UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di BIOMEDICINA COMPARATA E ALIMENTAZIONE

SCUOLA DI DOTTORATO DI RICERCA IN: SCIENZE ANIMALI E AGROALIMENTARI

INDIRIZZO: ALLEVAMENTO, ALIMENTAZIONE, AMBIENTE,

BENESSERE ANIMALE E QUALITA' DEI PRODOTTI

CICLO XXV

NUTRITIONAL VALUE OF CANOLA EXPELLERS PRODUCED "ON FARM" BY COLD EXTRACTION OF OIL USED AS BIO FUEL

Direttore della Scuola : Ch.mo Prof. Martino Cassandro

Coordinatore d'indirizzo: Ch.ma Prof.ssa Lucia Bailoni

Supervisore :Ch.ma Prof.ssa Lucia Bailoni

Dottorando : Matteo Guadagnin

Con il sudore della mia fronte

ho annaffiato le mie idee!

Table of Contents

Summary

The outline of the thesis

The present PhD thesis includes a general introduction about the importance of a new approach to animal feeding reducing competition with energy production crop and food production. The use of canola expellers (CE) in this contest can be interesting. So the contributes of thesis were aimed to evaluate the nutritional aspects of CE obtained by cold extraction in small plants. In the first contribute the stability of residual oil in CE was evaluated at different temperatures and times of storage: In the second contribute CE was compared with other protein sources using a fully automated gas production system. In third and fourth contributes CE was included in ruminant diets and tested with two different *in vitro* methods.

Abbreviation:

aNDF = neutral detergent fibre

ADF = acid detergent fibre

CP = crude protein

CE = canola expeller obtained on farm by cold extraction

DM = dry matter

EE = ether extract

FA = fatty acids

GP = gas production

H = high level of supplement inclusion in the diets

L = low level of supplement inclusion in the diets

MN = microbial nitrogen

N = nitrogen

NAN = non ammonia nitrogen

NDF = neutral detergent fibre

NDFd = neutral detergent fibre degradability

SBM = soybean meal

SVO = straight vegetable oil

TDMd = true dry matter degradability

VFA = volatile fatty acids

WSS = whole soybean seed

Abstract

General aim of this thesis was to study canola expellers (CE) extracted by cold pressing in a small plant (on farm) and to evaluate the validity to use this by-product in ruminant feeding. The thesis includes the results of four experimental contributes: the first one aimed to evaluate the stability of CE at different times and temperatures of storage in order to determine if the conditions usually found in the farms, especially during the hot season, can result in changes in fatty acids profile and in some oxidative parameters. Results found that under different temperatures (12, 24, and 36°C) and times of storage (10, 20, and 30 d), CE maintained a good oxidative stability, as evidenced by low peroxide values (< 10 mEq/kg fat) in all samples collected, by negative response for Kreis test and by low changes in fatty acids profile. From these results it could be hypothesized that the storage of these by-products did not change the characteristics of the lipid fraction. In the second contribute *in vitro* gas production (GP) values obtained from the incubation of CE, whole soybean seed (WSS) and soybean meal (SBM) were compared, incubating feed samples with two *media* containing N-rich buffer or N-free buffer, in order to compare the effect of the availability of feed as unique protein source. Results of the experiment showed that CE is an easily degradable protein source. In the first hours of incubation with limiting N availability, higher gas production was recorded compared to the two soybeans. On this basis, CE could be interesting in diets at low protein content, currently suggested in order to reduce nitrogen excretion. No toxic effects on the microbial yield were observed during the fermentation of the three different feeds. The third contribute compared four diets formulated for beef cattle, based on corn silage and containing WSS or CE as protein sources at two different inclusion levels, in order to obtain 15 and 11 % CP of DM in the diets. Diets were tested using Rusitec fermenter. Compared to WSS, CE provided greater

NDF degradability ($P < 0.01$), produced less acetate and propionate ($P < 0.001$) but more butyrate and branched-chain VFA. The total VFA production was similar for the two protein sources. With regard to nitrogen balance, CE showed greater ^{15}N enrichment in the non-ammonia N ($P < 0.01$) and nominally lower values of microbial N derived from ammonia compared to WSS ($P = 0.06$). At high inclusion level, the ^{15}N enrichments for ammonia N, non-ammonia N and total bacteria N were also greater than observed at low inclusion levels ($P < 0.001$). In conclusion, the two feeds showed different fermentation patterns. The manipulation of dietary protein level seemed to lead primarily to a variation of bypass protein, without effects on the synthesis of microbial N.

In the fourth contribute the same diets tested in Rusitec fermenter (third contribute) were evaluated using RF system (Ankom Technology, Macedon, NY, USA) in order to evaluate their gas production kinetics. Results showed that both NDFd and TDMd values were greater ($P<0.05$) for CE diets compared to WSS, confirming the results obtained with Rusitec and as expected were lower ($P=0.04$) for L compared to H diets. Compared to WSS, CE inclusion in the diets increased the rate of GP ($P<0.05$;) but did not affect the total amount of GP. The reduction of CP level in the diets from 15 to 11% decreased the rate of GP without effects on total GP. Ammonia content increased ($P<0.01$), as expected, with the level of dietary CP. In conclusion, when diets with low CE levels are used, the inclusion of rapeseed cake in replacement to soybean seeds could improve the rate of degradation during the first hours of fermentation. In general CE obtained by cold extraction on farm could be an interesting feed in ruminant feeding with economical and environmental benefits.

Riassunto

Obiettivo generale di questa tesi è stato quello di studiare e valutare il panello di colza (CE) estratto a freddo in impianti aziendali di piccole dimensioni per un potenziale utilizzo nell'alimentazione dei ruminanti. Nella tesi sono riportati i risultati di quattro prove sperimentali: nel primo contributo è

stata valutata la stabilità della frazione lipidica del CE a temperature diverse e per diversi periodi di tempo al fine di valutare se la conservazione in condizioni anche particolari (durante la stagione estiva) in azienda, possa modificare il profilo degli acidi grassi e alcuni parametri di ossidazione lipidica. I risultati hanno evidenziato che a diverse temperature (12, 24, e 36°C) e tempi di stoccaggio (10, 20, e 30 d), CE ha mantenuto una buona stabilità ossidativa, come evidenziato dai bassi valori del numero di perossidi (<10 $mEqO_2$/kg grasso), dal test di Kreis sempre negativo, e dalle scarse variazioni del contenuto di acidi grassi. Da questi risultati preliminari si può ipotizzare che lo stoccaggio aziendale per i panelli sottoprodotti ottenuti dal colza in azienda, non determina grosse variazioni della componente lipidica.

Nel secondo contributo sono state valutate, *in vitro,* le produzioni di gas prodotti da campioni di CE e da semi di soia integrale incubando i questi alimenti con due differenti *media*: uno ricco in a N e uno senza N in modo da confrontare l'andamento delle fermentazioni quando l'unica fonte di N risulta l'alimento. I risultati hanno evidenziato che CE è una fonte proteica rapidamente degradabile; in caso di diete ipoproteiche come quelle suggerite per ridurre l'escrezione azotata, ,la sua inclusione potrebbe favorire l'attività microbica ruminale. Non sono sati rilevati effetti tossici sulla microflora ruminale durante la fermentazione dei due alimenti.

