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

Evaluating CWP model silages (Table 37), contrary to pre-soaked and remoistened silages a striking gas production and fungus contamination was observed. However,

in silages mixed with sorghum, those undesired processes were reduced compared to solely ensiled CWP.

The pH value remained unaltered from day 5 to 60 of storage time (P>0.05), whereas in the control and CWP+LAB the highest pH values were found with 4.61 and 5.24, respectively. Mixed CWP+SOR silages produced the lowest pH, as well as CWP+MOL and CWP+MOL+LAB. In CWP+LAB the highest percentage of losses was determined (4.2 % of FM).

Table 37: Influence of storage time and treatment on dry matter, pH and losses of ensiled pre-germinated cowpea sole or mixed with sorghum (n=3)

	DM (g kg^{-1})	**pH** (-)	**Losses**[1] (%)
Storage time (d)			
5	482^{a}	4.37	1.0^{a}
20	488^{b}	4.37	2.0^{b}
60	491^{b}	4.48	4.8^{c}
Ensiling treatment			
CWP	404^{b}	4.61^{a}	3.4^{b}
CWP+MOL	418^{c}	4.33^{c}	3.4^{b}
CWP+LAB	392^{a}	5.24^{b}	4.2^{a}
CWP+MOL+LAB	410^{c}	4.34^{c}	3.3^{b}
CWP+SOR	565^{e}	4.21^{c}	1.8^{c}
CWP+SOR+MOL	575^{f}	4.22^{c}	1.8^{c}
CWP+SOR+LAB	560^{e}	4.20^{c}	1.5^{c}
CWP+SOR+MOL+LAB	571^{f}	4.14^{c}	1.6^{c}
pooled SD	±5.41	±0.25	±0.40
P-values			
Storage time (ST)	<0.001	0.189	<0.001
Ensiling treatment (ET)	<0.001	<0.001	<0.001
ST*ET	0.635	0.121	<0.001

[a,b] Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] Weight losses in respect to the initial weight.
CWP, cowpea; DM, dry matter; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

The determination of fermentation products in the filtrates of pre-germinated JBN model silages (Table 38) revealed JBN+MOL, JBN+MOL+LAB, JBN+SOR+LAB and JBN+SOR+MOL+LAB as the treatments with the best fermentation quality. They showed the lowest pH values (4.32, 4.18, 4.08 and 4.06, respectively), the highest

lactic acid production (6.5, 7.5, 4.8 and 4.7 % DM, respectively) and the lowest acetic acid production (1.7, 1.1, 0.6 and 0.6 % DM, respectively). The content of alcohols was generally below 1 % DM and butyric, propionic, valeric and iso-valeric acids were not detected in any treatment.

Table 38: Fermentation products determined in model silages of pre-germinated Jack bean grains after 60 days of storage (n=3)

	DM (%)	pH (-)	LA (% DM)	AC (% DM)	Ethanol (% DM)	Butanediol (% DM)
1	43.0^{a} ±0.24	4.86^{e} ±0.13	4.5^{b} ±0.18	2.5^{d} ±0.40	0.8^{f} ±0.02	0.07^{a} ±0.02
2	45.2^{b} ±0.03	4.32^{c} ±0.03	6.5^{c} ±0.02	1.7^{c} ±0.06	0.5^{d} ±0.06	0.15^{b} ±0.05
3	43.5^{c} ±0.10	4.66^{d} ±0.01	4.7^{b} ±0.11	1.9^{c} ±0.04	0.7^{e} ±0.03	0.19^{bc} ±0.04
4	44.5^{d} ±0.22	4.18^{b} ±0.01	7.5^{d} ±0.25	1.1^{b} ±0.07	0.4^{c} ±0.03	0.23^{c} ±0.06
5	58.8^{e} ±0.07	4.35^{c} ±0.01	3.4^{a} ±0.28	0.9^{b} ±0.05	0.5^{d} ±0.04	0.24^{c} ±0.02
6	59.2^{f} ±0.09	4.40^{c} ±0.01	3.5^{a} ±0.17	1.1^{b} ±0.02	0.3^{b} ±0.02	0.07^{a} ±0.01
7	58.5^{e} ±0.36	4.08^{a} ±0.04	4.8^{b} ±0.15	0.6^{a} ±0.02	0.4^{c} ±0.02	0.23^{c} ±0.02
8	59.4^{f} ±0.31	4.06^{a} ±0.01	4.7^{b} ±0.08	0.6^{a} ±0.01	0.1^{a} ±0.01	0.03^{a} ±0.01

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, JBN; 2, JBN+MOL; 3, JBN+LAB; 4, JBN+MOL+LAB; 5, JBN+SOR; 6, JBN+SOR+MOL; 7, JBN+SOR+LAB; 8, JBN+SOR+MOL+LAB
AC, acetic acid; DM, dry matter; JBN, Jack bean; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

In CWP model silages (Table 39), the lowest pH was measured in CWP+MOL and CWP+MOL+LAB, as well as in CWP+SOR+LAB and CWP+SOR+MOL+LAB. Among these treatments, the ones with solely ensiled CWP produced more lactic acid (6.7 and 6.7 % DM, respectively) than those ones mixed with SOR (4.6 and 5.0 % DM, respectively), although the latter showed the lowest pH values (4.15 in CWP+SOR+LAB and 4.13 in CWP+SOR+MOL+LAB). Butyric acid was not detected in any of the CWP+SOR mixed silages, but in those of sole CWP. Contents of acetic acid remained below 1 % DM in the CWP+SOR mixed silages, but more than double the amount was produced in solely CWP silages. Neither propionic nor valeric or iso-valeric acids were detected in any treatment. With the exception of the control (CWP) the content of ethanol was below 0.5 % DM in all treatments, which also applied for the content of butanediol, including the control treatment.

Table 39: Fermentation products determined in model silages of pre-germinated cowpea grains after 60 days of storage (n=3)

	DM (%)	pH (-)	LA	AC	BA	Ethanol	Butanediol
			(% DM)				
1	40.9^{a} ±0.41	4.71^{b} ±0.17	3.8^{a} ±1.15	2.2^{c} ±0.44	0.6 ±0.92	0.75^{c} ±0.02	0.31^{ab} ±0.04
2	42.1^{b} ±0.19	4.43^{ab} ±0.17	6.7^{b} ±0.39	1.5^{b} ±0.33	0.2 ±0.18	0.34^{ab} ±0.03	0.45^{cd} ±0.05
4	41.5^{ab} ±0.67	4.73^{b} ±0.52	6.7^{b} ±1.69	1.7^{bc} ±0.61	0.4 ±0.34	0.43^{b} ±0.20	0.54^{d} ±0.12
5	57.1^{cd} ±0.17	4.11^{a} ±0.01	5.3^{a} ±0.22	0.6^{a} ±0.02	ND	0.39^{b} ±0.01	0.29^{ab} ±0.03
6	58.0^{e} ±0.19	4.13^{a} ±0.01	5.1^{a} ±0.11	0.9^{a} ±0.08	ND	0.23^{a} ±0.01	0.24^{a} ±0.03
7	56.6^{c} ±0.27	4.15^{a} ±0.03	4.6^{a} ±0.15	0.5^{a} ±0.02	ND	0.36^{ab} ±0.02	0.36^{bc} ±0.02
8	57.6^{de} ±0.25	4.13^{a} ±0.01	5.0^{a} ±0.13	0.6^{a} ±0.04	ND	0.23^{a} ±0.00	0.21^{a} ±0.02

^{a,b} Mean values with different superscripts in the same column differ significantly (P<0.05).
Treatments: 1, CWP; 2, CWP+MOL; 4, CWP+MOL+LAB; 5, CWP+SOR; 6, CWP+SOR+MOL; 7, CWP+SOR+LAB; 8, CWP+SOR+MOL+LAB
Treatment 3 (CWP+LAB) was lost during sampling.
AC, acetic acid; BA, butyric acid; CWP, cowpea; DM, dry matter; LA, lactic acid; LAB, lactic acid bacteria; MOL, molasses; ND, not detected

According to the fermentation pattern of pre-germinated JBN silages (Table 38), JBN+MOL, JBN+MOL+LAB, JBN+SOR+LAB and JBN+SOR+MOL+LAB showed the best fermentation quality when evaluating ammonia with 2.2, 1.1, 1.6 and 1.1 % NH_3-N/N_{total}, respectively (Table 40). Like in JBN, the treatments CWP+MOL, CWP+MOL+LAB, CWP+SOR+LAB and CWP+SOR+MOL+LAB had the lowest ammonia production ($P<0.05$).

Table 40: Ammonia production (% NH_3-N/N_{total}) in model silages with pre-germinated beans after 60 days of storage (n=3)

Grain	Treatments							
	1	2	3	4	5	6	7	8
JBN	5.5^{a} ±0.40	2.2^{b} ±0.11	2.9^{c} ±0.11	1.1^{d} ±0.08	3.1^{c} ±0.06	2.8^{bc} ±0.01	1.6^{e} ±0.11	1.1^{de} ±0.03
CWP	6.2^{e} ±0.62	4.0^{bc} ±0.23	*	2.5^{a} ±0.07	3.7^{d} ±0.09	3.1^{c} ±0.03	3.0^{bc} ±0.07	2.6^{ab} ±0.01

^{a,b} Mean values with different superscripts in the same row differ significantly (P<0.05).
Treatments: 1, LEG; 2, LEG+MOL; 3, LEG+LAB; 4, LEG+MOL+LAB; 5, LEG+SOR; 6, LEG+SOR+MOL; 7, LEG+SOR+LAB; 8, LEG+SOR+MOL+LAB
* CWP+LAB was lost during sampling.
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum

5.3.3 Anti-nutritional factors

5.3.3.1 Effect of pre-germination on selected anti-nutritional factors in not ensiled legume grains

Due to controversial results found in literature about the effect of pre-germination on TIA and canavanine the influence of this pre-ensiling treatment should be investigated.

Only in CWP there was an influence of the illumination regime on TIA. Grains pre-germinated under light regime showed a lower TIA (60.44 mg TI·g^{-1} DM) than those germinated in darkness (66.85 mg TI·g^{-1} DM). In both grains, an interaction between the illumination regime and pre-germination time was observed (Table 41).

Table 41: Effect of illumination regime and pre-germination time on trypsin inhibitory activity and canavanine in Jack bean and cowpea (n=3)

	Jack bean		**Cowpea**
	TIA (mg TI·g^{-1} DM)	Canavanine (g·100 g^{-1} DM)	TIA (mg TI·g^{-1} DM)
Illumination regime			
Light	41.43	3.54	60.44^{a}
Darkness	41.07	3.56	66.85^{b}
Pre-germination time (h)			
Raw grain	59.39	3.91	140.55
Soaked[1]	57.73	ND	86.23
12	24.53^{a}	3.35^{a}	39.29^{a}
24	37.93^{b}	3.62^{b}	64.40^{b}
48	47.57^{c}	3.64^{b}	74.00^{c}
72	47.98^{c}	3.58^{b}	76.88^{d}
96	48.23^{c}	3.57^{b}	-
pooled SD	±0.809	±0.095	±1.021
P-values			
Illumination regime (IR)	0.228	0.702	<0.001
Pre-germination time (GT)	<0.001	<0.001	<0.001
IR*GT	0.007	0.119	<0.001

^{a,b} Mean values with different superscripts in the same column differ significantly within each factor (P<0.05).
[1] According to the germination trial: after disinfection seeds were soaked for 9 h (see 4.3.4.2).
DM, dry matter; ND, not determined; TIA, trypsin inhibitory activity; TI, trypsin inhibited

The methodically necessary soaking proceeding previous to pre-germination apparently did not have an effect on TIA in JBN but led to a reduction of 39 % in pre-germinated CWP compared to the raw grain. Interestingly, already after 12 h of

germination the lowest TIA was detected with a reduction of 59 % and 72 % for JBN and CWP, respectively, compared to the raw grain. Thereafter, TIA increased gradually as the germination time increased until the end of the experiment, resulting in a total reduction of 19 % and 45 % after 96 h (JBN) and 72 h (CWP), respectively.
In JBN, the highest reduction of canavanine, likewise TIA, was produced after 12 h. Until the end of the trial canavanine increased again slightly, showing a total reduction of 9 % after 96 h compared to the raw grain. Neither an influence of the illumination regime nor an interaction between the factors illumination regime and pre-germination time was observed.

5.3.3.2 Effect of pre-germination on selected anti-nutritional factors in ensiled legume grains

5.3.3.2.1 Trypsin inhibitory activity (TIA)

In all treatments of both legume grains the highest TIA reduction was found after 5 days of storage (Table 42) with approx. 30 % in JBN+MOL and JBN+MOL+LAB. After 20 and 60 days of storage, a slight increase was produced. However, contents never exceeded the levels of the raw JBN.

Table 42: Trypsin inhibitory activity (mg TI·g^{-1} DM) in four silage treatments of pre-germinated Jack bean and cowpea grains sole or mixed with sorghum during three storage periods (n=3)

Grains	Storage time	Treatments			
	(d)	2	4	7	8
JBN	0[1]	59.73^{a} ±0.52	59.73^{a} ±0.52	25.98^{a} ±0.39	25.98^{a} ±0.39
	5	41.75^{b} ±0.66	41.66^{b} ±0.28	20.60^{b} ±0.37	20.70^{c} ±0.15
	20	46.66^{c} ±0.43	43.85^{c} ±1.02	24.24^{c} ±0.41	23.38^{b} ±0.78
	60	46.39^{c} ±0.92	43.56^{c} ±1.24	27.61^{d} ±0.73	25.33^{a} ±0.36
CWP	0[1]	140.55^{a} ±0.30	140.55^{a} ±0.30	71.98^{a} ±0.16	71.98^{a} ±0.16
	5	66.42^{b} ±0.82	70.93^{b} ±0.28	28.97^{b} ±0.43	28.11^{b} ±0.74
	20	42.16^{c} ±0.11	48.97^{c} ±0.83	18.20^{c} ±0.99	16.22^{c} ±0.74
	60	31.96^{d} ±0.37	39.77^{d} ±0.82	18.21^{c} ±0.24	14.63^{d} ±0.21

^{a,b} Mean values with different superscripts in the same column differ significantly within each grain (P<0.05).
[1] Raw LEG for treatments 2 and 4, raw LEG and sorghum mixture to reach 18 % CP in the DM for treatments 7 and 8.
Treatments: 2, LEG+MOL; 4, LEG+MOL+LAB; 7, LEG+SOR+LAB; 8, LEG+SOR+MOL+LAB
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum

In pre-germinated CWP silages TIA was reduced in all treatments under evaluation and was directly influenced by the storage time. After 60 days of storage, the lowest TIA ($P<0.05$) was found in CWP+MOL, CWP+MOL+LAB, CWP+SOR+LAB and CWP+SOR+MOL+LAB with reductions of 77, 72, 73 and 80 %, respectively, compared to the raw cowpea.

5.3.3.2.2 Hydrogen cyanide (HCN)

A reduction of HCN was mainly detected in sole JBN treatments with a reduction of 30 and 60 % ($P<0.05$) in JBN+MOL and JBN+MOL+LAB, respectively (Table 43). In contrast, JBN+SOR+LAB showed a slight increase of HCN after 60 days of storage (42.43 mg·100 g^{-1} DM) with respect to the raw grain (39.58 mg·100 g^{-1} DM). In JBN+SOR+MOL+LAB HCN was reduced by approx. 5 % ($P<0.05$) after 60 days storage time. The inclusion of SOR in the silages might have had an influence on the different extents of reduction of HCN compared to solely ensiled JBN.

Table 43: Hydrogen cyanide (mg·100g^{-1} DM) in four silage treatments of pre-germinated Jack bean and cowpea grains sole or mixed with sorghum (n=3)

Grain	Storage time	Treatments			
	(d)	2	4	7	8
JBN	0[1]	80.48^{a} ±0.28	80.48^{a} ±0.28	39.58^{a} ±0.20	39.58^{a} ±0.20
	60	56.63^{b} ±0.32	47.85^{b} ±0.28	42.43^{b} ±0.35	37.69^{b} ±0.13
CWP	0[1]	8.57^{a} ±0.18	8.57^{a} ±0.18	7.47^{a} ±0.02	7.47^{a} ±0.02
	60	15.98^{b} ±0.46	12.86^{b} ±0.34	21.91^{b} ±0.04	20.13^{b} ±0.44

^{a,b} Mean values with different superscripts in the same column differ significantly within each grain ($P<0.05$).
[1] Raw LEG for treatments 2 and 4, raw LEG and sorghum mixture to reach 18 % CP in the DM for treatments 7 and 8.
Treatments: 2, LEG+MOL; 4, LEG +MOL+LAB; 7, LEG+SOR+LAB; 8, LEG+SOR+MOL+LAB
CWP, cowpea; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; LEG, legume grain (CWP or JBN); MOL, molasses; SOR, sorghum

Analyzing CWP silages, no reduction of HCN was observed after 60 days of storage. On the contrary, an increase ($P<0.05$) of approximately 46, 33, 66 and 63 % was found in CWP+MOL, CWP+MOL+LAB, CWP+SOR+LAB and CWP+SOR+MOL+LAB, respectively (Table 43). However, as HCN was not

determined during the pre-germination trial, there is no certainty if the increase was really produced during silage. The addition of SOR in treatments of mixed CWP+SOR silages was associated to even higher HCN levels in the silages after 60 days of storage.

5.3.3.2.3 Canavanine

The content of canavanine was reduced in every evaluated treatment, whereas the highest reduction ($P<0.05$) compared to the raw grain was produced after 60 days in JBN+MOL with 24 % and JBN+MOL+LAB with 7 % of reduction (Table 44).

Table 44: Canavanine (g·100 g^{-1} DM) in four silage treatments of pre-germinated Jack bean grains sole or mixed with sorghum during three storage periods (n=3)

Storage time	Treatments			
(d)	2	4	7	8
0*	3.91^{a} ±0.04	3.91^{a} ±0.04	1.87^{a} ±0.02	1.87^{a} ±0.02
5	3.68^{b} ±0.03	3.77^{b} ±0.00	1.76^{c} ±0.02	1.58^{d} ±0.02
20	3.17^{c} ±0.03	3.71^{b} ±0.02	1.86^{b} ±0.03	1.65^{c} ±0.03
60	2.98^{d} ±0.05	3.63^{c} ±0.05	1.51^{d} ±0.00	1.78^{b} ±0.04

[a,b] Mean values with different superscripts in the same column differ significantly ($P<0.05$).
[1] Raw JBN for treatments 2 and 4, raw JBN and sorghum mixture to reach 18 % CP in the DM for treatments 7 and 8.
Treatments: 2, JBN+MOL; 4, JBN+MOL+LAB; 7, JBN+SOR+LAB; 8, JBN+SOR+MOL+LAB
CP, crude protein; DM, dry matter; JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum

In JBN+SOR+LAB after 60 days storage and in JBN+SOR+MOL+LAB after 5 days storage, the highest reduction of the canavanine content was produced with 19 % and 15 % difference, respectively, compared to the raw grain. In those treatments, no progressive influence of the storage time on the canavanine decrease was observed. Nevertheless, after every evaluated storage period, the content of canavanine was always lower ($P<0.05$) compared to the raw material.

5.4 Silages of Jack bean and cowpea mixed with sorghum as feedstuffs for growing pigs

5.4.1 Evaluation of feedstuffs used in the animal trials

In order to evaluate the nutritional value of legume grain silages mixed with sorghum as a feedstuff for growing pigs the best treatment of legume grain-sorghum silages in correspondence with the results shown in the chapters 5.1, 5.2 and 5.3 was chosen. Therefore, the addition of a lactic acid bacteria inoculant ($3 \cdot 10^5$ cfu·g^{-1} FM) and molasses (4 % FM) was favored. As a pre-ensiling treatment, a soaking of 24 h with a grain:water ratio of 1:4 (w/v) was selected prior to ensiling, not only considering its effect on ensilability and ANF reduction, but as well factors like the efficient use of water and storage space.

As expected, the different chemical composition of JBN and CWP affected the estimated metabolizable energy (ME), as ME was higher in CWP (14.88 MJ·kg^{-1} DM) than in JBN (13.39 MJ·kg^{-1} DM) (Table 45). However, the ME of the raw mixture used for ensiling was rather similar, being 15.52 MJ·kg^{-1} DM and 15.11 MJ·kg^{-1} DM in CWP+SOR and JBN+SOR, respectively (Table 46). Due to the higher CP content in JBN, more SOR was mixed to achieve 18 % CP in the mix, which increased ME as a consequence mainly because the high starch and low CF content in SOR.

Table 45: Chemical composition of feedstuffs under evaluation in the feeding trials

Grain	DM	OM	CP	AEE	CF	Starch	Ash	ME*
	(g kg^{-1})	(g kg^{-1} DM)						(MJ kg^{-1} DM)
JBN	888	963	287	27.4	106	378	37.1	13.39
CWP	902	955	261	24.2	58.7	456	45.1	14.88
Wheat	881	984	134	25.6	34.7	708	16.1	15.73
SOR	866	985	95.4	41.4	26.2	760	15.0	16.15

AEE, acid ether extract; CF, crude fiber; CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; OM, organic matter; SOR, sorghum
*Metabolizable energy estimated following GfE (2008) recommendations.

During the silage fermentation a drastic reduction of starch by 58 % and 50 % in JBN+SOR in CWP+SOR mixtures, respectively, was observed. As a consequence, the calculated ME was reduced in JBN+SOR and CWP+SOR silages to 14.61 and 14.90 MJ·kg^{-1} DM, respectively (Table 46).