Nel terzo contributo sono state confrontate *in vitro* quattro diete per bovini da carne a base di silomais con 2 livelli di inclusione di CE e WSS, in modo da ottenere un livello di proteina grezza paria al 15% e all'11% PG sulla sostanza secca, . In questa prova è stato utilizzato il sistema semicontinuo di fermentazione Rusitec. Le diete contenenti CE hanno mostrato una maggior (P <0.01) degradabilità dell'NDF , e prodotto meno (P <0.01) acetato e propionato ma più butirrato e acidi grassi ramificati. La produzione totale di AGV non è risultato diversa tra le due fonti proteiche. Il bilancio dell'N ha mostrato un maggior quantità (P <0.01) di arricchimento in ^{15}N nell'azoto non ammoniacale e valori tendenzialmente (P = 0.06) inferiori di N microbico derivato dall'uso di ammoniaca rispetto alle diete con inclusione di WSS. Nelle diete ad alto livello di inclusione i valori di arricchimento in ^{15}N delle varie frazioni azotate sono risultati, come atteso, più

alti (P <0.01) rispetto a quelle a basso livello di inclusione. In conclusione i due supplementi hanno mostrato andamenti fermentativi molto diversi. I due diversi livelli di inclusione hanno influito principalmente sulla disponibilità di proteina by pass senza effetti sulla sintesi microbica.

Nel quarto contributo sono state testate, con la tecnica della gas production, le stesse quattro diete usate nel precedente esperimento. E' stato utilizzato il sistema RF Ankom® per testare la cinetica della produzione di gas nel corso della fermentazione. I risultati hanno mostrato che sia i valori di degradabilità dell'NDF che della SS sono stati maggiori (P < 0.05) per le diete contenenti CE rispetto a quelle con WSS e, come atteso, sono risultati inferiori nella diete a basso livello di inclusione delle due fonti proteiche. Le diete CE sono state caratterizzate da una produzione oraria di gas superiore (P < 0.05) in, ma non è variata la quantità totale di gas prodotto. La riduzione del livello di CP da 15all' 11% SS ha diminuito il tasso di produzione di gas ma non la quantità totale.

Il contenuto di ammoniaca nel liquido ruminale al termine dell'incubazione è risultato più alto (P < 0.001) nelle diete ad alto livello di inclusione. Concludendo possiamo affermare che con diete a basso livello di proteina, l'uso di CE in sostituzione alla soia, può migliorare la velocità di degradazione durante le prime ore di fermentazione. In generale, il panello di colza ottenuto per estrazione a freddo in azienda potrebbe essere un alimento interessante nell'alimentazione dei ruminanti con effetti favorevoli sia dal punto di vista economico che ambientale.

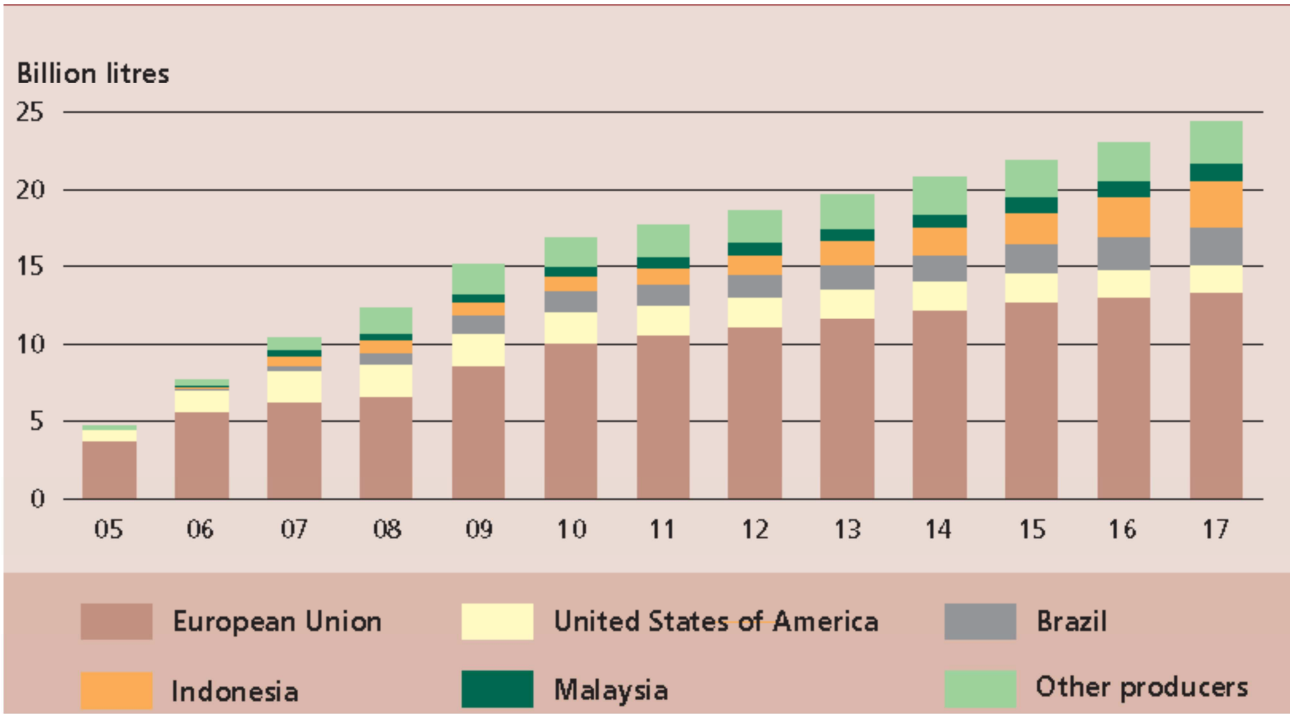
Chapter 1

General introduction

In 2030 the world will need produce 50% more energy than today, as argued by International Energy Agency report (IEA, 2007), especially due to the increasing demands of some countries, such as China and India. The most prediction models report that the peak of oil extraction has

already occurred (Duncan and Youngquist, 1999; Deffeyes, 2008), with only a few studies predicting that the peak will be occur in 2020 (Atabani *et al.*, 2012). Limited availability of oil, increasing demand for energy and growing problems related to environmental pollution have encouraged many governments in the world to support agriculture to grow crops for oil production. Biodiesel can be used in engines in substitution to diesel with minimal differences in performance reported and very low harmful emissions and when considering its whole life-cycle, the net emission of carbon dioxide is very low (Fazal *et al.*, 2011; Silitonga *et al.*, 2011). For these reasons, biodiesel crops are a very interesting economic aspect of agriculture (Carraretto *et al.*, 2004; Cetinkaya *et al.*, 2005). Production of biodiesel is growing in order to reduce environmental pollution and due to the increasing cost of petroleum derived fuels. In 2007, 10 billion litres of biodiesel were produced in the world, an increase of 1100% compared to 2000 (FAO, 2008). Europe is the world leader in biodiesel produced by oleaginous crops with over 10 billion of liters produced.

Figure 1. Biodiesel production in the world projected to 2017 (FAO, 2007)



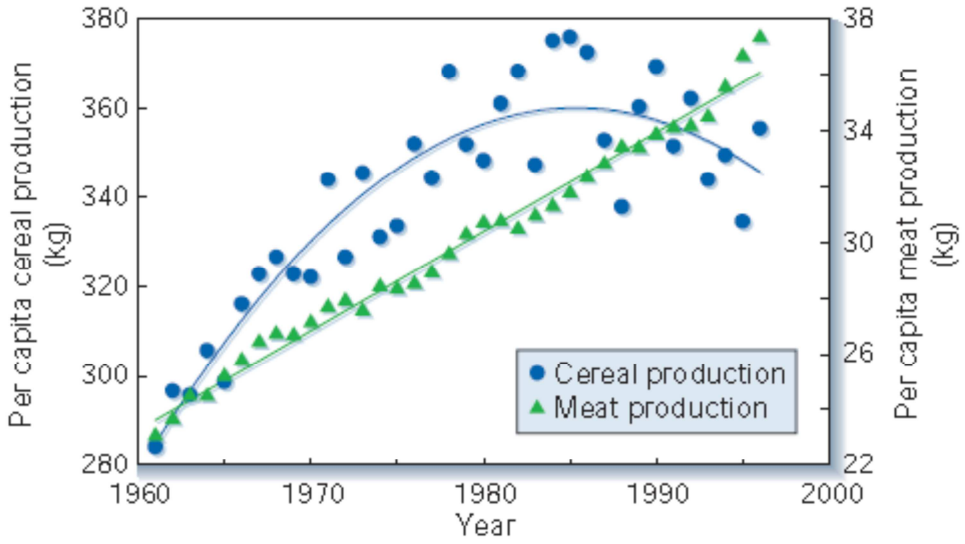
Due to the increasing price of fossil fuel and with concerns of public opinion about environmental pollution, the limit of biodiesel production is unknown. Some concerns were expressed by experts about its sustainability, especially when agriculture is converted to “energy production” from “food production”. Agriculture has historically produced the “fuel” to feed animals since man started to use animals as tools to work: two centuries ago, around 20 percent of the agricultural area in the United States of America was used to feed working animals (Sexton *et al*., 2007). Today agriculture is working again to produce energy as fuel for machines, but in this case it is the use of by-products that in many process of fuel production (i.e. oil extraction from oleagineuses) are obtained. Now the use of crops could not only be for energy production, but also for animal nutrition. The by-products obtained from bio-fuel production could be available for animal feeding. There are few studies published about this topic.

1.1 Food and feed demand

The processes of food production, preservation and distribution are very energy consuming and for this reason, they contribute to CO_2 emission. Moreover, consumers in developed countries demand safe and high quality food that has been produced with minimal impact on the environment (De Boer, 2003). The world population continues to grow and great pressure is being placed on arable land, water, energy and biological resources to provide an adequate supply of food whilst crucially maintaining the ecosystem (Poritosh *et al.,* 2009). More than 99% of the world’s food supply comes from land, while less than 1% is from water resources. In 2003, production of cereals, fruits and vegetables, and meat was reported to be 2,085,774, 1,345,056, and 253,688 thousand tons respectively. It is expected the world population will grow from 6.3 billion today to 8.9-12 billion in 2050 (Cohen, 2003). The question is: will the earth be able to provide enough resource and food to satisfy all the demands in the future? Demographic growth will consequently have an increasing food demand. As argued by Tilman *et al.* (2002) will result from a projected 2.4 fold increase in per

capita real income and from dietary shifts towards a higher proportion of meat (much of it grain fed) (Figure 2).

Figure 2. Long term trends, cereals and meat production (Tilman *et al.*, 2002)



Therefore the world will require an increased agricultural output. These will be major problems if there is competition between agriculture for food and for energy production, as already seen in developed countries. Competition and expansion of agriculture for bioenergy may lead great land use change resulting in loss of biodiversity (Volpi, 2010).

1.2 Technical aspect of bio-diesel and Stright Vegetable Oil (SVO) production

By-products from biodiesel extraction are produced by oil deprivation of oil seeds. The most common crops used for oil extraction are soybean and rapeseed. Soybean production averaged 175 million tons per year from 1998 to 2002, followed by 37 million tons of rapeseed, and 24 million tons of sunflower seeds (Mattson *et al.*, 2004). There are several methods to produce biodiesel: direct use and blending, micro-emulsions, thermal cracking (pyrolysis) and transesterification. The most commonly used process is transesterification of vegetable oils (Fangrui and Milford, 1999).

The most common method to extract oil is a mechanical-chemical method that usually includes several processes:

• seed cleaning

• seed pre-conditioning and flaking

• seed cooking

• pressing the flake to mechanically remove a portion of the oil

• solvent extraction of the press-cake to remove the remaining oil

• desolventizing and toasting of the meal.

Mechanical-chemical extraction of oil is a very efficient method because it leaves a very small amount of residual oil in the meal (less than 2%) and high protein content. The method of extraction has an effect on the quality of by-products, especially due to the use of high temperatures which have a role on degradation of anti-nutritional factors and on protein quality. Oil extraction by chemical methods do not permit the use of by-products (meals) in organic chains, due to the possibility of residual solvents.

Other methods to obtain oil and consequently by-products (expellers) for animal feeding are exclusively mechanical methods. Mechanical methods can be performed with or without pre-heating of seeds before pressing, and the seeds are usually single or double pressed. Pressing plants are usually of large scale when used in industrial systems and include the pre-heating of seeds. Small scale “on farm” plants usually employ cold pressing systems in order to obtain straight vegetable oil (SVO) without pre-heating of seeds.

Cold pressing is a relatively cheap process and can be implemented on the farm in order to use oil as fuel for modified tractor engines. However, the use of SVO in engines has resulted in mechanical problems limited of its use as a diesel fuel substitute. Developing new technologies in engine building has reduced the potentially damaging impacts of SVO in engines (Fore *et al.*, 2011). Modification of tractor engines is not very expensive and many companies are building several machine models “oil ready”.

Many researchers have studied the feasibility of an integrated on farm system of SVO production as an alternative to the use of biofuel produced by large scale plants as large scale production of biofuels is not sustainable and cannot be an alternative to fossil fuels. As mentioned above, the economic and environmental advantages are only possible using small scale plants, where SVO is produced and consumed, with low cost for the farmers (Grau *et al.,* 2010). Production of SVO is less expensive compared to biodiesel, due to several factors. The most important among these are the reduced chemical input as methanol (for chemical extraction), electricity and labor (Fore *et al.*, 2011).