Table 46: Chemical composition of ensiled and not ensiled legume-sorghum mixtures under evaluation in the feeding trials

		JBN+SOR		CWP+SOR	
		RMI	SIL	RMI	SIL
DM	(g kg^{-1})	607	596	882	540
OM		974	973	973	969
CP		182	182	174	177
AEE		31.5	32.5	28.8	28.2
CF		52.4	52.6	41.4	43.6
NDF_{OM}	(g kg^{-1} DM)	237	183	208	98.7
ADF_{OM}		97.1	91.7	78.6	66.4
ADL_{OM}		7.5	5.3	20.6	18.9
Starch		548	228	620	312.
Ash		26.4	27.0	27.0	30.6
ME*	(MJ·kg^{-1} DM)	15.11	14.61	15.52	14.90

ADF_{OM}, acid detergent fiber; ADL_{OM}, acid detergent lignin; AEE, acid ether extract; CF, crude fiber; CP, crude protein; CWP, cowpea; DM, dry matter; JBN, Jack bean; NDF_{OM}, neutral detergent fiber; OM, organic matter; RMI, raw mixture; SIL, silage; SOR, sorghum
*Metabolizable energy estimated following GfE (2008) recommendations.

It has to be emphasized that the NDF_{OM} content in CWP+SOR silage (SIL) was extremely reduced (by 52 %) compared to its corresponding raw mixture (RMI). Considering that only a small reduction of ADF_{OM} (15 %) and ADL_{OM} (8 %) was observed, obviously hemicelluloses (Hcell; NDF-ADF) were the most affected fraction, being degraded by more than 75 % in SIL compared to RMI in CWP+SOR mixtures. In JBN+SOR mixtures, ensiling reduced Hcell by 35 %.

The high quality of fermentation in silages can be evaluated by the parameters presented in Table 47. The lactic acid production was rather high and undesired fermentation products occurred in only little amounts. The pH value remained inside the wished range (≤ 4.0) at the silage opening.

Table 47: Selected fermentation parameters of legume-sorghum silages used in the feeding trials after 60 days storage (n=3)

Silage	pH	LA	AC	Ethanol	Bdiol	Propanol	NH_3-N/N_{total}
	(-)	(% DM)					(%)
JBN+SOR	4.0	4.9	0.4	0.3	0.1	0.02	1.0
CWP+SOR	4.0	5.7	0.4	0.5	0.2	0.02	2.0

AC, acetic acid; Bdiol, 2,3-butanediol; CWP, cowpea; LA, lactic acid; JBN, Jack bean; SOR, sorghum
Butyric, propionic, valeric and iso-valeric acids where not detected.

With regard to a future on-farm use of the legume-sorghum silages, the aerobic stability was as well of special interest to face the problem of storage. After determination of temperature rise, losses during aerobic storage and the pH value before and after aerobic storage in the aerobic stability test, remarkable differences between the legume grains were observed. While the temperature in JBN+SOR silage remained almost unaltered during the 7 days lasting assay, CWP+SOR silages started to be instable already after 66 h (Figure 10).

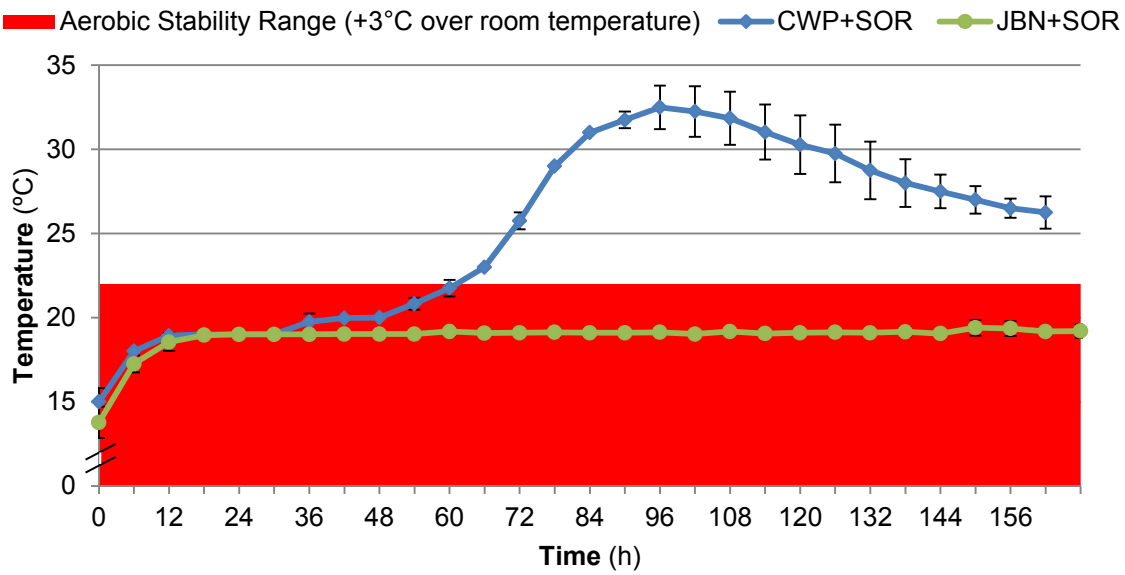


Figure 10: Temperature rise of Jack bean and cowpea silages mixed with sorghum used for feeding trials (n=4)

The evaluation of pH value and losses (Table 48) after aerobic exposure reflects the results of the temperature increase. Contrary to JBN+SOR silage, where the pH value remained unaltered (4.0), pH was increased from 4.0 to 7.4 at day 7 of aerobic exposure in CWP+SOR silages. This drastic change contributed to losses in the range of 2.6 % FM in CWP+SOR silage. The stable pH value in ensiled JBN+SOR could be the main reason, why only marginal losses (0.3 % FM) were detected in this silage.

Table 48: Changes in selected variables before and after the aerobic stability test in silages prepared for feeding trials

Silage	Moment of evaluation (with respect to aerobic stability test)	DM ($g\ kg^{-1}$)	pH (-)	Losses (% FM)
JBN+SOR	day 0	596	4.00	-
	day 7	596	4.00	0.3
CWP+SOR	day 0	540	4.01	-
	day 7	529	7.44	2.6

CWP, cowpea; DM, dry matter; FM, fresh matter; JBN, Jack bean; SOR, sorghum

The AA composition of grains used in the preparation of the diets for the feeding trials is shown in Table 49.

Table 49: Amino acid composition of not ensiled feedstuffs used in the feeding trials

Amino acids	Jack bean		Cowpea		Sorghum		Wheat	
	($g \cdot kg^{-1}$ DM)	($g \cdot 16\ g^{-1}$ N)	($g \cdot kg^{-1}$ DM)	($g \cdot 16\ g^{-1}$ N)	($g \cdot kg^{-1}$ DM)	($g \cdot 16\ g^{-1}$ N)	($g \cdot kg^{-1}$ DM)	($g \cdot 16\ g^{-1}$ N)
ASP	25.53	8.89	27.85	10.69	6.33	6.63	6.34	4.74
THR	10.56	3.68	9.48	3.64	3.10	3.25	3.71	2.77
SER	12.98	4.52	11.89	4.56	4.13	4.33	6.13	4.59
GLU	28.50	9.93	41.28	15.84	18.63	19.53	42.55	31.82
GLY	9.52	3.31	10.32	3.96	3.00	3.14	5.14	3.84
ALA	10.53	3.67	10.98	4.21	8.55	8.97	4.52	3.38
VAL	11.07	3.86	12.23	4.69	4.49	4.71	5.66	4.23
ILE	9.64	3.36	10.67	4.09	3.75	3.93	4.48	3.35
LEU	18.43	6.42	19.20	7.37	12.36	12.96	8.91	6.66
TYR	6.22	2.17	5.79	2.22	2.77	2.90	2.09	1.56
PHE	11.17	3.89	14.46	5.55	4.99	5.24	6.09	4.56
HIS	26.49	9.23	8.00	3.07	2.29	2.40	3.21	2.40
LYS	13.94	4.85	16.79	6.44	1.99	2.09	3.32	2.49
ARG	12.17	4.24	15.55	5.97	3.35	3.51	5.49	4.11
PRO	9.48	3.30	5.17	1.98	7.00	7.34	13.33	9.97
CYS	2.56	0.89	2.73	1.05	1.76	1.85	3.08	2.30
MET	3.00	1.04	3.74	1.44	1.67	1.75	1.98	1.48

Differences between JBN and CWP were observed mainly for glutamic acid with 41.28 $g \cdot kg^{-1}$ DM in CWP and 28.5 $g \cdot kg^{-1}$ DM in JBN. The content of histidine was more than three times higher in JBN (26.49 $g \cdot kg^{-1}$ DM) than in CWP (8.00 $g \cdot kg^{-1}$

DM). Lysine and phenylalanine were found to be higher in CWP (16.79 and 14.46 g·kg^{-1} DM, respectively) compared to JBN (13.94 and 11.17 g·kg^{-1} DM, respectively). Contents of cystine and methionine were rather similar in both legume grains.

Sorghum and wheat (basal feed ingredient) showed a similar AA composition, although subtle differences were found. In this regard glutamic acid has to be highlighted with more than double the amount in wheat (42.55 g·kg^{-1} DM) compared to SOR (18.63 g·kg^{-1} DM). Wheat was as well slightly higher in methionine (1.98 g·kg^{-1} DM and 1.67 g·kg^{-1} DM in wheat and SOR, respectively), in cystine (3.08 g·kg^{-1} DM and 1.76 g·kg^{-1} DM in wheat and SOR, respectively) and in lysine (3.32 g·kg^{-1} DM and 1.99 g·kg^{-1} DM in wheat and SOR, respectively). Furthermore, wheat was higher in other essential AA like arginine, histidine, phenylalanine, isoleucine, valine and threonine, although higher contents of leucine and tyrosine were found in SOR than in wheat.

In order to characterize raw and ensiled JBN+SOR and CWP+SOR, the AA composition of those mixtures was as well determined (Table 50).

Table 50: Amino acid composition of ensiled and not ensiled Jack bean-sorghum and cowpea-sorghum mixtures used in the feeding trials

	Raw mixture (CWP+SOR)		**Silage** (CWP+SOR)		**Raw mixture** (JBN+SOR)		**Silage** (JBN+SOR)	
	(g·kg^{-1} DM)	(g·16 g^{-1} N)	(g·kg^{-1} DM)	(g·16 g^{-1} N)	(g·kg^{-1} DM)	(g·16 g^{-1} N)	(g·kg^{-1} DM)	(g·16 g^{-1} N)
ASP	17.72	10.18	16.88	9.52	14.61	8.05	14.77	8.10
THR	6.62	3.80	6.35	3.58	6.44	3.55	6.50	3.56
SER	8.66	4.98	7.96	4.49	8.20	4.52	8.24	4.51
GLU	33.97	19.52	31.85	17.96	25.62	14.12	24.95	13.68
GLY	6.85	3.94	6.84	3.86	5.83	3.21	5.98	3.28
ALA	9.83	5.65	9.63	5.43	9.35	5.15	8.93	4.90
VAL	9.01	5.18	8.64	4.87	7.48	4.12	7.79	4.27
ILE	8.19	4.71	6.83	3.85	6.16	3.40	7.05	3.87
LEU	16.53	9.50	15.73	8.87	15.33	8.45	15.38	8.43
TYR	4.13	2.38	4.19	2.36	4.54	2.50	4.58	2.51
PHE	9.97	5.73	9.50	5.36	7.58	4.18	7.65	4.19
HIS	5.45	3.13	5.80	3.27	12.26	6.76	12.62	6.92
LYS	9.08	5.22	8.26	4.66	6.34	3.50	6.71	3.68
ARG	10.13	5.82	9.99	5.63	7.24	3.99	7.60	4.17
PRO	8.96	5.15	8.86	4.99	8.56	4.72	8.61	4.72
CYS	2.23	1.28	2.04	1.15	1.97	1.09	1.96	1.07
MET	2.76	1.58	2.54	1.43	2.22	1.22	2.14	1.17

Some changes were observed in the AA composition between raw mixtures and their corresponding silages. In CWP+SOR the losses due to ensiling in aspartic acid (4.7%), glutamic acid (6.2%), lysine (9.0%), glutamic acid (6.2%) and isoleucine (16.6%) contradict with what was observed between raw mixtures and silages of JBN+SOR.

As previously shown, ensiling had an effect on the reduction of TIA in both legume grain silages (Table 51).

Table 51: Content of selected anti-nutritional factors of ensiled or not ensiled legume-sorghum mixtures used in the feeding trials

Grain		TIA (mg TI·g^{-1} DM)	Canavanine (g·100 g^{-1} DM)	CT	TP (% DM)	NTP
JBN	RMI	25.98	1.58	0.33	0.98	0.62
	SIL	17.94	1.62	0.04	1.32	1.21
CWP	RMI	71.98	-	0.24	1.08	0.40
	SIL	31.08	-	0.15	1.50	0.99

CT, condensed tannins, CWP, cowpea; NTP, non-tannin phenols; RMI, raw mixture; SIL, silage; TP, tannin phenols; TIA, trypsin inhibitory activity

While the content of canavanine was not affected during the silage process, a reduction in the fraction of condensed tannins by 88 and 38 % in JBN+SOR and CWP+SOR silages, respectively, was observed. However, total phenols and non-tannin phenols increased considerably during ensilage by 35 and 95 % in JBN+SOR and by 39 and 148 % in CWP+SOR silages, respectively.

During the adaptation to the diets containing JBN+SOR silages, the animals under experiment reacted differently. Some pigs consumed the offered amounts without apparent reaction, others consumed it followed by vomiting and others refused it in any case. For CWP+SOR silages nothing similar was observed. In this case, every animal consumed the offered portions without any apparent negative reaction.
Considering the reaction of the animals to the JBN+SOR silage diets, a possible contamination with mycotoxins was assumed. In correspondence to the specific symptoms, deoxynivalenol (DON) was determined in the feedstuffs (Table 52).

Table 52: Content of deoxynivalenol in raw grains and mixed silages used in the feeding trials with pigs

Feedstuff	Deoxynivalenol ($ng \cdot g^{-1}$ DM)
Jack bean (JBN)	0.0
Cowpea (CWP)	0.0
Sorghum (SOR)	57.3
Silage (JBN+SOR)	0.0
Silage (CWP+SOR)	0.0

In the grains analyzed, only SOR was found to be contaminated with DON, showing a value that presumably is not high enough to hamper the animal's intake. Hence, the reason why the animals reacted in the described way to JBN containing diets has to be found elsewhere.

5.4.2 Standardized ileal digestibility of essential amino acids and apparent digestibility of proximate nutrients in raw cowpea-sorghum mixtures and corresponding silages

Only for methionine the standardized ileal digestibility (SID) was improved ($P<0.05$) by ensiling (Table 53), being approx. 24 % higher in silages compared to the raw mixture.

Table 53: Standardized ileal digestibilities (%) of N and essential amino acids of raw or ensiled cowpea-sorghum mixture

	Raw mixture	Silage	pooled SD	P
N	63.5	70.0	±6.44	0.204
THR	70.3	74.8	±4.03	0.165
VAL	66.0	71.8	±1.91	0.105
ILE	65.3	68.5	±3.91	0.284
LEU	72.5	76.5	±2.45	0.060
TYR	70.0	73.0	±3.42	0.261
PHE	70.8	73.3	±3.40	0.339
HIS	66.8	72.0	±4.18	0.126
LYS	72.0	71.5	±4.14	0.870
ARG	73.8	76.0	±3.29	0.370
CYS	64.8	63.0	±8.05	0.769
MET	56.5[b]	70.3[a]	±3.05	0.001

[a,b] Mean values with different superscripts show significant differences between the raw mixture and the silage ($P<0.05$).

For all other essential AA, SID increased likewise after ensiling (P>0.05), although not significant, which was as well the case for total N. Contrary to what was observed for methionine, SID in the other sulphurated AA cystine seemed to be unaffected by ensiling. It remains unclear why ensilage affected the AA in different ways.

Comparing silage and raw material of cowpea-sorghum mixtures, the apparent digestibility (AD) for OM, CP and CF was found not to be statistically different (Table 54). Worth mentioning, although not significant, AD of CF in silage was increased by approx. 25 % in respect to the raw mixture. Starch was considered to be completely digested in the raw mixture as well as in the silage. The AD of AEE was significantly increased through ensiling by approx. 26 %, so was the AD of ADF_{OM} (by 17 %) with respect to the raw material. Crude ash (CA) was the parameter, for which ensiling contributed most to increase (P<0.05) its AD by approx. 48 %. Contrary to what was assumed, the AD of NDF_{OM} was slightly depressed by approx. 18 %.

Table 54: Apparent fecal digestibilities (%) of selected nutrients of raw or ensiled cowpea-sorghum mixture

	Raw mixture	Silage	pooled SD	P
OM	86.3	89.8	±2.00	0.101
CA	44.6[b]	66.1[a]	±5.74	0.010
CP	75.9	77.0	±1.78	0.501
AEE	42.9[a]	54.1[b]	±2.83	0.009
CF	54.9	68.6	±7.12	0.078
NDF_{OM}	75.5[b]	61.8[a]	±3.63	0.010
ADF_{OM}	51.1[a]	59.7[b]	±1.58	0.003
Starch	99.9	100	±0.35	0.554

[a,b] Mean values with different superscripts show significant differences between the raw mixture and the silage (P<0.05).

ADF_{OM}, acid detergent fiber; AEE, acid ether extract; CA, crude ash; CF, crude fiber; CP, crude protein; OM, organic matter; NDF_{OM}, neutral detergent fiber

6 Discussion

6.1 Soaking and pre-germination as silage pre-treatments

6.1.1 Soaking

The buffering substances are primarily affected by the alkalinity of the minerals in the ash rather than by the protein and can be described through the following equation (Weissbach, 2011):

$BC = 0.092\ a + 0.442\ b - 19.5\ (5.88 - c)$ $\quad r^2 = 0.842$

a = N content (meq·100 g^{-1} DM)
b = ash alkalinity (meq·100 g^{-1} DM)
c = pH of the herbage

Playne & McDonald (1966) as well reported the incidence of the anion fractions on the total BC (influence of 68 – 80 % in fresh material and 73 – 88 % in silages). The higher the BC, the more lactic acid is needed to achieve the optimal silage acidity. Hence, a low BC is favored. Wilting for example is known to reduce the BC, probably by the reduction of organic acids (Playne & McDonald, 1966). But in ripe beans, where DM is above 85 %, wilting seems to be useless.

It is known that water soluble anion fractions can be leached out by gradient concentration through the highly permeable seed coat into the soaking water (Lee & Karunanithy, 1990; Bau *et al.*, 1997; Kaushik *et al.*, 2010). However, no previous experiments in literature were found, where soaking was used to reduce BC.

In the present study soaking was proved to reduce BC (see Table 22 and Table 23) and regression equations were produced to predict the BC in JBN and CWP in accordance to soaking time and grain:water ratio:

$BC_{(JBN)} = 0.804a - 0.180b$ $\quad r^2 = 0.678$ $\quad SE = 0.175$ $\quad n = 48$

$BC_{(CWP)} = 0.892a - 0.177b$ $\quad r^2 = 0.827$ $\quad SE = 0.151$ $\quad n = 48$

a = grain:water ration (g fresh beans·l^{-1})
b = soaking time (h)
BC = (g lactic acid·g^{-1} dry matter)

Unfortunately, other variables that might have an influence in how soaking affects BC were not considered for these equations. The hardness, acidity or temperature of the soaking water should be considered in future experiments. This would help to explain the process in a better way.

Soaking might cause as well undesired leaching of most of the water soluble nutrients. According to Kaushik *et al.* (2010) soaking soybeans for 12 h at room temperature in deionized water (1:5, w/v) resulted in a decrease of 9 % in K, 4 % in Ca, 5 % in Mg, 7 % in P and 3 % in Fe. Luo *et al.* (2009) concluded, that mineral losses due to soaking in faba beans can vary in a wide range (Ca: 20 - 61 %, Fe: 33 - 61 % and Zn: 8 - 51 %), depending of soaking conditions (water quality, acidity or temperature).

In the present study, soaking was found to affect the content of selected minerals of legume grains, compared to the corresponding raw and not soaked JBN or CWP (see Table 24). A reduction of 19, 33, 8, 59, 42 and 67 % was observed in CWP after 30 h and a grain:water ratio of 1:5 (w/v) for Ca, P, Na, K, Mg and Cl, respectively. In JBN, reductions of 4, 9, 15 and 7 % of Ca, P, K and Mg, respectively, occurred. Only in JBN, an apparent increase of 9 and 25 % for Na and Cl, respectively, was determined. Other studies reported similar apparent increases of Na. When soaking 9 h in 0.5 % $NaHCO_3$ solution, El-Adawy *et al.* (2000) found an apparent gain of 25 % in soaked soybean and lupin and 33 % in common bean, when the rest of macro- and microelements was reduced. An apparent increase was detected as well for Zn, Fe, Cu and Mn, when faba bean (Luo *et al.*, 2009) and soybean (Kaushik *et al.*, 2010) were soaked under different conditions. The reduction of other minerals could increase the percentage in relation to the total ash content of those ones presumably not leached like Na and Cl.