1.3 Characteristics of by-products

Many food-industry and bio-energy processes produce a large amount of by-products. By-product feedstuff is defined as a product that can be used in animal feeding and is obtained during the harvesting or processing of a commodity in which human food or fibre is derived. By-product feedstuffs can be obtained from plant or animal sources. Growing interest in identifying and quantifying by-product feedstuffs is due to the desire to understand and monitor environmental wastes in developed and developing countries (Fadel, 1999). There are many by-products derived by industries characterized by region and countries, including agricultural crops, remaining feedstuffs, human food and their production. The processing or harvesting of selected commodities were shown to generate tremendous tonnage of plant by-product feedstuffs. Animals can convert many by-product feedstuffs into human edible products and reduce animal waste. Assuming the by-products need to be disposed of and are generated even in the absence of an animal industry, thereby providing an important and sustainable use of natural resources, As showed in Table 1, by-products are produced in high quantities and they can be a massive source if fed to animals.

Table 1. By-product feedstuffs DM produced in 1993 expressed as 103 metric tons (MT) and kg/capita (Fadel, 1999)

By-product feedstuffs	Argentina	Egypt	Kenya	Mexico	Korea Rep	China	USA	World
Miscellaneous								
Almond hulls	1	0	0	0	0	31	668	2,251
Bagasse	1,229	895	345	3,442	0	4,307	2,311	80,252
Beet pulp	0	34	0	0	0	406	1,101	10,947
Brans	984	1,790	186	1,953	856	32,352	6,121	106,441
Brewers grains	38	1	13	161	13	466	872	4,005
Citrus pulp	78	70	0	122	24	240	517	2,300
Cottonseed, whole	0	0	0	0	0	2,506	2,067	5,656
Molasses	228	100	52	505	0	82	1,372	9,231
Miscellaneous total (10^3 MT)	2,557	2,890	596	6,183	893	40,389	15,030	221,084
Miscellaneous (kg/capita)	75	48	23	70	20	34	57	40
Cakes								
Soybean	80	383	2	1,933	1,281	6,682	20,254	67,097
Groundnut	26	0	0	10	2	1,123	146	4,472
Sunflower seed	139	21	7	67	22	256	368	7,303
Rape and mustard seed	10	0	0	238	477	2,199	596	12,002
Cottonseed	3	194	1	142	324	749	1,162	7,952
Palm kernel	0	0	0	2	0	21	0	1,971
Copra	0	0	1	56	39	6	1	1,681
Sesame seed	0	0	0	3	15	144	0	710
Miscellaneous cakes	10	0	23	116	150	652	219	7,408
Corn Germ Meal	13	0	0	63	73	57	289	2,441
Corn Gluten feed and meal	39	0	0	243	254	56	252	10,236
Soap stock oils	3	4	0	18	18	74	88	831
Cakes total (10^3 MT)	322	602	34	2,891	2,658	12,019	23,374	124,105
Cakes (kg/capita)	10	10	1	33	60	10	89	22
Crop Residues								
Wheat	4,413	2,208	69	1,637	1	48,608	29,798	257,892
Rice	332	2,268	28	157	3,546	97,983	3,859	287,483
Barley	87	25	12	102	84	785	1,639	32,147
Maize	1,699	785	326	2,825	13	16,068	25,082	73,976
Crop Residues total (10^3 MT)	6,530	5,286	434	4,720	3,643	163,444	60,377	651,498
Crop Residues (kg/capita)	193	88	17	54	83	137	230	118
Total (10^3 MT)	9,409	8,778	1,064	13,794	7,194	215,852	98,781	996,686
Total (kg/capita)	278	147	41	157	163	180	377	180

One of the most important sections of by-product production is the bio energy production, and especially bio-fuel production, such as bio-diesel produced by vegetable oil. In this thesis, the by-products obtained from SVO extraction with cold pressing method will be considered.

By-products obtained by pressing are richer in residual oil compared to by-products obtained by chemical extraction, therefore they have a higher energy content and do not have the limitations

related to chemical extraction. One of the most commonly used oil seed in cold pressing method is rapeseed. Rapeseed occupies a leading position in the production of oil crops, as it contains nutritionally highly valuable compounds (Gawrysiak-Witulska *et al*., 2012). Rapeseed production in Europe has increased two-fold in the last 10 years, and it is the second oil seed crop (after soybean) in the world with a production of 60.6 million metric tonnes (Table 2) in 2012 (Thoenes, 2012).

Table 2. World production of major oilseeds (Thoenes, 2012)

	2009/10	2010/11	211/12	Change 2011/12 over 2010/11 %
		Million metric tonnes		
Soybeans	259.7	265.3	240.0	-9.5
Rapeseed	61.4	60.9	60.6	-0.5
Cottonseed	40.4	43.5	46.6	7.1
Groundnuts (unshelled)	34.9	36.9	36.4	-1.6
Sunflower seed	32.8	33.2	38.0	14.5
Palm kernels	11.7	12.6	13.0	3.8
Copra	5.8	4.9	5.6	15.2
Total	446.3	457.3	440.2	- 3.7

1.4 Rapeseed

Taxonomy

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Brassicales*

Family: *Brassicaceae*

Genre: *Brassica*

Species: *Napus*

Rapeseed is a grass member of the cabbage family (*Brassicaceae* or *Cruciferaceae*), which also contains mustard, turnips, and kale. The name crucifer comes from the shape of flowers, with four diagonally opposite petals in the form of a cross. *B. Napus* has dark bluish green foliage, glaucous, smooth, or with a few scattered hairs near the margins, and partially clasping. The stems are well

branched, although the degree of branching depends on variety and environmental conditions; branches originate in the axils of the highest leaves on the stem, and each terminates in an inflorescence. The inflorescence is an elongated raceme, the flowers are yellow, clustered at the top but not higher than the terminal buds, and open upwards from the base of the raceme (Musil,1950). Rapeseed is grown in cold regions usually unsuitable for growing soybean and cottonseed, such as Northern Europe, China, India, Canada, higher and colder areas of South America. Rapeseed contains several anti-nutritional factors (glucosinolates, tannins, erucic acid, sinapine, phytic acid, mucilage), which can cause liver damage, decrease performance and feed consumption and can have a negative effect on reproductive performance (Mabon *et al.*, 2000).

Most observed anti-nutritional factors are glucosinolates because they are responsible for reduction in animal performance. In addition low palatability reduces the voluntary intake by animals. New varieties of rapeseed were developed by genetic selection in order to obtain plants with low contents of erucic acid and glucosinolates. These new varieties are currently the most commonly grown and are called “00” (0 erucic acid and 0 glucosinolates). The canola CP content can range from 41 to 45 % DM. The protein fraction was found to have a high digestibility value and high biological value that was comparable to soybean, but with a lower lysine and greater methionine content (Boss *et al.*, 2007; Glencross *et al.*, 2004). Canola fat is rich in unsaturated fatty acids that have a beneficial effect on cardiovascular dysfunction and other pathologies present in the developed countries (Magné et al., 2009). Canola contains a high level of sulphur that has to be considered when included in diets for animals as this can unbalance the cationic and anionic ratio. The fiber content (NDF or ADF) is higher compared to other seeds and was found to limit nutrient utilization of canola meal, which can be reduced by dehulling (Mustafa *et al.*, 1996). A chemical composition of Canola seeds is highlighted in table3:

Table 3. Chemical composition of canola seeds (Sauvant *et al.*, 2004)

Main Constituents			Aminoacids content			
	as feed	on DM		as feed	on DM	% CP
Dry matter (%)	92.2	100				
Crude Protein (%)	19.1	20.7	lys	11.9	12.9	6.2
Gross Cellulose (%)	8.2	8.9	thr	9.1	9.9	4.8
Lipids(%)	42	45.6	met	4.2	4.6	2.2
Ash (%)	4	4.3	cys	4.7	5.1	2.5
Insoluble Ash (%)	0.3	0.3	met+cys	8.9	9.7	4.7
NDF (%)	17.6	19.1	trp	2.5	2.7	1.3
ADF (%)	12.4	13.4	ile	7.7	8.4	4
ADL (%)	5.5	6.0	val	10	10.8	5.2
Starch (%)	0.0	0.0	leu	12.2	13.2	6.4
Gross Energy (MJ/kg)	26.4	28.6	phe	7.2	7.8	3.8
Ca (g/kg)	4.7	5.1	tyr	5.5	6.0	2.9
P (g/kg)	6.6	7.2	phe+tyr	12.8	13.9	6.7
Fatty acids (g/kg)			his	5.1	5.5	2.7
Myristic, C14:0	0.4	0.4	arg	11.4	12.4	6
Palmitic, C16:0	16.8	18.2	ala	8.6	9.3	4.5
Palmitoleic, C16:1	1.6	1.7	asp	13.5	14.6	7.1
Stearic, C18:0	7.2	7.8	glu	28.6	31.0	14.9
Oleic, C18:1	231.3	250.9	gly	8.9	9.7	4.6
Linoleic, C18:2	81.8	88.7	ser	8.5	9.2	4.5
Linolenic, C18:3	39.1	42.4	pro	12.4	13.4	6.5
Mineral elements (g/kg)						
Calcium	4.7	5.1				
Phosphosus	6.6	7.2				
Magnesium	2.4	2.6				
Potassium	7.8	8.5				
Sodium	0.9	1.0				
Chlorine	3.3	3.6				
Sulphur	184.0	199.6				
Manganese	34.0	36.9				
Zinc	40.0	43.4				
Copper	3.0	3.3				
Iron	216.0	234.3				
Selenium	0.8	0.8				
Vitamin E	117.0	126.9				
Phytate P/ total P (%)	70.0					

1.5 Canola by-product of oil extraction by cold pressing oil extraction

As discussed previously, the cold pressing system is a method that is of increasing interest for its positive effect on the environment and lower cost compared to other more efficient large scale methods. The expeller obtained from cold extraction method, especially when the extraction is produced in small plants, had a highly variable chemical composition in different experiments: Leming and Lember (2002) obtained canola cake expeller 20% of residual oil, Thacker and Petri (2009) with 27%, Spragg and Mailer (2007) with 13%. The variability of residual oil values is probably due to different conditions, pressing varieties and temperature. Compared to the canola meal, canola expellers have higher residual oil and therefore, a higher energy content. This increases the interest not only as protein sources but also as a high energy feed, especially when used in monogastric diets. In contrast, the effect of canola expellers inclusion in diet for ruminants is still poorly studied and many doubts have been put forward about the stability of lipid fraction during storage, the effect of lipids on rumen microbial population and consequently on its degradability. In addition to which cold-pressed rapeseed oil has been shown to be more susceptible to autoxidation than refined rapeseed oil. The latter has been attributed to the higher hydroperoxide and free fatty acid contents of the unrefined oil, compared to refined oil, where the refining process reduces tocopherols and phenols that protect lipids from oxidation (Koski *et al*., 2002). High residual oil could be responsible for some negative effects in the rumen, in particular when used in high inclusion levels in diets for ruminant. The inclusion of lipids can inhibit rumen microbial activity, and especially for cellulolytic (fibrolytic) bacteria, with negative effects on fibre degradation. Lipids in the rumen reduce fermentable matter and hydrogen consumption by bio-saturation of unsaturated fatty acids from methanogenic bacteria (Van Nevel and Demeyer, 1988). In addition to which polyunsaturated fatty acids drastically reduce protozoa number and consequently their endo/ectosymbiotic relationship with methanogenic *archea* (Newbold and Chamberlain, 1988). Consequently inclusion of canola cake in ruminant feeding could be a strategy to reduce methane production in the rumen. In order to evaluate the effect of canola expellers at

different levels in the rumen, two different experiments were conducted using different *in vitro* methods.

1.6 Evaluation of energy value of ruminant feeds

In vivo methods for the estimation of the nutritional value of ruminant feeds requires surgically modified animals with ethical problems and high costs, limited analytical capacity and inability to evaluate many types of feeds. For these reasons *in vivo* techniques tend be unattractive (Mould *et al.*, 2005) and the use of *in vitro* methods has increased. *In vitro* techniques are more rapid, precise and have greater repeatability and reproducibility with the degree of variation related to the methodology adopted. In order to evaluate the nutritional aspect of canola cake, two different *in vitro* methods were used:

RUSITEC (RUmen SImulation TEChnique), developed by Czerkawski and Breckeridge (1977), is an apparatus to allow the microbial growth whilst reproducing the conditions of inside the rumen. Rusitec can maintain these ideal conditions over a prolonged time. Rusitec permits the collection of rumen fluid samples, evaluation of degradability of feed, analysis of gas produced, end products of fermentation and analyses of rumen microbial activity. Fermentative units are filled with artificial saliva as buffer to control pH variation, rumen fluid and two filter-bags containing the feed to analyse. The bags are replaced alternatively by a new bag after 48 hours of fermentation. During the experimental period artificial saliva is infused continuously using a peristaltic pump, in order to control pH inside the vessel, while the filter-bags containing feeds are gently agitated by a mechanical system. The vessels are kept at 39ºC. Via the artificial saliva it is possible to infuse a microbial marker in order to monitoring activity. Collection of microbial samples for molecular analyses is possible directly from the inside of the fermenter vessels. Rusitec is a complete system because it allows numerous molecular analyses on microbial populations evolution and protein

synthesis during the experimental period due to the semi-continuous system in which bacteria can live over long time.

RF system (Ankom technology®) is a fully automated system that permits the measurement of gas production at different times, its kinetics and determination of incubation residual products. RF system consist of bottles, each equipped with an electronic module with a pressure detector that transmit the variations of pressure to a computer by wireless connection. The pressure is recorded at set time intervals and gas accumulated in the head space of the bottles is automatically released by an electronic valve when previously determined pressure is reached. The fermentation bottles are filled with a buffered solution and rumen fluid, to act as a bacterial inoculum. Rumen fluid can be collected by oral probe from cows as donors or directly at the slaughterhouse. RF allows the measurement of feed degradability and residual products of incubation (VFA, NH_3), whilst many other parameters of fermentation could be detected when the protocols are developed.