The WSC as well are susceptible to washing during soaking. Longland *et al.* (2011) soaked 7 different kind of hays for up to 16 h in water (8º C) and 27 % in average (ranging from 6 to 54 %) of the hay´s WSC were leached into the soaking water. In beans like cowpea, Akinlosotu & Akinyele (1991) proved that soaking in water led to a decrease of verbascose (49.4 %), stachyose (29.8 %) and raffinose (1.0 %). Nevertheless, sucrose and fructose increased by 41.9 % resp. 43.0 %, and glucose and galactose decreased by 55 %. When grains were dehulled, a further decrease of verbascose (76.4 %) and raffinose (56 %) as well as an increase of sucrose (45.9 %)

and glucose and galactose (63.6 %) was observed. In the present study, the effect of soaking on WSC was not evaluated mainly for the low levels observed in these grains (see Table 12). As it is reported by literature, soaking could contribute to increase certain WSC fractions. Soaking involves the entry of water into the legume kernels, wetting and dissolving of soluble nutrients, which makes all starch fractions more susceptible to enzymatic hydrolysis (Eyaru *et al.*, 2009). Further experiments should be conducted in this sense.

Protein (N x 6.25) was not found to be lost during the soaking experiment, which is in accordance to other studies (El-Adawy *et al.*, 2000; Longland *et al.*, 2011). Fernandes *et al.* (2010) cited authors that confirmed that the protein content in lentils, chick peas, red kidney beans, white kidney beans and black grams (*Vigna mungo*) may not be affected by soaking, discarding the soaking water and cooking.

Special attention should be paid to other variables that could affect soaking effects on the chemical composition of the beans. Not only their intrinsic physical characteristics (*e.g.* hull thickness and permeability), but their chemical composition itself: the distribution in the seed and the enzyme battery. Other variables, like ANF, are known to influence minerals and enzyme activity. Phytic acid, dietary fiber and polyphenols, among others, are affected by soaking (Ekholm *et al.*, 2000; El-Adawy *et al.*, 2000; Lestienne *et al.*, 2005a; Eyaru *et al.*, 2009). This could explain why JBN and CWP had a different performance during soaking.

The negative influence of soaking on water soluble nutrients like minerals could be assumed to overshadow its real effectiveness on BC. But the loss of certain minerals can be compensated by increasing bioavailability of the remaining ones (Lestienne *et al.*, 2005b; Fernandes *et al.*, 2010).

Moreover, soaking reduces ANF in the plant material (see 2.3.2) by the activation of preexisting enzymes in the seeds and/or the diffusion through the grain endosperm (Vijayakumari *et al.*, 1998). Similar explanations gave Khokhar & Chauhan (1986), highlighting the influence of the gradient´s concentration mediated by a change in the permeability of the seed coat. For example, the activity of ANF decreased as soaking time was increased in JBN (Belmar *et al.*, 1999).

6.1.2 Pre-germination

Changes in the chemical compositions during germination of JBN and CWP have been extensively reported (Ibrahim *et al.*, 2002; Camargos *et al.*, 2004; Ghavidel & Prakash, 2007; Martin-Cabrejas *et al.*, 2008). Diaz *et al.* (2007), when germinating CWP under different conditions observed no influence for CP, exceptionally in total darkness, where CP increased up to 34.5 % DM, representing 25 % more compared to the control. Under total darkness NDF_{OM} increased (57 % compared with the control), as well as ADF_{OM}, cellulose, and lignin as the germination process advanced in the different trials. As the most abundant element, K increased by 25 % followed by P, Mg and Ca. It was concluded that total darkness was superior to the light variants. In contradiction to these authors, no influence of the illumination regime was observed in the CP content for JBN and CWP, but an effect was found for the germination time. After 72 h of germination an increase ($P<0.001$) of nearly 7 % CP of the DM was observed for CWP, much less than observed by Diaz *et al.* (2007) in the same CWP variety (25 %). Akinlosotu & Akinyele (1991) reported a rise of 12 %, which is more in accordance with our results. In JBN, an increment of 3 and 4 % CP of the DM after 72 and 96 h was observed, respectively. Akpapunam & SefaDedeh (1997b) reported for germinated JBN a reduction in CP after 72 h from 23 to 20 %.

As assumed, germination increased the WSC content. But the drastic reduction after 12 h of germination in JBN (57 %) compared to raw JBN was not observed in CWP. At the end of the experiment WSC increased ($P<0.05$) in JBN (25 %) and CWP (321 %) after 96 and 72 h, respectively. Akinlosotu & Akinyele (1991) observed nearly the same WSC increase (294 %) in CWP after 72 h.

6.1.3 Pre-ensiling treatments and selected anti-nutritional factors

The amount of trypsin inhibitors detected in different legume seeds varies with legume species and variety (Guillamon *et al.*, 2008). During germination they are transformed in a different way from one legume to another (Muzquiz *et al.*, 2004). Furthermore, comparisons between results of different authors are frequently limited by the different inhibition units used. There is a general consensus that TIA can be reduced through germination. Akpapunam & SefaDedeh (1997b) reported after 72 h of germination a reduction of 79 % in JBN. In the present work the highest reduction

of TIA was detected after 12 h (59 %). A higher pre-germination temperature of 29 ºC vs. 25 ºC and oven temperature of 100 ºC (6 h) vs. only 60 ºC in the referred study could be among the reasons for such a high TIA reduction. In CWP, 12 h of germination produced as well the highest reduction of TIA (72 %), which was higher than reported by Ibrahim *et al.* (2002) after 48 h (54 %). The reduction of TIA can be associated with enzymatic degradation (Khokhar & Chauhan, 1986), which seems to be higher in the first 12 h.

Definite conclusions are difficult to draw, when still there is not a specific and reliable method to determine canavanine. Dmello *et al.* (1988) stated, that alternatives to the PCAF technique like HPLC or IEC have to be improved. Some reports refer to the use of HPLC with this purpose (Acamovic & Dmello, 1990; Viroben & MichelangeliVargas, 1997; Ekanayake *et al.*, 2007), but still there remains uncertainty due to the cross-reactivity with histidine or products of the canavanine hydrolysis, which hardly can be avoided even with advanced techniques (Viroben & MichelangeliVargas, 1997).

After Bell (1960) suggested germination to reduce canavanine, different variants like in our experiment (illumination regime, germination time) have been tested by several authors. In addition, metabolism of canavanine and its distribution across the plant during its growth have been studied (Rosenthal, 1970; 1972; Rosenthal, 1982; Rosenthal & Berge, 1989; Hwang *et al.*, 1996). Dmello *et al.* (1988) reported a reduction of 24 % compared to the not germinated variant after 24 h of germination in the darkness. In the present work, no influence of the illumination regime was detected. However, after 12 h a pronounced reduction was determined (14 %) with a further increase after 24 h.

6.2 Effect of different pre-ensiling treatments, additives and mixing with sorghum on selected fermentation parameters in Jack bean and cowpea silages

There are many reasons why silages with high DM contents are preferred. Apart from using storage space more efficiently in silos, it is even of more relevance, that the DM of the ensiled crop is inversely proportional to the fermentation losses (McDonald *et al.*, 1991).

As a methodically consequence, the DM of soaked (SOK) and pre-germinated (PRG) grains was considerably reduced, which influenced the silage DM. Compared to moisture re-constituted (to approx. 65 % DM, MOR) silages, the DM of SOK and PRG silages was lower ($P<0.05$), independent of the type of grain and the additive used. When SOK and PRG silages were mixed with SOR, the DM was increased and no differences ($P>0.05$) were found with MOR silages (Figure 11). Nevertheless, there remained the differences ($P<0.05$) with those treatments when SOR was not mixed independent of the additive and bean used (see Table A3 in the appendix).

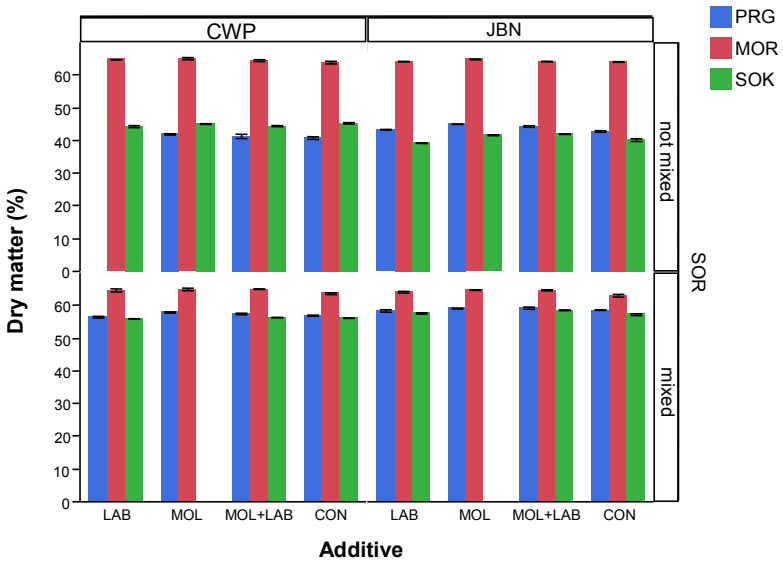


Figure 11: Dry matter content in grain model silages sole or mixed with sorghum after 60 days of storage submitted to different pre-ensiling treatments and additives (n=3)

CON, control; CWP, cowpea; MOL, molasses; MOR, moisture reconstituted; LAB, lactic acid bacteria; PRG, pre-germinated; SOK, soaked; SOR, sorghum
Each error bar is constructed using 1 standard deviation from the mean.

Several studies, summarized by Jones (1988), reported about the effect of different cereals when added to grass silages. In every case, DM was increased and lower amounts of VFA, ammonia and losses were reported. In Figure 12 (see as well Table A4 in the appendix) losses for every treatment of grain model silages are shown. Outstanding is the amount of losses observed in PRG silages of CWP when no SOR was mixed. Unfortunately, a detailed study during germination, if CWP was contaminated with microbes, which might have had an effect on fermentation losses, was not conducted.

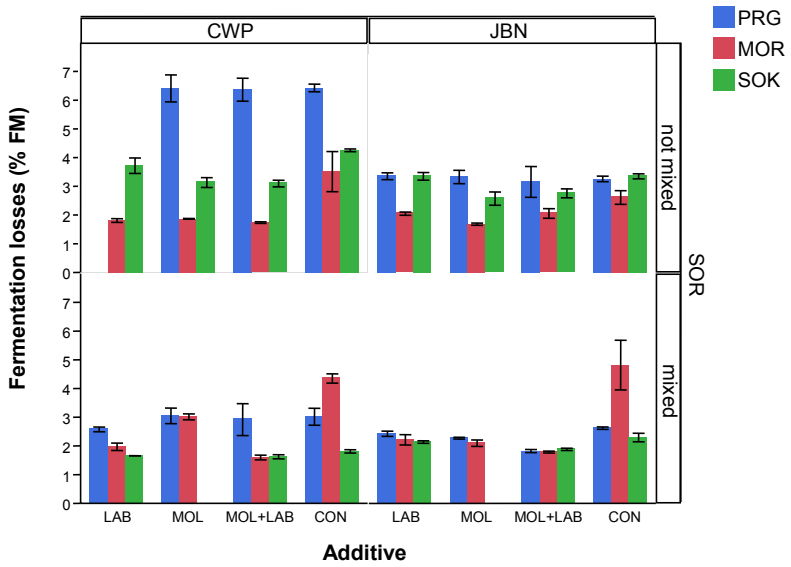


Figure 12: Losses in grain model silages sole or mixed with sorghum after 60 days of storage submitted to different pre-ensiling treatments and additives (n=3)

CON, control; CWP, cowpea; MOL, molasses; MOR, moisture reconstituted; LAB, lactic acid bacteria; PRG, pre-germinated; SOK, soaked; SOR, sorghum

Each error bar is constructed using 1 standard deviation from the mean.

Silage fermentation reaches its climax not only when the supply of available substrate has been exhausted or the pH of the crop mass has decreased to the point at which microbial growth is inhibited, but also when there is a lack of available water in the crop (a_w), which hampers the growth of microbes (Wilkinson & Davies, 2013).

The lower the DM and the higher the yeast counts (> $10^5 \cdot g^{-1}$ FM), the more likely is the increase of DM losses (Muck, 1988; McDonald *et al.*, 1991; Borreani & Tabacco, 2010). The DM affects as well the plant enzymes' activity, depending of the silage conditions. Enzymes can remain active during ensilage, hence contributing to its deterioration. The activity of proteolytic enzymes correlates linearly and negatively with a silage DM > 50 % and almost no activity is detected > 75 % DM (Muck, 1988). In most of the pre-treated silages where SOR was not mixed, DM was < 50 %, which increased losses compared to the rest.

The pre-treating conditions can contribute as well to an increase of the microbial population. In nature, seeds are colonized by several bacteria (Darrasse *et al.*, 2010) and fungi (Houssou *et al.*, 2009) and they persist during all plant vegetative stages (Darrasse *et al.*, 2010). Remains of soil and crop often appear mixed with grains after harvesting, like it was found in CWP and JBN. This is a common way soil-borne

microorganism including coliforms, clostridia and fungi get into the silo. The kind of microbial species does not only depend on the environment the seeds (soil) come from, but on the particular seeds´ exudates associated to the germination process (Ofek *et al.*, 2011). Even a disinfection of grains (see 4.3.3.2) is not totally effective (Miche & Balandreau, 2001; Ding *et al.*, 2013). Particularly in the soaking pre-ensiling treatment, no disinfection proceeding was run and evidences of microbial growth were as well observed. In any case, CWP seemed to be the most affected grain, which suggests a higher initial contamination compared to JBN.

The initial plant material contamination should be reduced drastically when undissociated organic acids (lactic and acetic acids) are sufficiently produced during ensilage (Muck *et al.*, 1991). However, in the model built by the same author, the pH was found to affect yeast growth only marginally and mould growth was not affected at all. The production of lactic acid (Figure 14) was much higher when JBN and CWP were ensiled alone, independent of the use of additives (see as well Table A6 in the appendix). Nevertheless, when mixed with SOR, no variation in pH was observed. Only in soaked JBN with the addition of LAB and soaked JBN without additives pH (Figure 13) was found to be lower ($P<0.05$) in mixed silages than in grains without SOR (see Table A5 in the appendix).

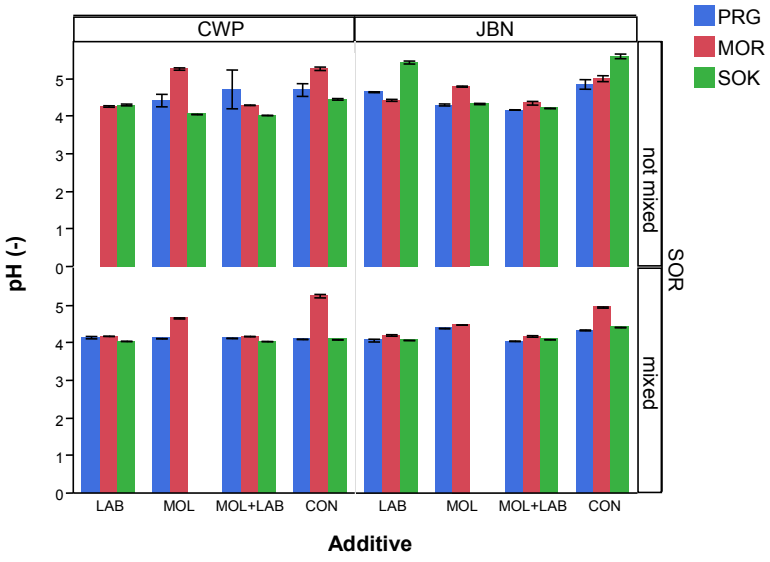


Figure 13: The pH in grain model silages sole or mixed with sorghum after 60 days of storage submitted to different pre-ensiling treatments and additives (n=3)

CON, control; CWP, cowpea; MOL, molasses; MOR, moisture reconstituted; LAB, lactic acid bacteria; PRG, pre-germinated; SOK, soaked; SOR, sorghum

Each error bar is constructed using 1 standard deviation from the mean.

The inclusion of SOR increased the DM in pre-treated silages. At a higher concentration of DM there is restricted lactic acid fermentation (Pieper *et al.*, 2010; Inoue *et al.*, 2013; Wilkinson & Davies, 2013). The higher BC of sole JBN and CWP compared with mixed SOR silages could be among the reasons, why solely ensiled grains, even with a higher lactic acid production, showed much lower pH values.

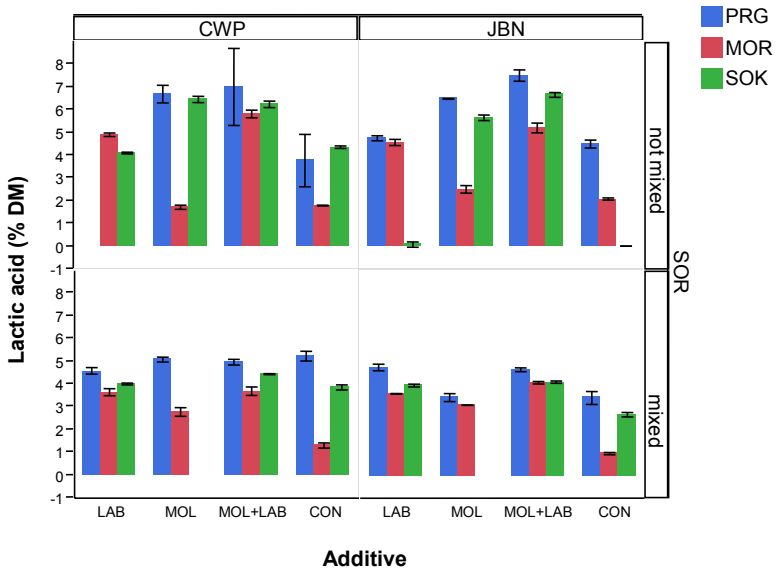


Figure 14: Lactic acid content in grain model silages sole or mixed with sorghum after 60 days of storage submitted to different pre-ensiling treatments and additives (n=3)

CON, control; CWP, cowpea; MOL, molasses; MOR, moisture reconstituted; LAB, lactic acid bacteria; PRG, pre-germinated; SOK, soaked; SOR, sorghum

Each error bar is constructed using 1 standard deviation from the mean.

The high BC of legumes implies a higher risk of butyric acid fermentation during ensiling. Studies made in Germany, Sweden and Finland achieved only slight fermentation improvements when wilting lucerne, galega, and red clover to 25 % DM. However, when wilted to 40 % DM butyric acid fermentation was prevented (Huhtanen *et al.*, 2012). Furthermore, mixing legumes with cereals seems to alleviate the negative effect on fermentation quality (Pursiainen & Tuori, 2008). In the present study, mixing with SOR prevented butyric acid fermentation, as butyric acid could not be detected in any of the treatments for both legumes. The use of SOR increased the DM which seemed to enhance fermentation quality in pre-treated silages. The depressing effect of DM on butyrogenesis was proved in MOR silages. As the DM was set to approx. 65 %, neither in mixed nor in not mixed silages, butyric acid was detected in any case, which is in accordance with ensiled peas, beans and lupines

with a similar DM (Gefrom *et al.*, 2013) and ensiled triticale, barley and wheat grains with 65 and 75 % DM (Pieper *et al.*, 2010). The DM in silages is negatively correlated to clostridal development, which is usually inhibited in silages > 45 % DM (Pahlow *et al.*, 2003).

As expected, ethanol was found in ensiled grains in levels not higher than 2 % of the DM (Buchanan-Smith *et al.*, 2003). Ethanolic fermentation was as well detected in most of the silages in the present study, but barely exceeded 1 % of the DM (Figure 15). Therefore, the risk of yeast contamination during pre-ensiling treatments that could affect the silage quality was minor. Only in silages without additive ethanol was higher ($P<0.05$) compared to the rest (see Table A7 in the appendix).

The high DM of the silages did not inhibit ethanolic fermentation to the same extend like in the case of butyrate. Although Pieper *et al.* (2010) found in their experiments a correlation between the DM (65 and 75 %) and ethanol, they reported that ethanol never exceeded 1 % of the DM. A low ethanol production was as well observed when ensiling legume grains (Gefrom *et al.*, 2013). Presumably, apart from the DM more variables have to be considered, when acidic and alcoholic fermentation in grain silages shall be explained, as grains have not only a different chemical structure than forages, but as well a different epiphytic micro-flora.

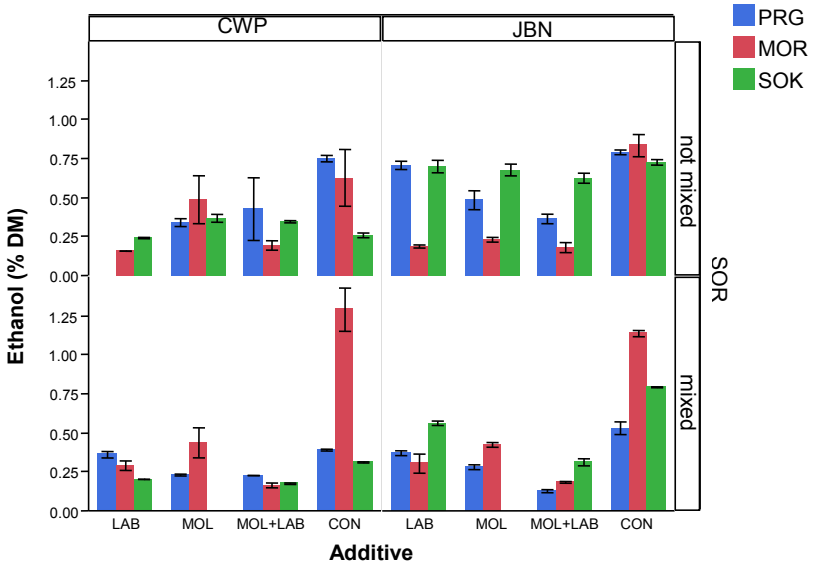


Figure 15: Ethanol content in grain model silages sole or mixed with sorghum after 60 days of storage submitted to different pre-ensiling treatments and additives (n=3)

CON, control; CWP, cowpea; MOL, molasses; MOR, moisture reconstituted; LAB, lactic acid bacteria; PRG, pre-germinated; SOK, soaked; SOR, sorghum

Each error bar is constructed using 1 standard deviation from the mean.

Enterobacteria are usually the second most numerous bacterial group in the epiphytic microflora (Pahlow *et al.*, 2003). They ferment carbohydrates, producing a wide range of final products (*e.g.* 2,3-butanediol). The 2,3-butanediol is part of the so called neutral fermentation, and therefore not desired in silage fermentation, what Rooke & Hatfield (2003) described through the following stoichiometric equation:

4 pyruvate + 4 NADH → 1 (2,3-butanediol) + 1 lactate + 1 ethanol + NAD + 3 CO_2

In the present study, 2,3-butanediol was determined in meager quantities and only in the case of the control treatments without any pre-ensiling treatment it was found to be higher (> 2 % DM). In silages without any additive alcohols can be found in much higher amounts like in untreated Italian ryegrass silage, for example, where the sum of ethanol and 2,3-butanediol content at day 14 was about 7 times higher than that of lactic and volatile fatty acids (Yan-bing & Nishino, 2012).
Furthermore, the differences in contents of this fermentation products are remarkable comparing our silages with whole plant legume silages. Eriksson & Ericson (2012) reported values between 0 and 4.1 % of the DM in 60 different not treated silages of timothy grass and red clover, where DM ranged between 13 and 75 %. Other studies report about higher amounts in lucerne (7.5 % DM) and red clover (5.4 % DM) (Hymes-Fecht *et al.*, 2012).
We observed that in general additives depressed 2,3-butanediol production. The addition of LAB contributes to suppress alcoholic fermentation when acetates or lactates are mainly produced (Yan-bing & Nishino, 2012). Presumably, the possible contamination with enterobacteria was not produced by the pre-ensiling treatments that far as it was feared.
Except ethanol, other alcohols are seldom referred to in literature about silage. One reason could be their low contents and toxicity, like it is the case for propanol. Like ethanol, propanol is produced mainly by yeasts and interferes with the lactic acid production due to its neutral nature and aerobic stability (Rooke & Hatfield, 2003). In the present study contents of propanol were generally negligible.

Table 55 summarizes the source of variation of the main parameters affecting fermentation quality. As just discussed, ethanol was not affected by the pre-ensiling treatment, neither it was by the interactions LEG*SOR, PET*LEG*SOR and LEG*ADT*SOR. The pH value varies in relation to PET, LEG, ADT and if SOR was

mixed or not, which was as well the case for every presented interaction (P<0.001). Similar effects were observed for DM, lactic acid and NH_3-N/N_{total}, although, like in the case of ethanol, the interaction LEG*SOR did not produce any effect on lactic acid. The losses in ROMOS were closely and significantly related to every factor (PET, LEG, ADT, SOR) and their interactions, except the not significant interactions PET*LEG*ADT and PET*LEG*ADT*SOR.

Table 55: Source of variation in fermentation parameters after 60 days storage in Jack bean and cowpea model silages using a model with pre-ensiling treatment, grain type, additive and sorghum inclusion as fixed effects

	DM	Losses	pH	LA	AC	Ethanol	NH_3-N/N_{total}
PET	***	***	***	***	***	n.s.	***
LEG	**	***	***	***	***	***	***
ADT	***	***	***	***	*	***	***
SOR	***	***	***	***	***	***	***
PET*LEG	***	***	***	***	**	***	***
PET*ADT	***	***	***	***	***	***	***
PET*SOR	***	***	***	***	***	***	***
LEG*ADT	***	***	***	***	n.s.	***	***
LEG*SOR	***	***	***	n.s.	n.s.	n.s.	***
ADT*SOR	*	**	***	***	***	***	***
PET*LEG*ADT	***	n.s.	***	***	**	***	***
PET*LEG*SOR	***	***	***	***	n.s.	n.s.	***
PET*ADT*SOR	***	***	***	***	**	***	***
LEG*ADT*SOR	**	*	***	***	n.s.	n.s.	***
PET*LEG*ADT*SOR	***	n.s.	***	***	n.s.	***	***

AC, acetic acid; ADT, additive, DM, dry matter; PET, pre-ensiling treatment; LA, lactic acid; LEG, legume (Jack bean or cowpea); SOR, sorghum
n.s. = not significant, * P<0.05, ** P<0.01, *** P<0.001

6.3 Effect of different pre-ensiling treatments and additives on selected anti-nutritional factors in Jack bean and cowpea silages mixed or not mixed with sorghum

The most commonly studied Trypsin inhibitor (TI, Kunitz) was described in soybean as a protein globulin type (Kunitz, 1945; 1946). Other protein-like molecules, but with less active inhibitory capacity (Bowman-Birk), were later isolated (Bowman, 1948; Birk, 1985). Irrespective of whether a TI is classified as the Kunitz or Bowman-Birk

family, they vary from one bean to another (Gatehouse *et al.*, 1980), even in relation to stress conditions and maturity stage (Benjakul *et al.*, 2000). Nowadays, heat treatment remains as the most efficient TIA deactivation method. But even the expected thermolability of TI is strongly connected to the grain's origin (Benjakul *et al.*, 2000). Some TI are extremely thermostable, like those in lima bean (*Phaseolus lunatus*), keeping 95 % of the inhibitory capacity after 30 min autoclaving (15 lb) (Sohonie & Ambe, 1955). Others like in chick pea retained all of their activity after being heated (80 °C, 5·min^{-1}) and only 50 % were inactivated by boiling (100 °C, 5·min^{-1}) or roasting (130 °C, 8·min^{-1}). The TI in faba beans still kept 20 % of their original activity after boiling in water for 60 min (Richardson, 1981), while in soybean it was destroyed completely at 92 °C (5·min^{-1}) (Benjakul *et al.*, 2000). Hence, differences in the origin of the TI could have caused the different effects of ensilage on TIA, which were more pronounced in CWP silages than in JBN (Figure 16).

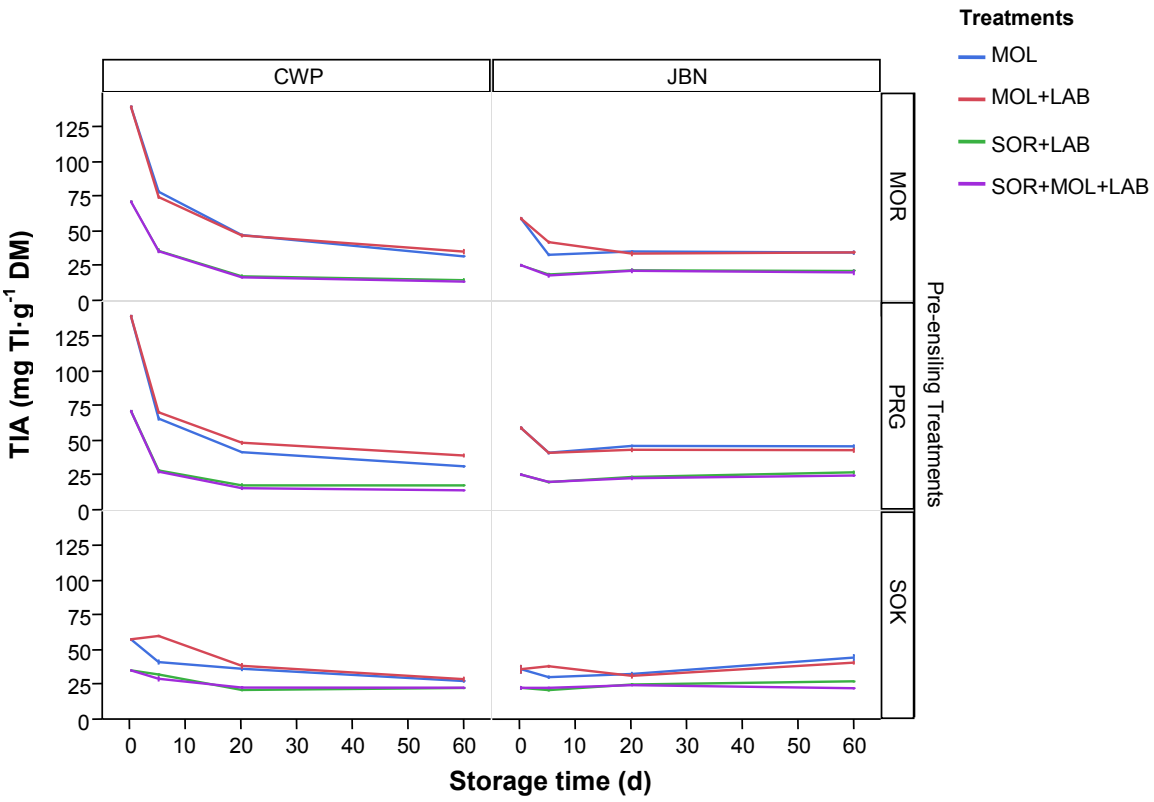


Figure 16: Trypsin inhibitory activity (TIA) measured after 5, 20 and 60 days of storage in four model silage treatments of Jack bean and cowpea after three pre-ensiling treatments (n=3)

CWP, cowpea; MOL, molasses; LAB, lactic acid bacteria; SOR, sorghum
Each error bar is constructed using 1 standard deviation from the mean

Figure 16 shows that TIA was affected ($P<0.05$) as the storage time was prolonged for every treatment, independent of JBN and CWP pre-ensiling treatment. In JBN, TIA was lower ($P<0.05$) after 60 days in MOR compared to SOK and PRG silages for every treatment, but not between SOK and PRG silages. Only in the case of treatments MOL and SOR+MOL+LAB, MOR was not different to SOK. No influence of the pre-ensiling treatment was noticed in the case of CWP silages after 60 days of storage (see Tables A8 and A9 in the appendix).

The activity of proteases in LAB is known to play a relevant role in transforming proteins during fermentation (Arendt *et al.*, 2011; Moslehishad *et al.*, 2013). However, other workers found that the transformation is more associated to a pH-induced protein shift rather than to protein hydrolysis (Loponen *et al.*, 2007), whereas LAB fermentation is generally associated to TIA reduction (Azeke *et al.*, 2005; Granito & Alvarez, 2006; Khalil, 2006; Fernandez-Orozco *et al.*, 2007; Silva *et al.*, 2013; Starzynska-Janiszewska *et al.*, 2014). However, the references about the role of silages on TIA reduction are meager.

Unexpectedly, HCN increased in every evaluated silage treatment where CWP was used (Figure 17). In silages using JBN, HCN content varied ($P<0.001$) after 60 days of storage in close relation to the pre-ensiling treatment used (see Table 56). In JBN silages, HCN was reduced by approx. 17 % in the treatment JBN+LAB in moisture reconstituted (65 % DM) silages and by approx. 41 % in JBN+MOL+LAB in pre-germinated silages. For every pre-ensiling treatment, the addition of SOR increased HCN by 10 - 25 %. Only in JBN+SOR+MOL+LAB, with pre-germinated JBN, HCN was slightly reduced (5 %). It was in silages with pre-germinated JBN sole or mixed with SOR with the addition of MOL+LAB, where the lowest HCN was measured with 47.85 and 37.69 $mg \cdot 100\ g^{-1}$, respectively. For all detected differences see as well Tables A10 and A11 in the appendix.

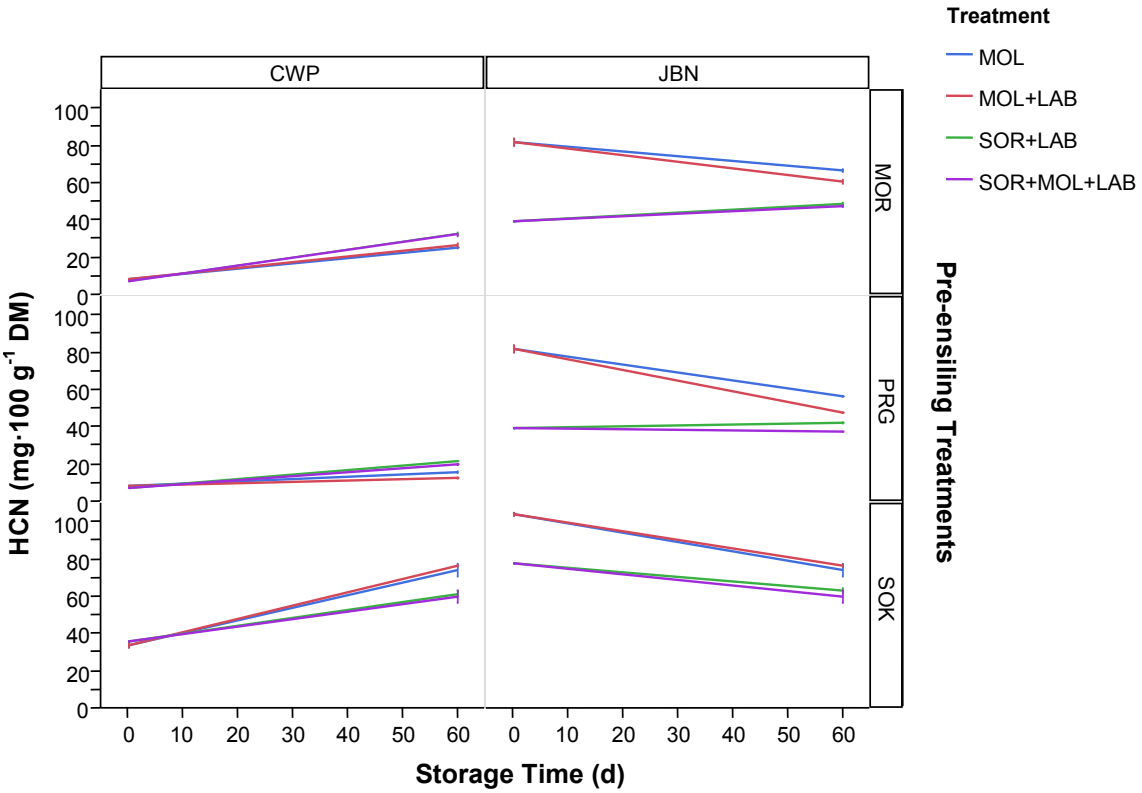


Figure 17: Hydrogen cyanide content after 5, 20 and 60 days of storage in four model silage treatments of Jack bean and cowpea after three pre-ensiling treatments (n=3)

CWP, cowpea; MOL, molasses; LAB, lactic acid bacteria; SOR, sorghum
Each error bar is constructed using 1 standard deviation from the mean

Literature refers to a HCN reduction, when fermentation takes place (Reddy & Pierson, 1994; Azeke *et al.*, 2005; Azeke *et al.*, 2007). As previously reported in 2.3.1, HCN is liberated from CG after enzymatic reaction by naturally occurring plant enzymes or preferably basic hydrolysis over acid. Furthermore, *Lactobacillus spp.* has been reported to synthesize β-glycosidase related to HCN releasing from CG (Lei *et al.*, 1999; Kostinek *et al.*, 2005; Nwokoro & Anya, 2011). However, it remains unclear what the necessary conditions in silages to favor LAB detoxifying capacity are. Contradictory appears the fact that there is a β-glucosidase activity peak at pH 4.0 - 6.2, which seems to be not affected under acid silage conditions. But, in the case of α-hydroxy nitrile lyase with an optimum at pH 5 - 6.5, silages are not the best environment. Considering that non-enzymatic detoxification is fast at alkaline pH and negligible below pH 5.5, the role of silages and LAB inoculants on the HCN reduction should be further investigated.

The pre-ensiling treatments influenced (P<0.05) the canavanine content for every selected treatment (Figure 18 and Table A12 in the appendix).

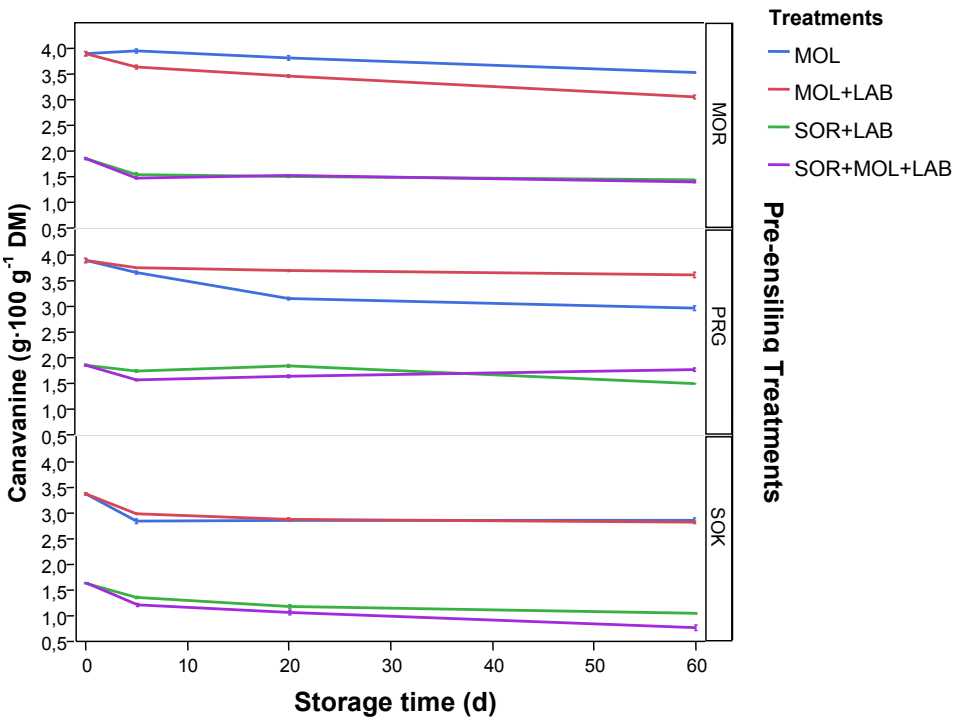


Figure 18: Canavanine content measured after 5, 20 and 60 days of storage in four model silage treatments of Jack bean after three pre-ensiling treatments (n=3)

CWP, cowpea; MOL, molasses; LAB, lactic acid bacteria; SOR, sorghum
Each error bar is constructed using 1 standard deviation from the mean

In SOK silages canavanine was reduced already at day 5 and a further decrease was caused until day 60. Hereby, in the treatments with SOR included, the reduction was even more pronounced. Unfortunately, no further analyses of remains (degradation products) of canavanine could be conducted to determine *e.g.* L-canaline, a L-canavanine cleavage product known to compete with ornithine in the arginine urea cycle (Sridhar & Seena, 2006), which would be highly interesting for future experiments.

The used pre-ensiling treatment (PET) as well as the silage treatments (TTM) and the storage time (STI) affected the content of ANF analyzed (Table 56). The interaction between PET*STI influenced the content of every ANF evaluated differentially. Only in the case of canavanine, no effect of the interactions PET*STI, STI*TTM and PET*STI*TTM was observed.

Table 56: Source of variation in anti-nutritional factors in Jack bean and cowpea model silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

	TIA		HCN		Canavanine[1]
	JBN	CWP	JBN	CWP	
PET	***	**	***	***	**
TTM	***	*	***	***	**
STI	***	**	***	***	*
PET*TTM	***	*	***	***	*
PET*STI	***	**	***	***	n.s.
STI*TTM	***	*	***	***	n.s.
PET*STI*TTM	***	n.s.	***	***	n.s.

CWP, cowpea; HCN, hydrogen cyanide; JBN, Jack bean; PET, pre-ensiling treatment; LEG, legume (Jack bean or cowpea); STI, storage time; TIA, trypsin inhibitory activity; TTM, treatment
n.s. = not significant, * P<0.05, ** P<0.01, *** P<0.001
[1] Corresponding only to model silages (ROMOS) where JBN was used as grain.

6.4 Silages of Jack bean-sorghum and cowpea-sorghum mixtures as feedstuffs for pigs

The refusal of the pigs to eat the diets containing Jack bean has given rise to the question for possible explanations. An approach was that the grains might have been contaminated with mycotoxins, especially with deoxynivalenol (DON). Pigs react highly sensitive to this mycotoxin, decreasing drastically the food intake. From the practical point of view it is commonly detected at levels high enough to cause adverse effects. Furthermore, the trivial name “vomitoxin” has also been assigned to DON because of its acute ability to induce vomiting (Pestka, 2007; Döll & Dänicke, 2011).

Surprisingly, no DON was detected in the beans or in any of their corresponding silages (Table 52). Only in SOR a small amount (57.3 $ng \cdot g^{-1}$ DM) was found, which was far below the critical value of 400 $\mu g \cdot kg^{-1}$ DM in feed materials and feedstuffs accepted by the European Union (EFSA, 2004).