In contrast to the RUSITEC, the RF system is not semi-continuous and the fermentations are time limited. Following the standard protocols of incubation, it is hard to determination of the microbial growth derived by fermentation of protein in the feed and not from N content in the rumen fluid. However, using a N-free buffer and N-depleted rumen fluid it is possible to evaluate the effect of nitrogen content in the feed analysed (Cone *et al.,* 2009). Employing Rusitec and RF to test the effect a feed is possible have more complete information on nutritional aspects of feed tested.

1.7 References

- Atabani, A.E., A.S. Silitonga, I. A. Badruddin, T. M. I. Mahlia, H. H. Masjuki, and S. Mekhilef. 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews. 16(4): 2070-2093.

- Bos, C., G. Airinei, F. Mariotti, R. Benamouzig, S. Bérot, J. Evrard, E Fénart, D. Tomé, C. Gaudischon. 2007. The poor digestibility of rapeseed protein is balanced by its very high metabolic utilization in humans. J. Nutr. 137(3): 594-600.

- Carraretto, C., A. Macor, A. Mirandola, A. Stoppato, S. Tonon. 2004. Biodiesel as alternative fuel: experimental analysis and energetic evaluations. Energy Convers Manage;29(12‑15):2195‑211.

- Cetinkaya, M, Y. Ulusoy, Y. Tekın, F. Karaosmanĩglue. 2005. Engine and winter road test performances of used cooking oil originated biodiesel. Energy Convers Manage. 46(7‑8):1279‑91.

- Cohen, J. E. 2003. Human population: The next half century. Science. 302(5648): 1172-1175.

- Cone, J. W., M. A. M. Rodrigues, C. M. Guedes, M. C. Blok. 2009. Comparison of protein fermentation characteristics in rumen fluid determined with the gas production technique and the nylon bag technique. Anim. Feed Sci. Technol. 153(1-2): 28-38.

- Czerkawski, J. W., G. Breckenridge. 2007. Design and development of a long-term rumen simulation technique (rusitec). Br. J. Nutr. 38(03): 371.

- De Boer, I. J. M. 2003. Environmental impact assessment of conventional and organic milk production.80: 69-77.

- Deffeyes, K.S. 2008. Hubbert's Peak: The Impending World Oil Shortage (New Edition). 1: 1-13

- Duncan R.C., Youngquist W. 1999. Encircling the Peak of World Oil Production. Natural Resources Research. 8 (3): 219-232

- Fadel, J. G. 1999. Quantitative analyses of selected plant by-product feedstuffs, a global perspective. Anim. Feed Sci. Technol. 79(4): 255-268.

- Fangrui, M., Milford A. H. 1999. Biodiesel production: A review. Bioresour. Technol. 70(1): 1-15.

- FAO, 2008. Biofuels: Prospects, Risks and Opportunities. The State of Food and Agriculture. FAO, Rome,. 2:10-22.

- Fazal, M. A., A.S.M.A. Haseeb, H. H. Masjuki. 2011. Biodiesel feasibility study: An evaluation of material compatibility; performance; emission and engine durability. Renewable and Sustainable Energy Reviews. 15(2): 1314-1324.

- Fore, S. R., W. Lazarus, P. Porter, N. Jordan. 2011. Economics of small-scale on-farm use of canola and soybean for biodiesel and straight vegetable oil biofuels. Biomass Bioenergy. 35(1): 193-202.

- Gawrysiak-Witulska, M., M. Rudzińska, J. Wawrzyniak, A. Siger. 2012. The effect of temperature and moisture content of stored rapeseed on the phytosterol degradation rate. J. Am. Oil Chem. Soc. 89(9): 1673-1679.

- Glencross, B., W. Hawkins, J. Curnow. 2004. Nutritional assessment of australian canola meals. II. evaluation of the influence of the canola oil extraction method on the protein value of canola meals fed to the red seabream (pagrus auratus, paulin). Aquacult. Res. 35(1): 25-34.

- Grau, B., E. Bernat, R. Antoni, R. Jordi-Roger, P. Rita. 2010. Small-scale production of straight vegetable oil from rapeseed and its use as biofuel in the spanish territory. Energy Policy. 38(1): 189-196.

- International Energy Agency (IEA). World energy outlook 2007. Available from: http://www.iea.org/textbase/nppdf/free/2007/weo 2007.pdf; 2007

- Koski, A., E. Psomiadou, M. Tsimidou, A. Hopia, P. Kefalas, Wähälä Kristiina et al. 2002. Oxidative stability and minor constituents of virgin olive oil and cold-pressed rapeseed oil. European Food Research and Technology. 214(4): 294-298.

- Leming, R., A. Lember. 2002. Chemical composition of expeller-extracted and cold-pressed rapeseed cake. Production. (1995): 103-109.

- Mabon, N., S. N. M. Mandiki, G. Derycke, J. Bister. 2000. Chemical changes and in and influences of rapeseed antinutritional factors on lamb physiology and performance. 3. antinutritional factors in plasma and organs. Anim. Feed Sci. Technol. 85: 111-120.

- Mattson, J., C. Sun, W. Koo. 2004. Analysis of the world oil crops market. Agribusiness and Applied Economic Report n.529, 1:10

- Magné, J., Huneau, J. F., Tsikas, D., Franc, J., Rochette, L., Tome, D. 2009. Rapeseed Protein in a High-Fat Mixed Meal Alleviates Postprandial Systemic and Vascular Oxidative Stress and Prevents Vascular Endothelial Dysfunction in Healthy Rats. j. Nutr. 139:1660-1666

- Mould, F., K. Kliem, R. Morgan, R. Mauricio. 2005. In vitro microbial inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123-124: 31-50.

- Musil, A.F. (1950) Identification of Brassicas by seedling growth or later vegetative stages. USDA circular. 857. pp. 26

- Mustafa, A. F., D. A. Christensen, J. J. McKinnon. 1996. Chemical characterization and nutrient availability of high and low fiber canola meal. Canadian Journal of Animal Science. 76(4): 579-586.

- Newbold, C.J., D.G. Chamberlain, 1988. Lipids as rumen-defaunating agents. Proc. Nutr. Soc. 47, 154A.

- Poritosh, R., N. Daisuke, T. Orikasa. ,. Q. Xu, H. Okadome, N. Nakamura, T. Shiina. 2009. A review of life cycle assessment (LCA) on some food products. J. Food Eng. 90(1): 1-10.

- Sexton, S., D. Rajagopal, D. Zilberman, D. Roland-Holst. 2007. The intersections of energy and agriculture: implications of rising demand for biofuels and the search for the next generation. ARE Update, 10(5): 4-7.

- Silitonga, A.S., A.E. Atabani, T. M. I. Mahlia, H. H. Masjuki, I. A. Badruddin, and S. Mekhilef. 2011. A review on prospect of jatropha curcas for biodiesel in Indonesia. Renewable and Sustainable Energy Reviews. 15(8): 3733-3756.