In the literature reviewed, there were no reports about JBN or CWP contaminated with DON. This mycotoxin occurs predominantly in cereals, but SOR is excluded from reports of contamination with *Fusarium* mycotoxins (Placinta *et al.*, 1999; Döll & Dänicke, 2011). The European Food Safety Authority (2013) cited, that it is less often reported. Some workers refer to the effect of LAB fermentation on DON and binding as the most probable removal mechanism, which leads to a nearly 100 % reduction

(Niderkorn *et al.*, 2006; Niderkorn *et al.*, 2007; Boudra & Morgavi, 2008; Arendt *et al.*, 2011). Other workers observed an increase or no effect on DON during silage (Kristensen *et al.*, 2010; Teller *et al.*, 2012). They considered that factors like silage DM, level of pre-harvest mycotoxin contamination or LAB strain could influence the effects on DON so that it is not possible to rely on ensilage completely.

The real cause why the inclusion of JBN in diets reduced so drastically the pigs' intake remains unclear. It has been proved that only 5 % of raw JBN in diets for pigs hamper the intake extremely (Michelangeli *et al.*, 2004b; Torres *et al.*, 2013). The single action of an ANF or the combination of some of them remains as strong argument. Authors like Enneking *et al.* (1993) observed intake suppressions when fed canavanine dihydrochloride to pigs mixed in a canavanine free diet. Belmar & Morris (1994a) reported about a decrease in feed intake when canavanine was added to a maize-soybean diet at only 0.8 $g \cdot kg^{-1}$. The inactivation (toasting: 194 °C for 18 min) of canavanine (95 % compared to that in raw beans) improved intake in pigs, but with a lower performance compared to the control (Michelangeli *et al.*, 2004a; Michelangeli *et al.*, 2004b). The same authors considered that an acceptable intake can be achieved when the content of canavanine is 0.06 % of the DM or lower. From that point of view, the canavanine content is still too high (1.62 % DM) in JBN+SOR mix silages (see Table 51), explaining why some animals refused JBN containing diets. Contradictory appear other experiments (Yin *et al.*, 1993; Yin *et al.*, 2002). Among many other feedstuffs, they offered raw or autoclaved JBN. No negative pig behavior was reported and a rather exceptional ileal and fecal digestibility of proximate nutrients and AA was shown.

6.4.1 Standardized ileal digestibility of essential amino acids in raw cowpea-sorghum mixtures and corresponding silages

Due to their high BC and protein content, legumes are especially susceptible to proteolysis during silage. The combined action of both, plant and silage microbial enzymes, can reduce the true protein content of the original herbage by up to 80 % by the end of the conservation period (Borreani *et al.*, 2006). Furthermore, this can change from one plant species to another. In tannin-containing species, the protein degradation process during ensiling is reduced in comparison to non-tannin

containing species (Albrecht & Muck, 1991). As in JBN and CWP not only tannins but other ANF were observed in different quantities (see Table 51), the effect on protein conservation was expected to have some variations.

The AA composition of mixed silages is shown in Table 57. In CWP+SOR, losses affected most of the essential AA under evaluation, only TYR (+1.5 %) and HIS (+6.4 %) were observed to increase relatively compared to the raw mixture. In contradiction, higher amounts of most AA were observed in JBN+SOR silages than in the not ensiled mixture. Only for GLU (-2.6 %), ALA (-4.5 %), CYS (-0.5 %) and MET (-3.6 %) a reduction was observed.

Table 57: Losses and gains of amino acids comparing raw and ensiled mixtures of cowpea or Jack bean with sorghum

AA	CWP+SOR			JBN+SOR		
	Raw mixture	Silage	Losses (-) or gains (+)	Raw mixture	Silage	Losses (-) or gains (+)
	(g·kg^{-1} DM)	(g·kg^{-1} DM)	(%)	(g·kg^{-1} DM)	(g·kg^{-1} DM)	(%)
ASP	17.72	16.88	-4.7	14.61	14.77	+1.1
THR	6.62	6.35	-4.1	6.44	6.50	+0.9
SER	8.66	7.96	-8.1	8.20	8.24	+0.5
GLU	33.97	31.85	-6.2	25.62	24.95	-2.6
GLY	6.85	6.84	-0.1	5.83	5.98	+2.6
ALA	9.83	9.63	-2.0	9.35	8.93	-4.5
VAL	9.01	8.64	-4.1	7.48	7.79	+4.1
ILE	8.19	6.83	-16.6	6.16	7.05	+14.4
LEU	16.53	15.73	-4.8	15.33	15.38	+0.3
TYR	4.13	4.19	+1.5	4.54	4.58	+0.9
PHE	9.97	9.50	-4.7	7.58	7.65	+0.9
HIS	5.45	5.80	+6.4	12.26	12.62	+2.9
LYS	9.08	8.26	-9.0	6.34	6.71	+5.8
ARG	10.13	9.99	-1.4	7.24	7.60	+5.0
PRO	8.96	8.86	-1.1	8.56	8.61	+0.6
CYS	2.23	2.04	-8.5	1.97	1.96	-0.5
MET	2.76	2.54	-8.0	2.22	2.14	-3.6

The addition of tannic acid to alfalfa silages by Guo *et al.* (2008) resulted in a reduction ($P<0.05$) of ARG (-67.4 %), HIS (-3.9 %), LYS (-5.6 %) and THR

(-48.0%), but showed an increase of ILE (+21.7 %), LEU (+9.8 %) and VAL (+11.7 %), whereas MET and PHE remained unaltered. The authors stated that the silage quality (low pH) corresponded to a higher AA protection. But as the pH values in JBN+SOR and CWP+SOR mix silages were rather similar (4.01 and 4.00, respectively), ANF could play a decisive role.

Several workers claim that legume silages are typically more stable on exposure to air compared to grass silage, because they might contain a natural compound that inhibits the growth of spoilage microorganisms (Wilkinson & Davies, 2013). Further researches should be conducted in order to test the role of other ANF than tannins on the preservation of proximate nutrients during silage fermentation.

Paradoxically, what could be seen as beneficial during silage fermentation and aerobic stability represents a drawback, when silages with remains of naturally occurring ANF are fed to the animals. Certainly, it is the inactivation of naturally occurring ANF what improves animal performance.

The content of ANF in the diet depresses ileal CP and AA digestibility (Schulze *et al.*, 1997; Myrie *et al.*, 2008; Jezierny *et al.*, 2010; Jezierny *et al.*, 2011; Gilani *et al.*, 2012). Yin *et al.* (2002) observed an increase of the apparent ileal digestibility of CP and AA ($P<0.01$) as a result of heat treatment in both common soybean and black soybean varieties. They stated that the trypsin inhibitor deactivation was the main cause. In the present work, it was expected that most essential AA will improve SID in CWP+SOR mix silages. Unexpectedly, only for MET a significantly increased ($P<0.01$) SID was observed (see Table 53). However, most of the other essential AA under evaluation showed an increase of SID as a tendency. The reduction of TIA (56.8 %) and CT (37.5 %) throughout fermentation could be among the reasons.

Contradictory, not always a reduction of ANF improves ileal AA digestibility. Other authors were unsatisfied when no positive effect on the coefficients of the SID of proteins and AA in germinated yellow and blue lupines was observed, despite a decrease of the concentrations of the raffinose family oligosaccharides and alkaloids in the sprouts (Chilomer *et al.*, 2013). When ensiling wheat and barley grains, Hackl *et al.* (2010) speculated that the increase ($P<0.05$) of SID for LYS, MET, THR, LEU, PHE and ARG in wheat was due to the breakdown of non-starch-polysaccharides during ensiling, but they failed to explain the reduction of SID for LYS and HIS in the case of barley.

Apparently, several variables are influenced while naturally occurring ANF are deactivated by any method (see 2.3.2). The variation produced by a certain deactivation method in SID of AA seems to be multifactorial. Some particular examples are presented by Gilani *et al.* (2012). Furthermore, the individual animal response plays a relevant role, which is rarely mentioned in the literature. This factor could lead to considerable differences (Qin *et al.*, 1996). In a certain way, this explains the inhomogeneous reaction of the animals observed when consuming JBN+SOR silage.

6.4.2 Apparent digestibility of proximate nutrients in raw cowpea-sorghum mixtures and corresponding silages

As presented in Table 54, ensiling increased ($P<0.05$) the apparent digestibility (AD) of AEE (26.0%), ADF_{OM} (16.8%) and CA (48.2%), whereas the digestibility of NDF_{OM} decreased (18.2%, $P<0.05$).

The AD of AEE was increased ($P<0.05$) after ensiling grains of wheat (51.6 %), triticale (46.0 %) and rye (19.3 %) (Hackl *et al.*, 2010) and in high moisture content silages of triticale and wheat (Pieper *et al.*, 2010), although the authors could not explain the reasons. Presumably, the AD of AEE was affected by the fatty acid transformation that may occur during ensiling (Alves *et al.*, 2011). It is known that LAB play an active role in the isomerization, hydration, dehydration, saturation and free fatty acid production (Ogawa *et al.*, 2005; Yadav *et al.*, 2007). However, the increase of free fatty acids corresponds to a linear decrease of fat digestibility in the same way when saturated fatty acids increase in relation to unsaturated fatty acids (NRC, 2012). About 1 % of additional CF in the diet decreases the AD of AEE from 1.3 to 1.5 % (NRC, 2012), but CF was rather the same in the raw mixture and in silages.

The reduction of ANF (see Table 51) during ensiling, particularly CT, seems to explain better the increase of AD for AEE. As it was previously documented in the present work, polyphenols (tannins) play a relevant role in interfering enzymatic activity. Tea polyphenols produce an inhibition of lipase (Gondoin *et al.*, 2010) reaching up to 54 % in a concentration of 0.05 $mg \cdot ml^{-1}$ tea (He *et al.*, 2007). Recently, Wang *et al.* (2014) reported a 34 % inhibitory rate of proanthocyanidins

(PC) on porcine lipase with a tendency to increase in proportion to PC concentration. The hydrolysis of CT when ensiling concerns not only polyphenol oxidase activity but as well microbial enzymes. *Lactobacillus* is well known as silage inoculant to improve lactic acid production. However, limited is the information concerning the role of LAB on ANF deactivation. The detanification capacity of *L. plantarum* (Rodriguez *et al.*, 2009; Natarajan & Rajendran, 2012; Ren *et al.*, 2013*),* for example, could be of better use. The influence of other ANF, not determined in the present study but known to affect enzymatic activity, could contribute to improve AD of AEE. Since they are expected to be reduced when ensiling, further researches should be conducted in this direction.

The increase of the ADF/NDF ratio due to hemicelluloses (Hcell) breakdown might explain the decrease of NDF digestibility in the silage compared to the raw mixture. Jaurena & Pichard (2001) agree with our report assuming Hcell as the most widely hydrolyzed fiber fraction when ensiling lucerne with the addition of barley or maize. They found NDF to reduce digestibility and assume the increase of ADF/NDF ratio as the main reason. McDonald *et al.* (1991) cited several authors that found as much as half of Hcell could be degraded during ensilage. They were persuaded that the hemicellulases present in the original herbage or in bacteria, as well as the hydrolysis by organic acids produced during fermentation are the possible reasons for Hcell breakdown. However, since it has been failed to demonstrate hemicellulase activity in bacteria isolated from silages and the sensibility of plant hemicellulases to low pH and temperature, the acid hydrolysis seems the most appropriate explanation. Whether the concentration of natural occurring acids in silages produce any Hcell breakdown could not be sustained (McDonald *et al.*, 1991). The capacity of *Lactobacillus spp.* to use the most common occurring Hcell (*e.g.* xylan, arabinoxylan and β-glucan) seems less probable (Crittenden *et al.*, 2002). However, it has been proved that they can metabolize hydrolysis products of hemicelluloses, e.g xylooligosaccharides and β-glucooligosaccharides, but not arabinoxylooligo-saccharides (Crittenden *et al.*, 2002; Falck *et al.*, 2013). More studies should be conducted to understand Hcell fermentation in silages.

Ensilage did not produce any effect ($P<0.05$) on OM digestibility. It is possible that labile fractions in NDF after hydrolysis during silage fermentation were conserved and later recovered as cellular content (Jaurena & Pichard, 2001).

Starch was highly digestible in the raw mixture and in the silage. It was remarkable how much starch was fermented during ensilage. In JBN+SOR and CWP+SOR silages were found about half the amount than in the raw mixture (Table 46). Other studies reported similar occurrences in cereal and grain silages (Hackl *et al.*, 2010; Pieper *et al.*, 2010; Gefrom *et al.*, 2013). Gefrom *et al.* (2013) assumed that LAB were the main responsible due to the significant starch reduction in inoculated treatments compared to not inoculated variants. Indeed, apart of plant amylases being involved in starch hydrolysis (Muck, 1988), amylolytic LAB have been identified, specifically *L. plantarum* (Reddy et al., 2008). Paradoxically, Pieper *et al.* (2010) stated that the strains of *L. plantarum* used, which were the same used by Gefrom *et al.* (2013) and Hackl *et al.* (2010) and in the present study, have not a proved starch hydrolytic capacity. Natural occurring fermentations in common beans and lentils report that starch reduction is associated to lower pH values (Granito *et al.*, 2002). Further studies should be conducted to identify natural occurring starch hydrolytic enzymes in JBN, CWP and SOR or in the associated epiphytic flora in JBN+SOR and CWP+SOR mixed silages.

7 Conclusions

Based on the methodological studies about ensiling tropical legume grains sole or mixed with sorghum, the following conclusions can be drawn, that are of special relevance for the future use of these feedstuffs for growing-finishing pigs:

1. The grains of Jack bean (JBN, Canavalia ensiformis [L.] DC) and cowpea (CWP, Vigna unguiculata [L.] Walp) var. INIFAT-93 have good nutritional characteristics but like most legumes they are high in buffering capacity (BC) and low in water soluble carbohydrates (WSC), which makes them difficult to ensile. Sorghum (SOR, Sorghum bicolor [L.] Moench) as a cereal grain has a more favorable ensilability due to a low BC, but the amount of WSC is apparently not high enough to obtain a sufficient WSC content in the tested mixtures of legume grains and SOR for achieving good ensilability characteristics.
2. According to the Rostock Fermentation Test (RFT), an in vitro test on ensilability, and Rostock model silages (ROMOS) JBN and CWP, either alone or mixed with SOR, show good fermentation qualities and can therefore be conserved as silages. However, the addition of LAB is decisive to improve silage quality, independent of the used pre-ensiling treatment.
3. Soaking as a pre-ensiling treatment reduces the BC in JBN as well as in CWP. Thereby, the values for BC are lower the longer the soaking time is and the bigger the soaking water volume is in respect of the grains. What remains unclear is the entire effect of the BC reduction on the ensilability provoked by soaking. As grains increase their moisture content passing the pre-ensiling treatment of soaking, high moisture silages are produced (DM < 65 %), what in general might be beneficial for ensilage due to a higher water activity compared to remoistened silages with a DM of 65 %.
4. Soaking leads to an undesired reduction of minerals. In both legume grains Ca, P, K and Mg are slightly leached by soaking, whereas Na and Cl apparently only decrease in CWP, but increase in JBN. As soaking is commonly reported to increase the bioavailability of minerals, the apparent drawbacks of soaking on the content of minerals should be studied more exactly.
5. Pre-germination prior to ensiling increases the content of water soluble carbohydrates (WSC), reaching a peak after 96 h in JBN and after 72 h in CWP. The illumination regime produces marginal differences in the WSC content and no

differences in the trypsin inhibitory activity (TIA) and the content of canavanine. The highest reduction of TIA and canavanine in the legume grains is observed already after 12 h and independent of the illumination regime.

6. Although a general reduction of anti-nutritional factors (ANF) due to the silage process can be expected, ensiling affects the content of the selected analyzed ANF in different ways. While a reduction of TIA is accompanied by an increasing storage time in CWP silages, the effect of ensilage on a TIA decrease is meager in JBN silages. On the contrary, HCN is reduced in JBN silages, but increased in every evaluated treatment of CWP silage after 60 days of storage. Canavanine is reduced in JBN silages with soaking as pre-ensiling treatment, whereas the effects of remoistening or pre-germination on the canavanine content are only marginal.
7. It is known that due to a wide range of ANF present in the grains of JBN the proportion of this legume has to be restricted in the diet in particular for monogastrics. However, it is expected that ensilage has a positive effect on the reduction of several ANF. Thus the rejection of the silages of soaked JBN mixed with sorghum by the pigs of the digestibility trials could indicate, that the extent of reduction of ANF in JBN through ensiling was insufficient.
8. In silages of soaked CPW mixed with SOR (to achieve 18 % CP of DM in the mix) ensiled with additives (MOL+LAB), only methionine showed a significantly higher ($P<0.05$) standardized ileal digestibility (SID) compared to the corresponding raw mixture. For the majority of the other tested amino acids and nitrogen an increase of SID was observed as a tendency ($P>0.05$), whereas lysine and cystine seemed to be unaffected.
9. Ensiling a mixture of pre-soaked CWP and SOR causes a remarkable increase of the digestibility of crude ash as well as AEE and ADF_{OM}, whereas the digestibility of OM and CP remains fairly unaffected. The contradictory effect of ensilage on the digestibility of NDF_{OM} needs to be further clarified.

8 Summary

8.1 Summary

The developing countries in tropical regions face a feed scarcity that negatively affects their livestock industry. The necessity to import most of the feedstuffs represents a barrier against the sustainability of the animal production. Many alternative feedstuffs have been widely studied, but until now they are only partial solutions.

Due to their agro-technical and nutritional characteristics, grains of Jack bean (JBN), cowpea (CWP) and sorghum (SOR) are assumed to be alternatives to conventional feedstuffs in pig feeding. However, as they are well known to contain anti-nutritional factors (ANF), their efficient use in diets for pigs is hampered. To face the problem of feed storage, cost-effective methods like ensilage are preferred, but are limited due to the generally low ensilability especially of the legume grains. For that reason, the aim of the present study was to find out if ensiling JBN and CWP grains sole or mixed with SOR would be an effective method, not only to guarantee that the nutritional value is preserved but moreover is improved for pig feeding by reducing the main ANF they contain.

Therefore, the ensilability of JBN and CWP grains was, as a first step, evaluated through an *in vitro* rapid fermentation test (RFT). Two mixtures with sorghum (to reach 20 or 24 % crude protein [CP] in the dry matter [DM]) were used and the effect of additives (lactic acid bacteria inoculant [LAB] and molasses [MOL]) was tested.

It was assumed that increasing the low content of water soluble carbohydrates (WSC) and decreasing the high buffering capacity (BC) in JBN and CWP would improve ensilability, whereas pre-germination (PGR) and soaking (SOK) as pre-ensiling treatment (PET) were thought to increment WSC and to reduce BC, respectively. The illumination regime and PGR time, in the case of PGR and soaking time and grain:water ratio (w/v) in the case of SOK were used as fixed factors.

Lab scale silages (ROMOS) were prepared to measure the effect of additives and mixing with SOR (18 % CP of DM) on the pH value and the main fermentation products (lactic acid, volatile fatty acids, alcohols and ammonia). As PET, moisture reconstitution to 65 % DM (MOR) as well as PGR and SOK were evaluated. Furthermore, the impact of storage time (STI) on the reduction of trypsin inhibitory activity (TIA), HCN (hydrogen cyanide) and canavanine (only for JBN-containing

silages) was determined in selected treatments with the best fermentation quality after 60 days.

Considering the results of model silages and some relevant issues under practical conditions, soaked grains (grain:water ratio of 1:4 [w/v]) and 24 h soaking) of JBN or CWP mixed with SOR (18 % CP of DM) with the addition of MOL and LAB were ensiled in plastic tons (120 l volume) for 60 days to be used in animal feeding trials to determine the standardized ileal digestibility (SID) and the apparent digestibility. For SID, minipigs with an ileal-rectal anastomosis were allocated in a Latin square design. The SID of essential amino acids in silages was calculated through a regression method and compared with the corresponding raw mixture. The determination of the apparent digestibility (AD) of selected nutrients (organic matter [OM], crude ash [CA], crude protein [CP], acid ether extract [AEE], crude fiber [CF], neutral detergent fiber [NDF_{OM}], acid detergent fiber [ADF_{OM}] and starch) was conducted with growing-finishing pigs, as well allocated in a Latin square design.

The main results can be summarized as follows:

1. The content of WSC increased as PGR time increased, independent of the illumination regime. After 96 h in JBN and 72 h in CWP, WSC contents were increased by 25 and 321 %, respectively. SOK reduced the BC in both JBN and CWP, which was most pronounced the longer the time and the higher the water volume for SOK were.
2. As a consequence of soaking both legume grains, Ca, P, K and Mg were slightly leached, whereas Na and Cl apparently only decreased in CWP, but increased in JBN. The apparent drawbacks of SOK on the content of minerals should be studied in more detail.
3. Analyzing the effect of PGR on TIA and canavanine, the highest reduction of TIA and canavanine occurred after 12 h independent of the illumination regime, which was in contrast to the WSC content.
4. The evaluated fermentation parameters in RFT and ROMOS revealed a good fermentation quality of JBN and CWP either alone or mixed with SOR, independent of the PET used. However, the use of LAB is recommended to guarantee an optimal fermentation.

5. In CWP silages an increasing STI was accompanied by a reduction of TIA, but the effect of ensilage on a TIA decrease was meager in JBN silages. On the contrary, HCN was reduced in JBN silages, but increased in every evaluated treatment of CWP silages after 60 days of storage. Canavanine was reduced in JBN silages with soaking as pre-ensiling treatment, whereas the effects of remoistening or pre-germination on the canavanine content were only marginal.
6. It is assumed that the extent of ANF reduction through ensiling JBN on larger scale in plastic tons was insufficient compared to model silages, as the animals refused the offered JBN diets during the experiments of SID and AD. However, the CWP ton silages were well consumed.
7. The SID of amino acids in silages compared to the corresponding raw mixture was found to be significantly higher ($P<0.05$) only for methionine. However, for the other tested amino acids and nitrogen an increase of SID was observed as a tendency ($P>0.05$), whereas lysine and cystine seemed to be unaffected.
8. Remarkable increases of the AD in silages compared to the raw mixture were found for CA as well as for AEE and ADF_{OM}, whereas the digestibility of OM and CP remained fairly unaffected by the ensiling process. Contradictory was the reduction of the NDF_{OM} digestibility after fermentation. Further experiments should be conducted to give an explanation for this finding.

8.2 Zusammenfassung

Die Entwicklungsländer der tropischen Gebiete sehen sich mit einem Mangel an Futtermitteln konfrontiert, der ihre Viehhaltung negativ beeinflusst. Die Nachhaltigkeit der Tierproduktion wird dabei maßgeblich durch die Notwendigkeit des Futtermittel-imports eingeschränkt. Die Vielzahl untersuchter alternativer Futterressourcen stellt bis zum heutigen Tag nur eine unzureichende Lösung dar.

Aufgrund ihrer agrotechnischen und ernährungsphysiologischen Eigenschaften werden die Körner von Jackbohne (JBN), Kuhbohne (CWP) und Sorghum (SOR) als Alternativen zu den konventionellen Futtermitteln in der Schweineernährung angesehen. Allerdings wird ihr effizienter Einsatz in Rationen aufgrund der Gehalte an anti-nutritiven Inhaltsstoffen (ANF) erschwert. Um dem Problem der Futterlagerung dieser Körner zu begegnen, sind kostengünstige Verfahren wie die

Silierung zu bevorzugen. Die im Allgemeinen geringe Silierbarkeit, insbesondere der Leguminosenkörner, steht dem jedoch im Wege. Daher war es das Ziel der vorliegenden Arbeit zu untersuchen, ob die Silierung von JBN und CWP Körnern alleine oder gemischt mit SOR eine geeignete Methode ist, um den Futterwert nicht nur zu erhalten, sondern darüber hinaus durch die Reduzierung der hauptsächlich vertretenen ANF zu verbessern.

In einem ersten Schritt wurde die Silierbarkeit von JBN und CWP Körnern in einem *in vitro* Fermentationstest (RFT) bestimmt. Dafür wurden zwei Mischungen mit SOR verwendet (um jeweils 20 oder 24 % Rohprotein [CP] in der Trockenmasse [DM] zu erzielen) und der Einfluss von Zusätzen (Milchsäurebakterienpräparat [LAB] und Melasse [MOL]) geprüft.

Es wurde davon ausgegangen, dass eine Erhöhung des geringen Gehaltes wasserlöslicher Kohlenhydrate (WSC) und eine Reduzierung der hohen Pufferkapazität (BC) in den Körnern von JBN bzw. CWP die Silierbarkeit verbessert, wobei angenommen wurde, dass als Vorbehandlungen (PET) die Vorkeimung (PRG) den Gehalt an WSC erhöht und das Einweichen (SOK) die BC senkt. Als fixe Effekte wurden bei PRG das Lichtregime und die Dauer der PRG und bei SOK das Verhältnis von Körnern zu Einweichwasser (w/v) und die Einweichdauer verwendet.

Um den Einfluss von Zusätzen und der Mischung mit SOR (18 % Rohprotein in der Trockenmasse) zu untersuchen wurden Silagen auf Labormaßstabsebene (ROMOS) angelegt und pH-Wert und Fermentationsprodukte (Milchsäure, flüchtige Fettsäuren, Alkohole und Ammoniak) bestimmt. Neben PRG und SOK als PET wurde eine Rückbefeuchtung der Körner auf 65 % DM geprüft. Weiterhin wurde in ausgewählten Silagevarianten mit den besten Fermentationsqualitäten nach 60 Tagen Lagerung der Effekt der Lagerdauer (STI) auf die Reduzierung der trypsinhemmenden Aktivität (TIA), HCN und Canavanin (nur in JBN Silagen) bestimmt.

Ausgehend von den Ergebnissen der Modellsilagen und praxisrelevanter Aspekte wurden in größerem Maßstab eingeweichte Körner (Körner:Wasser-Verhältnis von 1:4, 24 h eingeweicht) von JBN oder CWP gemischt mit SOR (18 % Rohprotein in der Trockenmasse) unter Zusatz von MOL und LAB in Plastiktonnen (120 l Volumen) für 60 Tage siliert, um in Fütterungsversuchen die praecaecale Verdaulichkeit (SID) und die scheinbare Verdaulichkeit (AD) zu bestimmen. Für die Bestimmung der SID wurden mit einer ileorektalen Anastomose ausgestattete Minipigs in einem

lateinischen Quadrat angeordnet. Die SID der essentiellen Aminosäuren der Silagen wurde mittels Regressionsmethode berechnet und mit der korrespondierenden nicht silierten Mischung verglichen. Die Ermittlung der AD ausgewählter Inhaltsstoffe (organische Substanz [OM], Rohasche [CA], Rohprotein [CP], Rohfett [AEE], Rohfaser [CF], neutrale Detergentienfaser [NDF_{OM}], saure Detergentienfaser [ADF_{OM}] und Stärke) erfolgte mit Mastschweinen, welche ebenfalls in einem lateinischen Quadrat angeordnet waren.

Die wesentlichen Ergebnisse können wie folgt zusammengefasst werden:

1. Mit zunehmender Dauer der PGR erhöht sich der Gehalt an WSC unabhängig vom Lichtregime, wobei die WSC Gehalte nach 96 h bei JBN um 25 % und nach 72 h bei CWP um 321 % zunahmen. Durch SOK verringerte sich die BC sowohl in JBN als auch in CWP. Die Reduzierung war ausgeprägter je länger die Einweichdauer und je größer die Wassermenge zum Einweichen waren.
2. In Folge des Einweichprozesses beider Leguminosenkörner wurden Ca, P, K und Mg geringfügig ausgewaschen, wohingegen der Gehalt an Na und Cl sich offensichtlich nur in CWP verringerte und dagegen in JBN zunahm. Die scheinbaren negativen Auswirkungen von SOK auf den Gehalt an Mineralstoffen sollten noch eingehender untersucht werden.
3. Bei der Beurteilung von PGR als Vorbehandlung trat nach 12 h unabhängig des Lichtregimes die größte Abnahme des Gehaltes an TIA und Canavanin auf, wohingegen sich der WSC Gehalt gegenläufig verhielt.
4. Die untersuchten Fermentationsparameter im RFT und in ROMOS bestätigten eine gute Fermentationsqualität von JBN und CWP sowohl alleine als auch im Gemisch mit SOR, unabhängig der angewendeten PET. Allerdings wird zur Sicherung einer optimalen Vergärung der Zusatz von LAB empfohlen.
5. Bei CWP Silagen ging eine steigende Lagerungsdauer einher mit einer Verringerung der TIA, wohingegen der Effekt der Silierung bei JBN Silagen gering war. Im Gegensatz dazu wurde der Gehalt von HCN in JBN Silagen reduziert, jedoch in jeder untersuchten Variante der CWP Silagen nach 60 Tagen Lagerdauer erhöht. Der Canavaningehalt wurde in JBN Silagen mit SOK als Vorbehandlung verringert, MOR und PRG hatten dagegen nur einen sehr geringen Einfluss.

6. Es wurde angenommen, dass das Ausmaß der Reduzierung der ANF in JBN Silagen, die im größeren Maßstab in Plastiktonnen hergestellt wurden, im Gegensatz zu den Modellsilagen unzureichend war, da die Tiere in den Fütterungsversuchen zur SID und AD die Aufnahme der Rationen verweigerten, die JBN enthielten. Die in Plastiktonnen silierten CWP wurden jedoch gut aufgenommen.
7. Die SID von Aminosäuren in Silagen war im Vergleich zur SID in der korrespondierenden nicht silierten Mischung nur für Methionin signifikant höher ($P<0.05$). Bei den weiterhin untersuchten Aminosäuren und bei Stickstoff wurde in der Tendenz eine Erhöhung festgestellt, wobei Lysin und Cystin scheinbar unbeeinflusst waren.
8. Bei der AD von CA, AEE und ADF_{OM} wurden bemerkenswerte Zunahmen in Silagen im Vergleich zu der korrespondierenden nicht silierten Mischung festgestellt, wobei die Verdaulichkeiten von OM und CP durch den Silierprozess offenbar nicht beeinflusst wurden. Widersprüchlich erscheint die Reduzierung der Verdaulichkeit von NDF_{OM} nach der Silierung. Um einen Erklärungsansatz zu finden, sollten hierzu weiterführende Untersuchungen durchgeführt werden.

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10 Table index

11 Figure index

12 Appendix

Table A1: The pH kinetic during incubation of cowpea grains sole or mixed with sorghum grains in the Rostock Fermentation Test (n=3)

	0 h	14 h	18 h	22 h	26 h	38 h
CWP	6.37^{abA} ±0.00	5.77^{cB} ±0.06	5.80^{dB} ±0.07	5.57^{dC} ±0.09	5.31^{dD} ±0.13	5.50^{cC} ±0.06
CWP+MOL	6.40^{cA} ±0.04	5.84^{cB} ±0.05	5.77^{dB} ±0.05	5.22^{cC} ±0.02	5.00^{bcD} ±0.06	4.99^{bD} ±0.10
CWP+SOR (20 %)	6.38^{bcA} ±0.00	5.79^{cB} ±0.04	5.59^{cB} ±0.04	5.04^{bC} ±0.06	4.92^{bC} ±0.17	4.95^{bC} ±0.25
CWP+SOR (24 %)	6.38^{abA} ±0.01	5.89^{cB} ±0.13	5.77^{dB} ±0.09	5.66^{dB} ±0.22	5.17^{cdC} ±0.30	4.97^{bC} ±0.01
CWP+LAB	6.36^{abA} ±0.00	5.18^{aB} ±0.06	4.45^{aC} ±0.06	4.29^{aD} ±0.03	4.18^{aE} ±0.02	4.02^{aF} ±0.02
CWP+LAB+MOL	6.37^{abA} ±0.00	5.37^{bB} ±0.07	4.58^{bC} ±0.04	4.24^{aD} ±0.02	4.19^{aD} ±0.02	4.04^{aE} ±0.01
CWP+LAB+SOR (20 %)	6.35^{aA} ±0.00	5.18^{aB} ±0.04	4.46^{aC} ±0.04	4.24^{aD} ±0.01	4.10^{aE} ±0.01	3.99^{aF} ±0.01
CWP+LAB+SOR (24 %)	6.36^{abA} ±0.01	5.10^{aB} ±0.07	4.46^{aC} ±0.03	4.30^{aD} ±0.03	4.17^{aE} ±0.03	4.01^{aF} ±0.03

^{abc} Mean values with different superscripts in the same column differ significantly (P<0.05).
^{ABC} Mean values with different superscripts in the same row differ significantly (P<0.05).
CWP, cowpea; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum; 20 %, addition of sorghum to reach 20 % crude protein of DM in the mix; 24 %, addition of sorghum to reach 24 % crude protein of DM in the mix

Table A2: The pH kinetic during incubation of Jack bean grains sole or mixed with sorghum grains in the Rostock Fermentation Test (n=3)

	0 h	14 h	18 h	22 h	26 h	38 h
JBN	6.10^{cA} ±0.01	5.99^{dAB} ±0.02	5.73^{dB} ±0.04	5.15^{dC} ±0.30	4.89^{cCD} ±0.29	4.62^{D} ±0.20
JBN+MOL	6.14^{fA} ±0.00	6.02^{dA} ±0.01	5.73^{dA} ±0.22	5.10^{dB} ±0.49	4.77^{bcB} ±0.31	4.51^{C} ±0.25
JBN+SOR (20 %)	6.13^{eA} ±0.00	5.91^{cB} ±0.04	5.55^{cC} ±0.04	4.99^{cdD} ±0.07	4.58^{abE} ±0.04	4.50^{F} ±0.04
JBN+SOR (24 %)	6.11^{dA} ±0.00	5.99^{dB} ±0.01	5.73^{dC} ±0.03	5.28^{dD} ±0.05	4.78^{bcE} ±0.02	4.56^{F} ±0.01
JBN+LAB	6.07^{aA} ±0.01	5.93^{cB} ±0.01	5.51^{cC} ±0.02	4.89^{bcdD} ±0.05	4.63^{abcE} ±0.02	4.51^{F} ±0.02
JBN+LAB+MOL	6.09^{bA} ±0.00	5.92^{cB} ±0.03	5.43^{cC} ±0.08	4.65^{abcD} ±0.06	4.43^{aE} ±0.02	4.35^{F} ±0.00
JBN+LAB+SOR (20 %)	6.11^{cdA} ±0.01	5.74^{aB} ±0.03	4.91^{aC} ±0.02	4.48^{aD} ±0.02	4.43^{aE} ±0.01	4.33^{F} ±0.01
JBN+LAB+SOR (24 %)	6.10^{cA} ±0.00	5.78^{bB} ±0.03	5.07^{bC} ±0.01	4.58^{abD} ±0.02	4.44^{aE} ±0.01	4.37^{F} ±0.01

^{abc} Mean values with different superscripts in the same column differ significantly (P<0.05).
^{ABC} Mean values with different superscripts in the same row differ significantly (P<0.05).
JBN, Jack bean; LAB, lactic acid bacteria; MOL, molasses; SOR, sorghum; 20 %, addition of sorghum to reach 20 % crude protein of DM in the mix; 24 %, addition of sorghum to reach 24 % crude protein of DM in the mix

Table A3: Differences (P<0.05) in silage dry matter content using a model with pre-treatment, legume grain, additives and sorghum inclusion as fixed effects

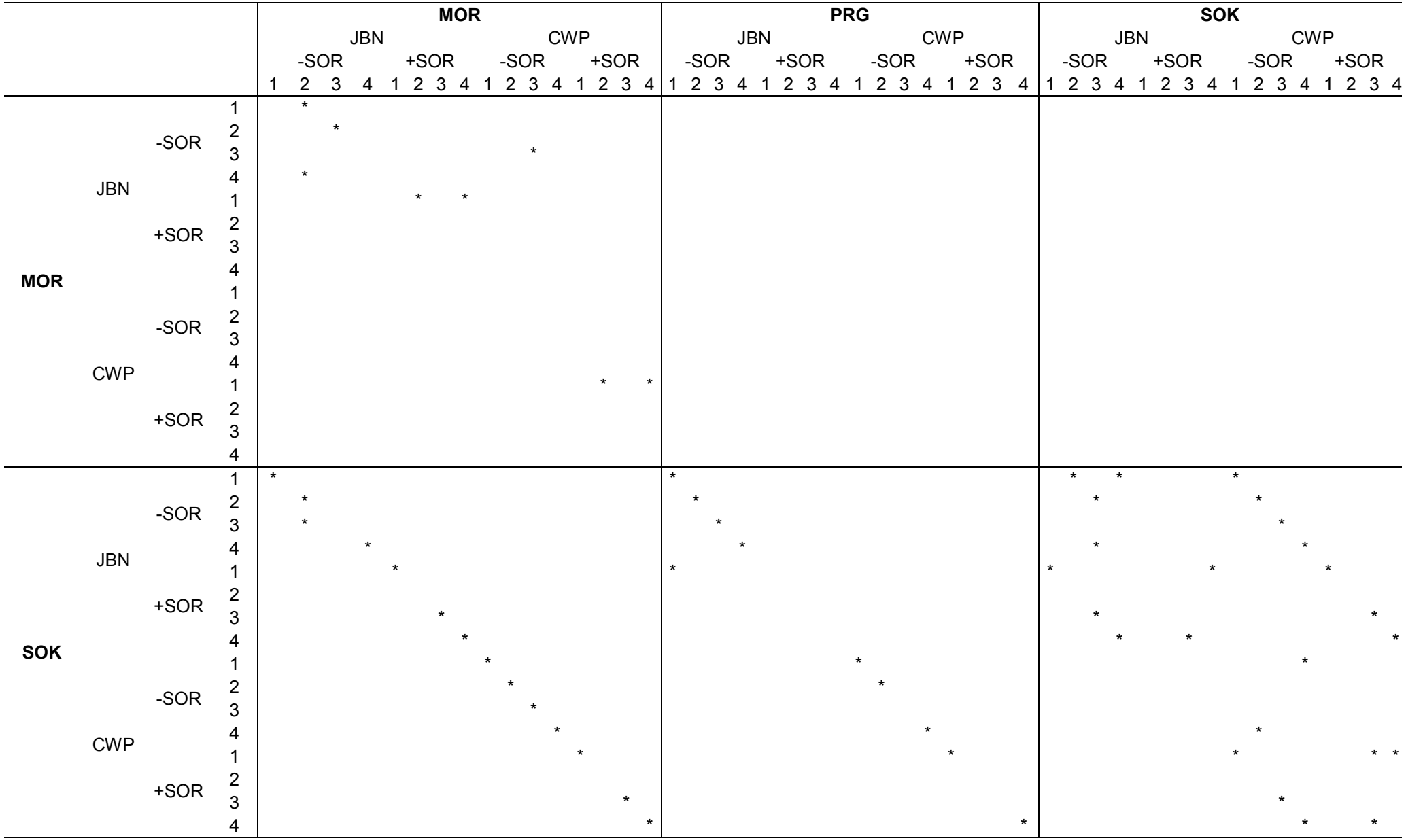


Table A3 (continued)

				MOR																PRG												SOK							
				JBN								CWP								JBN								CWP				JBN							
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR				-SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PRG	JBN	-SOR	1	*																*		*																	
			2		*																				*	*													
			3			*																																	
			4				*														*				*		*												
		+SOR	1					*												*				*				*											
			2						*												*								*										
			3							*												*									*								
			4								*											*							*										
	CWP	-SOR	1									*																											
			2										*																											
			3																																				
			4												*																								
		+SOR	1													*									*														
			2														*								*														
			3															*																					
			4																*																				

* Significant difference for P<0.05.

Treatments: 1, control; 2, molasses; 3, lactic acid bacteria; 4, molasses and lactic acid bacteria

CWP, cowpea; JBN, Jack bean; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; -SOR, without the addition of sorghum; +SOR, with sorghum in the mix

Table A5: Differences (P<0.05) in silage pH value using a model with pre-treatment, legume grain, additives and sorghum inclusion as fixed effects

				MOR																PRG												SOK							
				JBN								CWP								JBN								CWP				JBN							
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR				-SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
MOR	JBN	-SOR	1			*	*																																
			2			*							*																										
			3																																				
			4		*																																			
		+SOR	1						*	*	*					*																							
			2		*																																			
			3																																				
			4						*																															
	CWP	-SOR	1											*	*																								
			2											*																										
			3																																				
			4										*																										
		+SOR	1																																				
			2										*																										
			3																																				
			4																																				
SOK	JBN	-SOR	1	*																*													*		*					
			2		*																													*						
			3			*																*																		
		+SOR	1						*																									*						
			2																																					
			3																															*						
			4																																					
	CWP	-SOR	1					*																												*		*		
			2										*													*														
			3																																					
			4																								*													
		+SOR	1												*																									
			2																																					
			3																																					
			4																																					

Table A5 (continued)

				MOR								PRG								SOK							
				JBN				CWP				JBN				CWP				JBN				CWP			
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
			1										*		*												
		-SOR	2		*									*													
			3																								
			4											*					*								
	JBN		1					*				*															
		+SOR	2														*										
			3											*													
			4														*										
PRG			1								*																
		-SOR	2																								
			3																								
			4																								
	CWP		1													*											
		+SOR	2														*										
			3																								
			4																*								

* Significant difference for P<0.05.