- Sauvant, D., J. Perez, Tran, G. 2004. Tables of composition and nutritional value of feed materials. Pigs, poultry, cattle, sheep, goats, rabbits, horses and fish. Ed. INRA, 160-162

- Spragg, J., Mailer, R. 2007. Canola meal value chain quality improvement A final report prepared for AOF and pork CRC. JCS solutions pty ltd., berwick, victoria, australia.(January 2009)

- Thacker, P. A., Petri, D. 2009. The effects of canola or mustard biodiesel press cake on nutrient digestibility and performance of broiler chickens. Asian-Aust J Anim Sci. 22(11): 1531.

- Thoenes, P. 2012. FAO - trade and markets division. Food outlook, may 2012. pp 35

- Tilman, D., Cassman, K.G. Matson, P. A. 2002. Agricultural sustainability and intensive production practices. Nature 418: 671-677

- Van Nevel, C.J., Demeyer, D.I. 1988. Manipulation of rumen fermentation. In: Ed. P.N. Hobson. The Rumen Microbial Ecosystem. Elsevier Applied Science, London, pp. 387–443.

- Volpi, G. 2010. Biofuels in brazil and land use change. Journal of Biobased Materials and Bioenergy. 4(3): 211-220.

Chapter 2

General aims

The general aim of the this thesis is the study of canola expellers (CE) extracted by cold method in small plant and evaluation of its use in ruminant nutrition. Four experimental contributes will be presented: in the first contribute, the conservation of CE was evaluated at different times and temperatures in order to determine if the storage conditions usually found in on the farm caused changes in volatile fatty acids composition profile. In the second contribute, *in vitro* gas production (GP) values were compared for CE, whole soybean seed and soybean meal by incubating feed samples with N-rich or N-free buffer in order to compare the effect of feed protein in rumen. The third contribute compared four diets *in vitro* for beef cattle containing whole soybean seed or canola expeller as protein sources at two inclusion levels for digestibility, gas production, end-products of fermentation, nitrogen balance and microbial activity using the Rusitec fermenter. Finally in fourth contribute the same diets tested in Rusitec fermenter (third contribute) were evaluated using RF system in order to evaluate their gas production kinetics.

Chapter 3.

(First contribute)

Effect of pressing, storage temperature and storage time on chemical composition and fatty acid profile of canola expellers

Matteo Guadagnin[1], Mirko Cattani[2] and Lucia Bailoni[1]

[1]Dipartimento di Biomedicina comparata e alimentazione, Università di Padova, Italy

[2]Dipartimento di Agronomia Animali Alimenti risorse Naturali e Ambiente Università di Padova, Italy

Corresponding author: Matteo Guadagnin, [1]Dipartimento di Biomedicina comparata e alimentazione, Università di Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy – Tel. +39.049.8272777

Abstract

This experiment aimed to investigate the effects of different temperatures and times of storage on chemical composition, fatty acid profile, and oxidative stability of canola expellers obtained from cold-pressing extraction of oil. Canola seeds were obtained by eight hybrid cultivars of canola seeds were single-crushed at moderate temperatures (60°C) using two presses equipped with a rotating screw shaft within an horizontal barrel. After each pressing session, 9 samples of canola expellers (100±1 g) were randomly collected, inserted into sealed bags, placed into three incubators (3 bags/incubator) set at different temperatures (12, 24 or 36°C), and stored over three periods of times (10, 20 or 30 d). The same analyses were conducted on samples (100±1 g) of canola seeds collected before each pressing session and at the end of each storage time. Chemical composition,

fatty acid profile, peroxide number and Kreis test in canola seeds and expellers, were determined. Canola expellers lipid content found in this trial was high, because of the efficiency of oil extraction from canola seeds, using the small plant, was low. Moderate temperatures (60°C) were applied for the extraction of oil. Before storage the fatty acid profile of canola seeds and expellers differed significantly, except for the content of myristic (P=0.18), palmitic (P=0.57), oleic (P=0.07), and α-linolenic acid (C18:3 n-3; P=0.45). Compared to the canola seeds, expellers showed greater content of saturated (SFA), poly-unsaturated (PUFA), and omega-6 fatty acids (P<0.01), and definitely higher peroxide values (P<0.01). Conversely, the content of mono-unsaturated fatty acids (MUFA) was significantly lower (P<0.01). In the current work, peroxide values provided by canola expellers averaged 4.22 and 4.11 mEq/kg fat before and after storage, respectively, Kreis test, resulted negative for all samples. Under different temperatures (12, 24 and 36°C) and times of storage (10, 20 or 30 d), canola expellers showed to maintain a good oxidative stability, as evidenced by low peroxide values (< 10 mEq/kg fat) and negative response for Kreis test. From these results it could be hypothesized that the storage of by-products derived from the productive cycle of biofuel is possible. Canola expellers tested in this experiment, despite great oil residual (17-19% EE on DM basis), could be potentially used as animal feeds, as the level of peroxides was definitely under the threshold supposed to impair rumen function.

Introduction

Canola represents the trademark of a particular rapeseed with low content of erucic acid (< 5% by weight, for EU) and glucosinolates (< 30 mmol/kg DM, for EU). In the last years an increasing number of farms has been equipping with small-sized facilities for mechanical extraction of oil from canola seeds. After the extraction, the oil is usually converted in biodiesel and utilized as fuel for tractors, whereas the resulting by-products (canola expellers) are included in ruminant diets as protein and fat sources. This productive cycle offers economical and environmental advantages, as it contributes to reduce the need for carburant fossil in agricultural industry and to recycle the resulting expellers as animal feeds (Baquero *et al.*, 2010; Esteban *et al.*, 2011). However, to date,

information about the nutritional and energy value of canola expellers are still limited. Literature indicates that oil content of canola expellers is extremely variable, with a fat percentage ranging from 8 to almost 30% DM, as the mechanical extraction is a poorly standardized and efficient method (Leming and Lember, 2005; Spragg and Mailer, 2007; Thacker and Petri, 2009). Further, the canola oil, as all vegetable oils, is characterized by a great degree of unsaturation and, thus, is susceptible to oxidation (O'Brien, 2008; Matthäus, 2012). This chemical profile could strongly reduce the possibility to store canola expellers at farm level and discourage their utilization in animal feeding. Thus, the identification of techniques to store canola expellers at farm level could represent a topic of public and scientific interest. This experiment aimed to investigate the effects of different temperatures and times of storage on chemical composition, fatty acid profile, and oxidative stability of canola expellers obtained from cold-pressing extraction of oil.