Treatments: 1, control; 2, molasses; 3, lactic acid bacteria; 4, molasses and lactic acid bacteria

CWP, cowpea; JBN, Jack bean; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; -SOR, without the addition of sorghum; +SOR, with sorghum in the mix

Table A6: Differences (P<0.05) in silage lactic acid content using a model with pre-treatment, legume grain, additives and sorghum inclusion as fixed effects

Lactic acid as

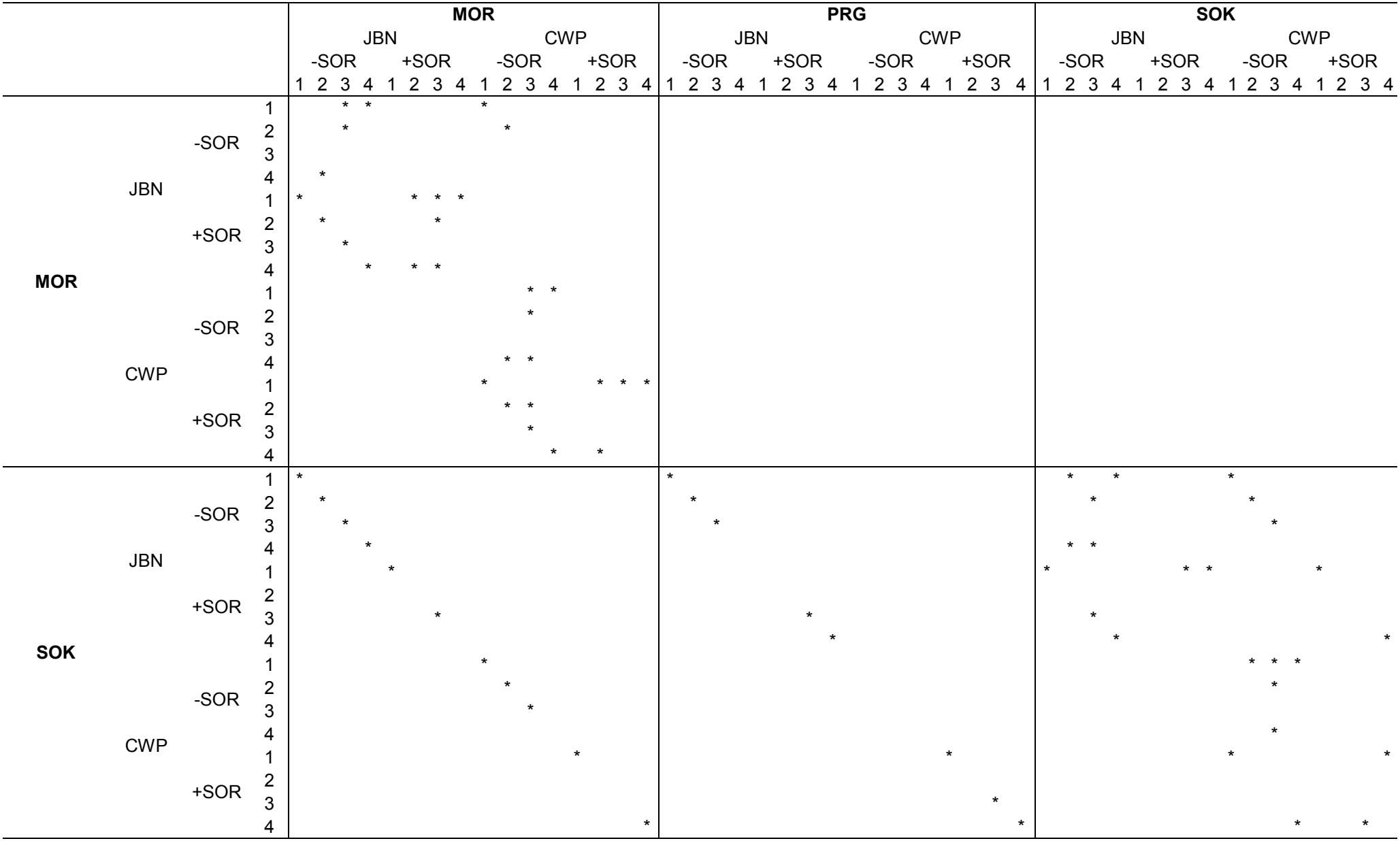


Table A6 (continued)

				MOR								PRG								SOK							
				JBN				CWP				JBN				CWP				JBN				CWP			
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PRG	JBN	-SOR	1	*									*		*												
			2		*									*													
			3																								
			4				*						*	*													
		+SOR	1					*									*	*									
			2										*				*										
			3							*																	
			4								*			*		*											
	CWP	-SOR	1																								
			2																								
			3																								
			4																								
		+SOR	1																								
			2																								
			3																								
			4																								

* Significant difference for P<0.05.

Treatments: 1, control; 2, molasses; 3, lactic acid bacteria; 4, molasses and lactic acid bacteria

CWP, cowpea; JBN, Jack bean; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; -SOR, without the addition of sorghum; +SOR, with sorghum in the mix

Table A4: Differences (P<0.05) in silage fermentation losses using a model with pre-treatment, legume grain, additives and sorghum inclusion as fixed effects

				MOR								PRG								SOK							
				JBN						CWP		JBN						CWP		JBN						CWP	
				-SOR				+SOR		-SOR		-SOR				+SOR		-SOR		-SOR				+SOR		-SOR	
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
MOR	JBN	-SOR	1		*								*																									
			2			*																																	
			3																																			
		+SOR	4																																			
			1								*																											
			2		*														*																			
			3																																			
	CWP	-SOR	4																																			
			1																																			
			2																																			
			3																																			
			4																																			
		+SOR	1																																			
			2																																			
			3																																			
			4																																			
SOK	JBN	-SOR	1																																			
			2		*																																	
			3			*																																
		+SOR	4																																			
			1																	*																		
			2																																			
			3																			*																
			4																		*																	
	CWP	-SOR	1																																			
			2								*																											
			3																																			
			4																																			
		+SOR	1																																			
			2																																			
			3																																			
			4																																			

Table A4 (continued)

				MOR								PRG								SOK							
				JBN				CWP				JBN				CWP				JBN				CWP			
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PRG	JBN	-SOR	1																								
			2		*																						
			3			*																					
			4																								
		+SOR	1									*					*		*								
			2										*														
			3											*													
			4														*	*									
	CWP	-SOR	1								*																
			2																								
			3																								
			4																								
		+SOR	1																								
			2																								
			3																								
			4																								

* Significant difference for P<0.05.

Treatments: 1, control; 2, molasses; 3, lactic acid bacteria; 4, molasses and lactic acid bacteria

CWP, cowpea; JBN, Jack bean; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; -SOR, without the addition of sorghum; +SOR, with sorghum in the mix

Table A7: Differences (P<0.05) in silage ethanol content using a model with pre-treatment, legume grain, additives and sorghum inclusion as fixed effects

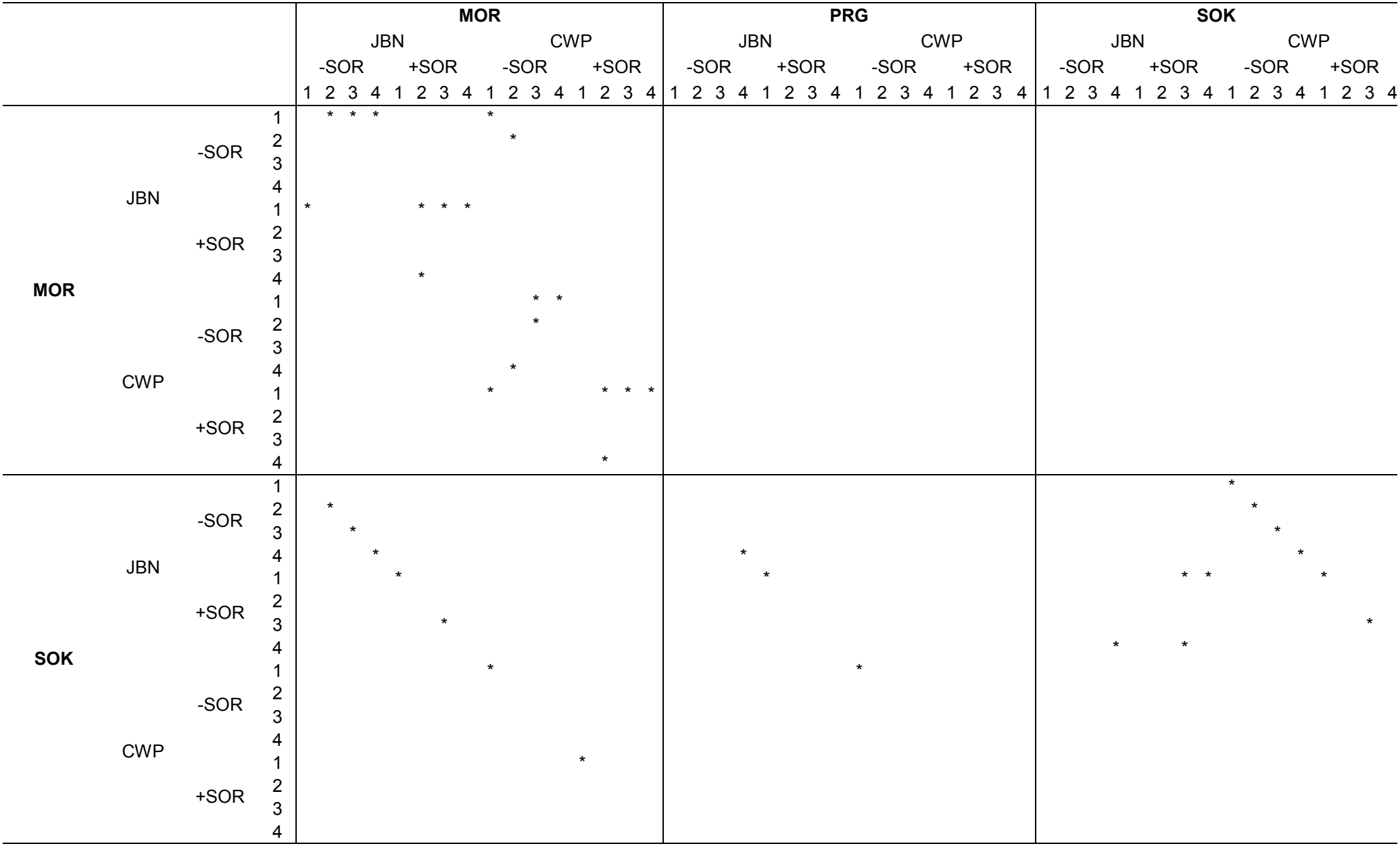


Table A7 (continued)

				MOR								PRG								SOK							
				JBN				CWP				JBN				CWP				JBN				CWP			
				-SOR				+SOR				-SOR				+SOR				-SOR				+SOR			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PRG	JBN	-SOR	1										*		*												
			2		*										*												
			3			*																					
			4												*												
		+SOR	1					*				*					*		*								
			2										*														
			3												*												
			4											*				*									
	CWP	-SOR	1																								
			2																								
			3																								
			4								*																
		+SOR	1																								
			2																								
			3																								
			4																								

* Significant difference for P<0.05.

Treatments: 1, control; 2, molasses; 3, lactic acid bacteria; 4, molasses and lactic acid bacteria

CWP, cowpea; JBN, Jack bean; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; -SOR, without the addition of sorghum; +SOR, with sorghum in the mix

Table A10: Differences (P<0.05) in HCN content of Jack bean silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

			MOR								PRG								SOK							
			LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB	
			0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60
MOR	LAB	0																								
		60	*																							
	MOL+LAB	0																								
		60		*	*																					
	SOR+LAB	0	*		*																					
		60		*		*	*																			
	SOR+MOL+LAB	0	*		*																					
		60		*		*			*																	
SOK	LAB	0	*								*								*							
		60		*								*								*						
	MOL+LAB	0			*								*													
		60				*								*							*					
	SOR+LAB	0					*								*				*		*					
		60						*								*				*		*	*			
	SOR+MOL+LAB	0							*								*		*		*					
		60								*								*		*		*			*	

Table A10 (continued)

			MOR								PRG								SOK							
			LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB	
			0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60
PRG	LAB	0																								
		60		*							*															
	MOL+LAB	0																								
		60				*						*	*													
	SOR+LAB	0									*		*													
		60						*				*			*											
	SOR+MOL+LAB	0									*		*													
		60								*		*			*		*									

* Significant difference for P<0.05.

LAB, lactic acid bacteria; MOL, molasses; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; SOR, sorghum

0, day 0 before ensiling; 60, day 60 of storage

Table A11: Differences (P<0.05) in HCN content of cowpea silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

			MOR								PRG								SOK							
			LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB	
			0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60
MOR	LAB	0																								
		60	*																							
	MOL+LAB	0																								
		60			*																					
	SOR+LAB	0																								
		60		*		*	*																			
	SOR+MOL+LAB	0																								
		60		*		*			*																	
SOK	LAB	0	*								*								*							
		60		*								*														
	MOL+LAB	0			*								*													
		60				*								*							*					
	SOR+LAB	0					*								*											
		60						*								*				*		*	*			
	SOR+MOL+LAB	0							*								*									
		60								*								*		*		*				*	

Table A11 (continued)

			MOR								PRG															
			LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB		LAB		MOL+LAB		SOR+LAB		SOR+MOL+LAB	
			0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60	0	60
PRG	LAB	0																								
		60		*							*															
	MOL+LAB	0																								
		60				*								*												
	SOR+LAB	0																								
		60						*				*				*		*								
	SOR+MOL+LAB	0																								
		60								*						*				*						

* Significant difference for P<0.05.

LAB, lactic acid bacteria; MOL, molasses; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; SOR, sorghum

0, day 0 before ensiling; 60, day 60 of storage

Table A8: Differences (P<0.05) in TIA of Jack bean silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

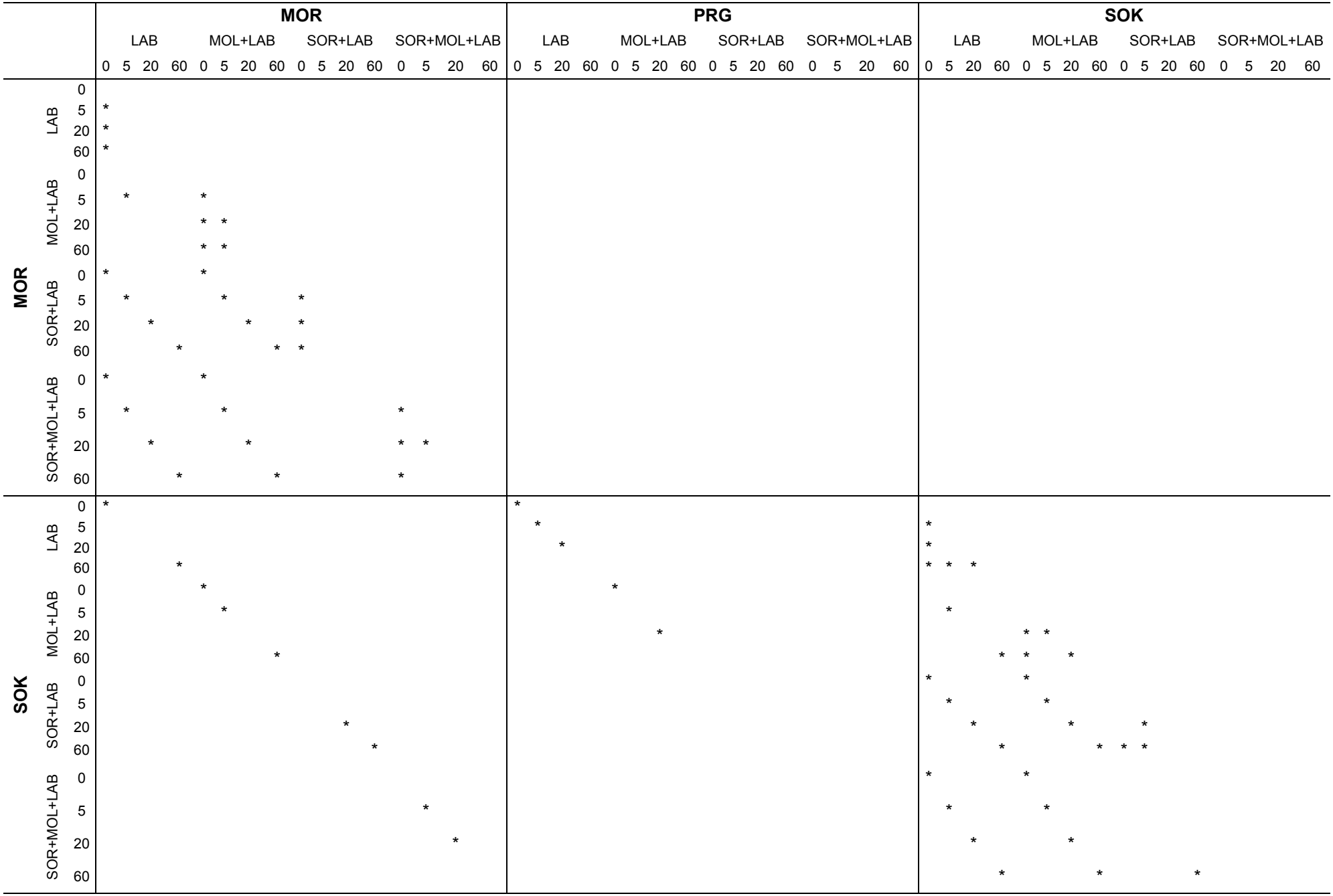
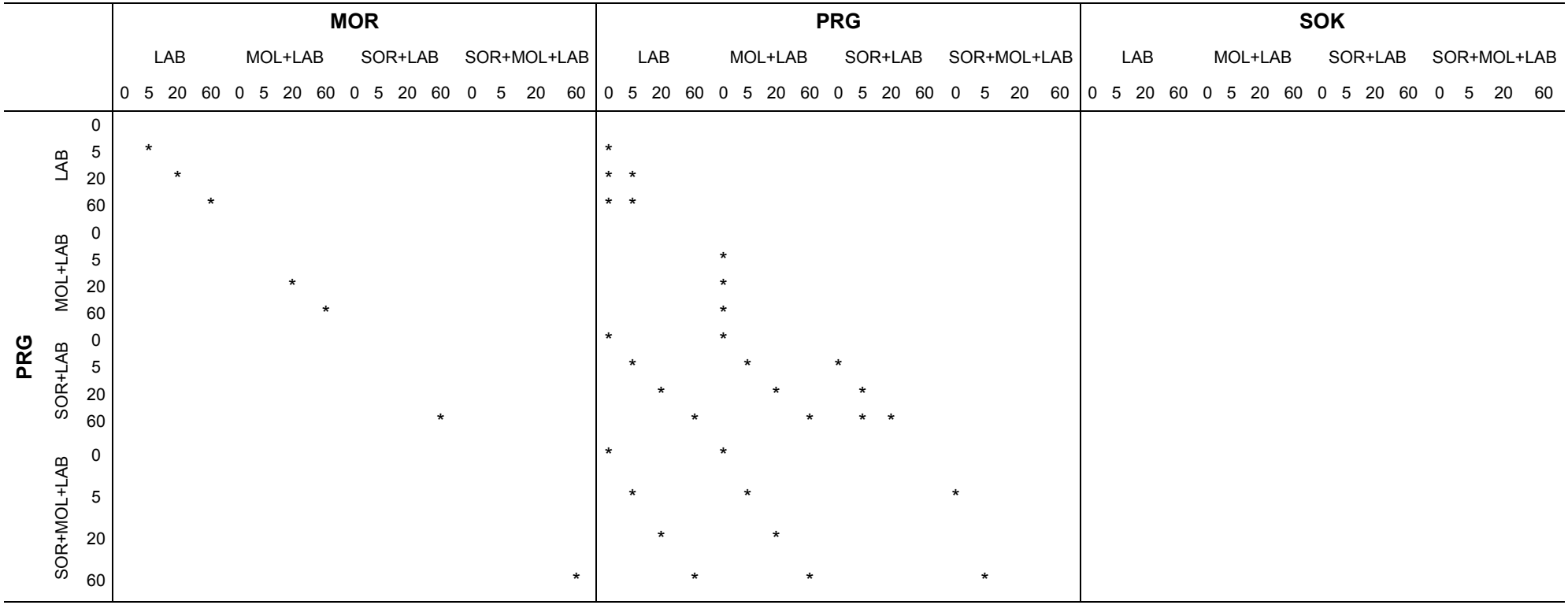


Table A8 (continued)



* Significant difference for P<0.05.

LAB, lactic acid bacteria; MOL, molasses; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; SOR, sorghum

0, day 0 before ensiling; 5, day 5 of storage; 20, day 20 of storage; 60, day 60 of storage

Table A9: Differences (P<0.05) in TIA of cowpea silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

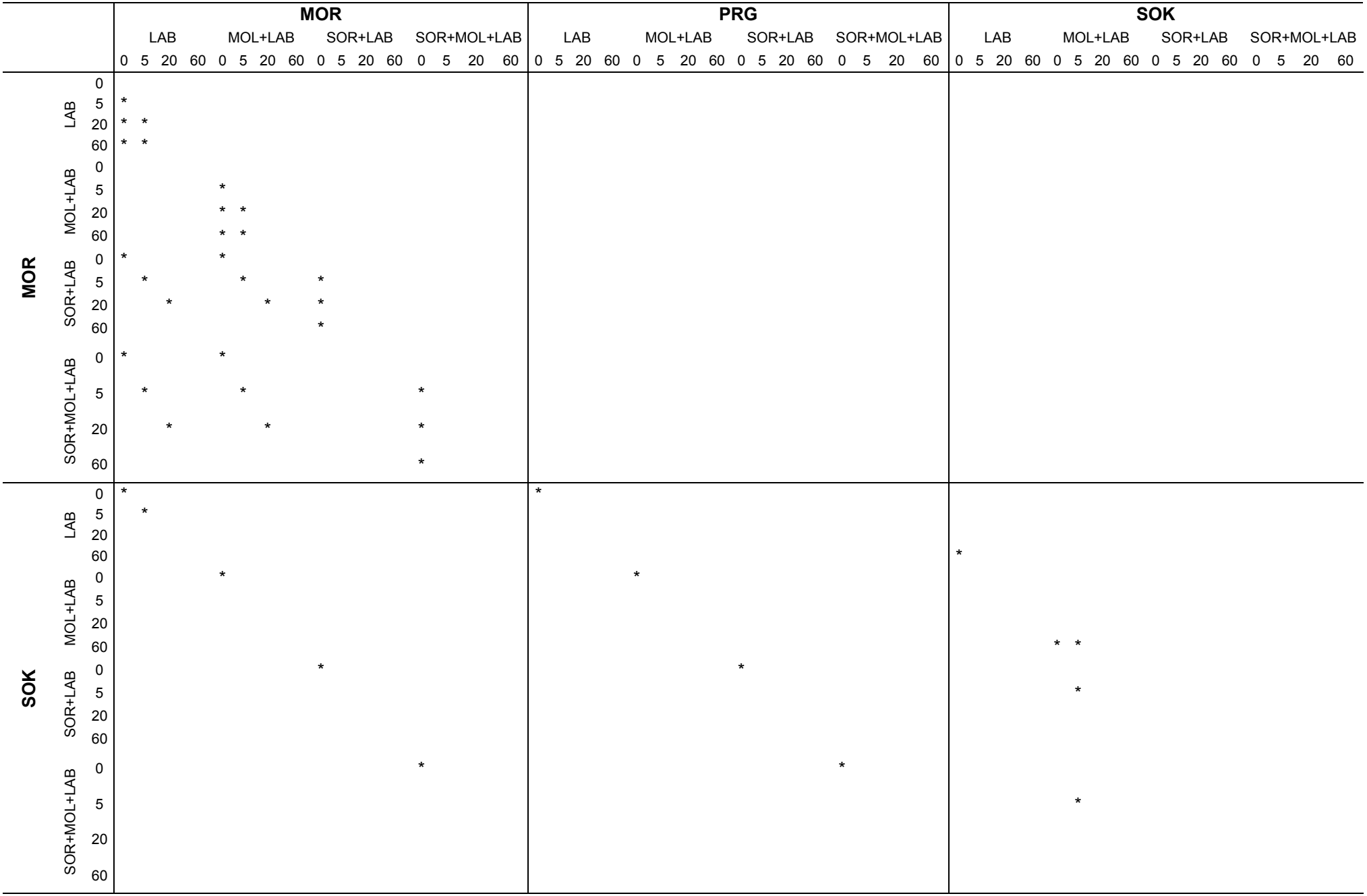


Table A9 (continued)

PRG		MOR																PRG																SOK															
		LAB				MOL+LAB				SOR+LAB				SOR+MOL+LAB				LAB				MOL+LAB				SOR+LAB				SOR+MOL+LAB				LAB				MOL+LAB				SOR+LAB				SOR+MOL+LAB			
		0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60	0	5	20	60
LAB	0																																													
	5																	*																													
	20																	*																													
	60																	*	*																												
MOL+LAB	0																																													
	5																					*																							
	20																					*																							
	60																					*	*																							
SOR+LAB	0																	*				*																								
	5																		*					*					*																	
	20																								*																				
	60																																													
SOR+MOL+LAB	0																	*				*																								
	5																		*					*																						
	20																								*																				
	60																																													

* Significant difference for P<0.05.

LAB, lactic acid bacteria; MOL, molasses; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; SOR, sorghum

0, day 0 before ensiling; 5, day 5 of storage; 20, day 20 of storage; 60, day 60 of storage

Table A12: Differences (P<0.05) in canavanine content of Jack bean silages using a model with pre-ensiling treatment, treatment and storage time as fixed effects

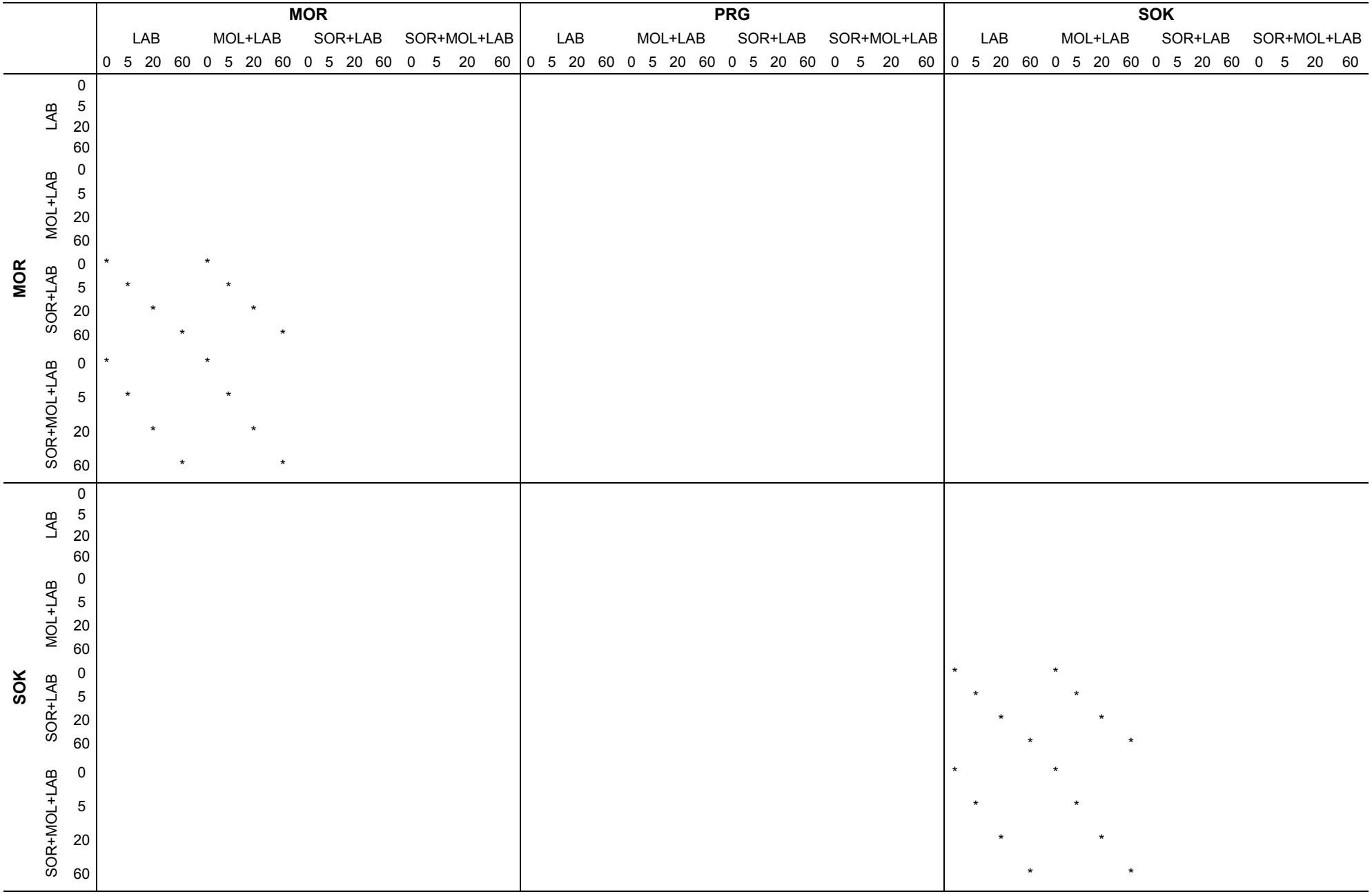
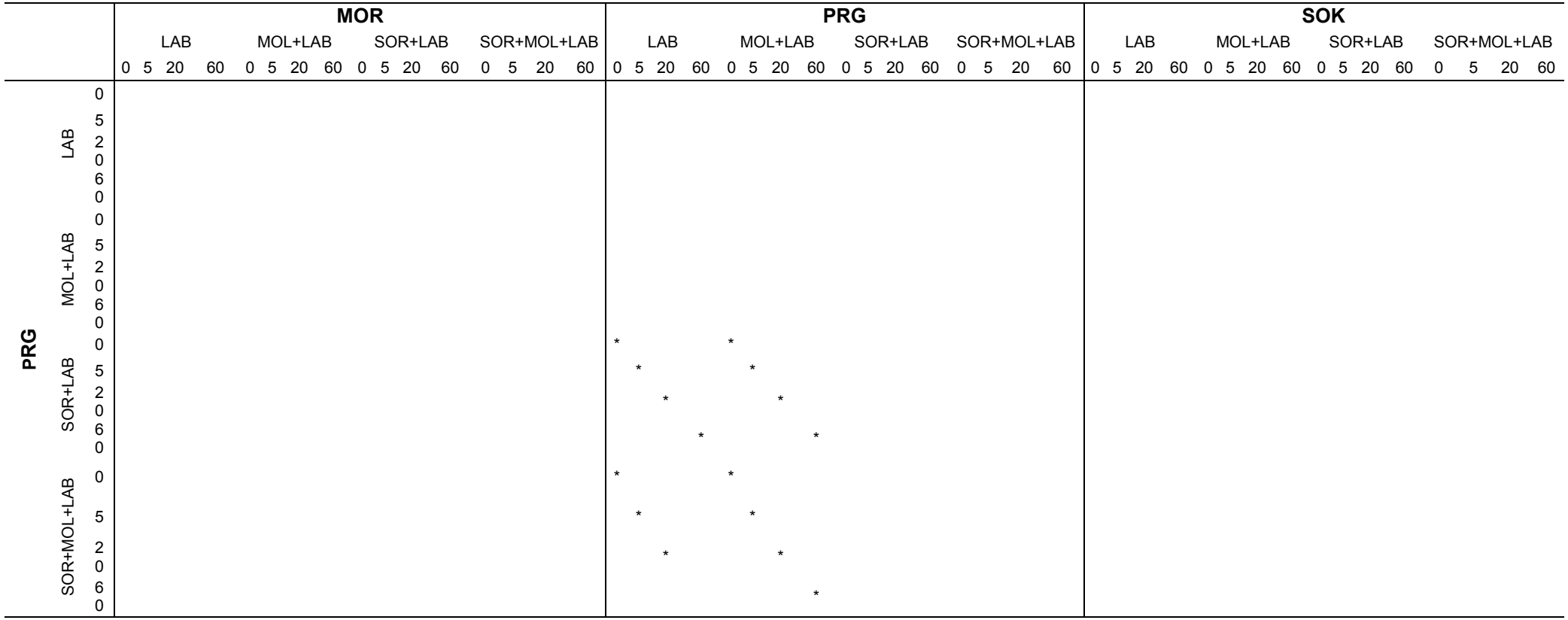


Table A12 (continued)



* Significant difference for P<0.05.

LAB, lactic acid bacteria; MOL, molasses; MOR, moisture reconstituted; PRG, pre-germinated; SOK, soaked; SOR, sorghum

0, day 0 before ensiling; 5, day 5 of storage; 20, day 20 of storage; 60, day 60 of storage

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel genutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, im September 2014

Acknowledgement

I could not imagine that this would be the most difficult chapter to write. I could fill pages with the names of people that in one way or another supported me in bringing this thesis to the end.

In first place, I would like to express my infinite gratitude to my former advisers Dr. Francisco Diaz Casas† (Pancho) and Dr. Leandro Marrero Suarez for leading me through the exciting universe of knowledge and science. I enjoyed every second you let me take part in your scientific discussions and I appreciate all the support, patience and advises you passed on me. You were the first to encourage me in starting this project and helped me in making it better in our frequent brainstorming meetings. Thanks to Dr. Andres Castro Alegría for continuing what they started.

I am grateful to Prof. Martin Gabel for offering me the opportunity to start my doctoral studies at the Chair for Nutrition Physiology and Animal Nutrition, University of Rostock and to Dr. Edda Sandmann and Dr. Wolfgang Hackl for their selfless help.

I also thank Prof. Annette Zeyner for allowing me to continue my PhD study and research and for assuming my tutorship. Her guidance helped me in all the time of research and writing of this thesis.

The support of Prof. Ellen Kienzle and Prof. Sven Dänicke in completing special lab analysis was of great value. Thanks go as well to Prof. Norbert Reinsch, Dr. Gerd Nürnberg and especially to Dr. Armin Tuchscherer for the immeasurable support in the statistical processing of the data.

I appreciate all the support I got from Sabine Bremer, Diana Gall and Margitta Beck in completing my chemical analysis. Thanks go as well to Dr. Kirsten Büsing, Heike Riese and Heino Marx for the assistance with the animal experiments. My German was a mess, so was your Spanish language, but seemed to work in the end. I want to thank Helga Strehlow and Anja Kofahl for making me feel at home. Thanks to the rest of the staff of the professorship for their encouraging words, they were of good help.

I acknowledge my colleagues at the Centro de Investigaciones Agropecuarias and experimental stations (Universidad Central de Las Villas) for their technical support and many others that helped with planting, keeping, harvesting and transporting the plant material used in the experiments. We all know how hard those activities in our conditions are. Especially I would like to mention Dora Quintana† for showing me the shortcuts in the techniques of chemical analysis of feedstuffs and for being a friend.

I want to thank my parents and sisters for their unconditional love. Even when I was far apart from you, I could feel you pushing in my back. As well, I want to express my sincere gratitude to my wife. Only we both know how much love, healing words and support from your side were necessary to keep me in one piece and get the power to bring this project to the end.

Theses

“Ensilage of Jack bean and cowpea grains sole or mixed with sorghum to improve their nutritional value as feedstuff for growing-finishing pigs”

submitted by Dipl. vet. med. Luis Alberto González Díaz

Research objectives

1. Due to the high prices of feedstuffs in the world market, local and profitable solutions should be suggested to recover the weakened swine husbandry in tropical developing countries, especially Cuba.

2. Until today, alternative strategies had failed in offering a feedstuff able to cover all the animals´ requirements. The protein content remains as the limiting nutrient, which requires supplementation with synthetic amino acids or other conventional sources. Furthermore, alternative feedstuffs often have low dry matter contents, which hinders their conservation and storage as drying is expensive.

3. Because of a high nutritional quality, native legume grains like Jack bean (JBN) and cowpea (CWP) and cereal grains like sorghum (SOR) are reliable ingredients in integral rations for pigs. A necessary condition is that most of the relevant anti-nutritional factors (ANF) present in those grains must be deactivated or at least reduced.

4. In contrast to the storage of air-dry grains, lactic acid fermentation in silages of JBN and CWP sole or mixed with SOR can contribute not only to prevent losses during storage (e.g. plagues and diseases) provoked by the high humidity and temperature in the tropics. Moreover, ensilage can contribute to increase the nutritional value of grains and additionally it has a beneficial effect on the reduction of ANF.

5. As is known, the low content of water soluble carbohydrates and the generally high buffering capacity of legumes lower their ensilability. Pre-ensiling treatments like pre-germination and soaking are assumed to have a positive effect by

increasing the content of water soluble carbohydrates and reducing the buffering capacity, respectively.

6. The use of silages in pig feeding is rarely reported. Hence, information is limited about the standardized ileal digestibility of essential amino acids and the apparent digestibility of selected nutrients, especially of ensiled JBN or CWP grains mixed with SOR.

Major outcomes and conclusions

7. Soaking in water was revealed as a simple method to reduce the buffering capacity in JBN and CWP grains. The buffering capacity is reduced by almost half in CWP and by approx. 30 % in JBN in respect of the not soaked grains. The physical characteristics of the seeds, mainly the hull, may play an important role for the final result. For this reason soaking apparently affected JBN and CWP in a different way.

8. As a consequence of soaking, the moisture content in JBN and CWP is drastically increased, although the crude protein content (% DM) is unaffected. Ash (% DM) is susceptible to be reduced the longer the soaking time and the higher the amount of water (grain:water ratio [w/v]) is. Equations for JBN and CWP describe well this phenomenon:

$BC_{(JBN)} = 0.804a - 0.180b$ $\quad r^2 = 0.678$ $\quad SE = 0.175$ $\quad n=48$

$BC_{(CWP)} = 0.892a - 0.177b$ $\quad r^2 = 0.827$ $\quad SE = 0.151$ $\quad n=48$

a = grain:water ratio (g fresh beans·l^{-1})
b = soaking time (h)
BC = buffering capacity (g lactic acid·g^{-1} dry matter)

9. Coinciding with the treatments where the buffering capacity was reduced most, a group of minerals was found to be leached by soaking (Ca, P, K, Cl, Na and Mg). However, it is not yet clear if that reduction might be compensated with an increase of mineral bioavailability that soaking is known to produce.

10. Pre-germination produces an increase ($P<0.05$) of the content of water soluble carbohydrates of 25 % in JBN and 321 % in CWP after 96 and 72 h, respectively. Therefore, pre-germination represents an alternative to the addition of a readily available source of water soluble carbohydrates like molasses, when legume grains like JBN and CWP are conserved by ensiling. The illumination regime during germination does not play a significant role in the final quantity of water soluble carbohydrates.

11. An expected reduction of canavanine, one of the major ANF in JBN with the highest influence on feed intake depression, was not observed in this study. There was no accordance with the literature that claimed pre-germination a means of reducing canavanine and that this effect is more visible under light regime. On the other hand, in model silages (ROMOS) with soaked JBN, canavanine was reduced as the storage time was prolonged. Outstanding was the treatment JBN+SOR+MOL+LAB (Jack bean mixed with sorghum and addition of molasses and a lactic acid bacteria inoculant) with a reduction of approx. 55 % after 60 days of storage compared with the corresponding not ensiled raw mixture. The question remains why in the same silage treatment when scaled up for the animal feeding trial, the content of canavanine remained on the same level after ensiling. It is assumed that the different storage conditions of the ton silages and ROMOS had an influence.

12. Of the analyzed ANF, the trypsin inhibitory activity (TIA) was the most susceptible to be reduced by ensiling. As storage time was prolonged from 5 to 20 and 60 days the lowest TIA was observed in CWP. In moisture reconstituted (65 % DM) silages and those with pre-germinated grains TIA was reduced by 72 to 80 % compared with not ensiled material. The treatment CWP+SOR+MOL+LAB (cowpea mixed with sorghum and addition of molasses and a lactic acid bacteria inoculant) showed the lowest TIA after 60 days. In JBN, remoistened silages showed the highest reduction (44 %) of TIA already after 5 days for JBN+MOL+LAB. In pre-germinated JBN silages TIA reduction was never higher than 30 % and even not altered in silages mixed with sorghum. In soaked JBN silages TIA increased, whereas the treatment JBN+SOR+MOL+LAB showed the lowest increase (18 %).

13. The reasons why JBN containing diets produce a poor animal performance are still controversial as pigs reject diets containing JBN even after deactivation of ANF took place. Presumably a low palatability is given higher priority rather than the later effects ANF produce in the animal. However, taste masking had failed in solving the problem. The content of HCN has as well been associated to feed intake depression, principally due to its bitter taste. In this work, there was the belief ensiling could be a way to reduce HCN and to improve palatability. Unfortunately, in the case of CWP the HCN content was higher for every evaluated treatment after ensiling. In JBN silages, HCN was reduced by approx. 17 % in the treatment JBN+LAB (Jack bean with addition of a lactic acid bacteria inoculant) in moisture reconstituted (65 % DM) silages and by approx. 41 % in JBN+MOL+LAB (Jack bean with addition of molasses and a lactic acid bacteria inoculant) in pre-germinated silages. The addition of SOR increased HCN by 10 - 25 %. Only in JBN+SOR+MOL+LAB (pre-germinated JBN) HCN was slightly reduced (5 %).

14. A taste masking effect, that lactic acid fermentation is expected to have, did not occur when JBN grains were ensiled, as animals under experiment refused to eat the diets containing JBN silages. Further studies should aim at finding the reasons for the low palatability of JBN.

15. Ensiling contributes to an increased standardized ileal digestibility for essential amino acids, although only for methionine a higher digestibility (increased by 24 %, $P<0.05$) compared to the corresponding raw mixture was observed, for which the reduction of ANF due to the ensiling process might be among the reasons. Still remains unclear why ensiling affected the digestibility of amino acids differently, as for example the standardized ileal digestibility of cystine seemed to be unaffected.

16. Ensiling a mixture of soaked CWP and SOR with the addition of molasses and a lactic acid bacteria inoculant increases ($P<0.05$) the apparent digestibility of crude ash, as well as of AEE and ADF_{OM}, whereas the digestibility of the organic matter and crude protein remains fairly unaffected. Contradictory seems to be the fact that the digestibility of NDF_{OM} was reduced after ensiling. This might be

explained by the hydrolysis of hemicelluloses that took place during ensiling, which correspond to the high digestible fiber fraction in pigs.

17. Both pre-ensiling treatments, soaking and pre-germination, can be seen as time-consuming and work-intensive in addition to what is already laborious itself, the ensiling process. Nevertheless, this is compensated by a rather simple technology and a low demand for infrastructure what makes the pre-ensiling treatments accessible to producers of all levels, what together with ensiling is a cost-effective alternative to the expensive and high energy-consuming conserving methods offered by the market.

Future outlook

18. In order to understand better how soaking affects the buffering capacity, other variables like pH, temperature and hardness of the water should be considered. As the available sources of water around the world have different physical characteristics, the variation produced by these parameters would affect the accuracy of the equations to predict the buffering capacity after soaking.

19. It is a concern to study the leaching effect soaking produces on minerals, although soaking water could be recycled as drinking water, so that leached minerals will be recovered. Further experiments should be conducted to understand how soaking transforms the bioavailability of minerals. Already some hints are given, as ensiling soaked CWP mixed with SOR improved the apparent digestibility of crude ash by approx. 48 % compared to the raw mixture.

20. The deactivation of selected ANF during pre-ensiling treatments or ensilage correlated to the time the grains were pre-treated or silages were stored. As the content of some ANF remained almost unaltered (canavanine in JBN) or even increased (HCN in CWP), the effect of longer pre-ensiling and/or storage time on the deactivation of these ANF might broaden the knowledge about the influence of ensiling.

21. Many of the decomposition products of ANF are well described in the literature. These substances can produce an anti-nutritive effect on the animal that in some

cases might be different to the effect of the original ANF. However, it is still unclear if the same decomposition products were formed during ensiling than described for other deactivation methods, which requires further experiments.

22. It was not possible to clarify why the aerobic stability of JBN+SOR silage prepared for animal trials was higher compared to CWP+SOR silage. As both silages showed a rather similar fermentation pattern, unidentified factors present in JBN or formed during ensiling could be an explanation. Therefore, the possible role certain ANF play in the aerobic stability, especially those ones in JBN that were not significantly affected by ensiling like canavanine, should be studied.