Material and methods

Experimental design and analytical procedures

Canola seeds were obtained by eight hybrid cultivars of canola. The hybrids were Dkexme, Excalibur, and Excel (Dekalb, Monsanto Agricoltura Spa, Lodi, Italy), PR46W10 (Pioneer Hi-Bred srl, Cremona, Italy), Pulsar and Tissot (Società Italiana Sementi Bologna, Italy), Toccata and Makila (Maisadour Semences Italia, Verona, Italy). Hybrids were grown and harvested at the pilot farm of the Veneto Agricultura Agency (Caorle, Venezia, Italy). After harvest, canola seeds were screened to remove extraneous material, artificial dried and stored until pressing for oil extraction. Three pressing sessions were conducted in three successive months, where canola seeds were single-crushed at moderate temperatures (60°C) using two presses equipped with a rotating screw shaft within an horizontal barrel (Mailca Srl, Modena, Italy). The two presses had an input flow rate of 120 kg/h of seeds, with a production of 40 kg/h of oil and 80 kg/h of expellers. After each pressing session, 9 samples of canola expellers (100±1 g) were randomly collected, inserted into sealed bags, placed into three incubators (3 bags/incubator) set at different temperatures (12, 24 or 36°C), and stored over three periods of times (10, 20 or 30 d). These temperatures and times were

chosen to simulate different storage conditions at farm level. Chemical composition and fatty acid profile of canola expellers, previously ground to 1 mm using a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, Germany), were determined before storage (t = 0) and at the end of each storage time (10, 20, and 30 d). The same analyses were conducted on samples (100±1 g) of canola seeds collected before each pressing session. Chemical analyses were performed in the same laboratory by the same technician. Samples were analysed for dry matter (DM; # 934.01; AOAC, 2003), crude protein (CP; # 976.05; AOAC, 2003), ether extract (EE; # 920.29; AOAC, 2003), ash (# 942.05; AOAC, 2003), and neutral detergent fibre (aNDF), as suggested by Mertens (2002). The aNDF fraction, inclusive of residual ash, was determined with α-amylase and sodium sulphite using the Ankom^{220} Fibre Analyzer (Ankom Technology®, Macedon, NY, USA). The fatty acid profile was determined in duplicate using a Thermo Finnigan Spectra System AS3000 autosampler (Thermo Electron Corporation, Waltham, MA, USA) equipped with a H_2SO_4 0.0025 N Bio-Rad HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA). After the extraction with chloroform and methanol (Folch *et al.*, 1957), the oil was analysed for peroxide number, using conventional iodometric titration with thiosulfate (Peroxide value of oils and fats, #965.33; AOAC, 2003), and for Kreis test (NGD, C 56, 1979;).

Statistical analysis

Experimental data were subjected to analysis of variance using the general linear model procedure (PROC GLM) of SAS (2005). For fatty acid profile provided by canola seeds and canola expellers before storage (t = 0) the model included the effects of feed and pressing session:

$$y_{ijkl} = \mu + F_i + P_j + FP_{ij} + \varepsilon_{ijk}$$

where: y_{ijk} = single observation, μ = overall mean, F_i = effect of feed (i = 1 to 2); P_j = effect of pressing session (j = 1 to 3) and ε_{ijk} = residual error. For chemical composition and fatty acid profile

of samples canola expellers collected after the different periods of storage at different temperatures, the model included the effects of pressing session, storage temperature, storage time, and the corresponding interactions.

$y_{ijkl} = \mu + P_i + T_j + L_k + PT_{ij} + PL_{ik} + TL_{jk} + \varepsilon_{ijkl}$

where: Y_{ijk} = single observation, μ = overall mean, P_i = effect of pressing session (i = 1 to 3); T_j = effect of storage temperature (j = 1 to 3); L_k = effect of storage length (k = 1 to 3); PT_{ij}, PL_{ik}, TL_{jk} = first order interaction and ε_{ijkl} = residual error. Significant differences were always accepted if $P \leq 0.05$.

Results and discussion

The proximate analysis of canola seeds and expellers after each pressing is given in Table 1, The differences (especially for lipid content) among pressing sessions could be due to sampling procedures. However, chemical values provided by canola seeds resulted in agreement with tabled data (NRC, 2001). In the current work canola expellers showed a similar CP content (from 30.6 to 30.9%DM), whereas lipid content (from 17.5% to 19.1% DM)varied notably among pressing sessions depending to the value of the seeds (from 42.2 to 46.3% DM) to . The highest value was in line with findings of Leming and Lember (2005), and intermediate between 13% DM reported by Spragg and Mailer (2007) and 27% DM observed by Thacker and Petri (2009). However, in relative terms, lipid content found in this trial must to be considered high, and this evidences that the efficiency of oil extraction from canola seeds, using the small plant, was low. Literature indicates that processing conditions, and especially the number of pressings, can influence largely the amount of residual oil in the expellers (Weigal, 1991; Glencross *et al.*, 2004). Currently, the common practice is to press seeds once or double times; the double pressing ensures a greater recovery of oil from seeds but also causes the generation of high temperatures inside the pressing system, that could reduce the storability of oil by increasing amount of free fatty acids (Matthäus, 2012). In the

present study canola seeds were subjected to single-pressing, as the purpose was to evaluate the possibility to store expellers with a great oil residual. Further, moderate temperatures (60°C) were applied for the extraction of oil, to avoid possible alterations of fatty acid profile. Before storage (t = 0), the fatty acid profile of canola seeds and expellers differed significantly, except for the content of myristic (P=0.18), palmitic (P=0.57), oleic (P=0.07), and α-linolenic acid (C18:3 n-3; P=0.45) (Table 2). Compared to the canola seeds, expellers showed greater content of saturated (SFA), poly-unsaturated (PUFA), and omega-6 fatty acids (P<0.01), and definitely higher peroxide values (P<0.01). Conversely, the content of mono-unsaturated fatty acids (MUFA) was significantly lower (P<0.01) in the expellers compared to the seeds. However, notable differences were found between the fatty acid profile of expellers and that of original seeds. Harris and James (1969) stated that the temperature controls the dehydrogenation of fatty acids by influencing the amount of available oxygen. More in detail, the dehydrogenation is usually limited at increasing temperatures, as the oxygen becomes less soluble and, hence, available in the cell cytoplasm. On this basis, it could be hypothesized that the decrease of MUFA content in expellers with respect to the original seeds was related with the effect of temperature during the pressing. Pressing session influenced significantly (P<0.01) only the SFA content and the ratio between SFA and unsaturated fatty acids (UFA), that resulted greater in the second session compared to the others. Results of this trial also revealed as the amount of peroxides markedly increased passing from canola seeds to expellers (Table 2). Läubli and Bruttel (1986) noted that formation of peroxides is enhanced by temperature, thus it could be hypothesized that the increased content of peroxides in canola expellers was related with heat arisen from the pressing of seeds. Considering the canola expellers after the different storage conditions, pressing session showed significant effects on the NDF (P<0.05) and ash (P<0.01) content (Table 3), but these differences were presumably related to the fact that expellers derived from various canola cultivars rather than to a real effect of pressing procedure. The temperature and time of storage affected significantly, as expected, the dry matter of expellers with a linear increment at increasing temperature and time. The lack of significant effects due to the pressing

session provides evidence that the pressing procedure had a satisfactory degree of standardization. On the other hand, the presence of notable effects due to the feed seems to suggest that the pressing procedure changed strongly the fatty acid profile of expellers compared to that of original seeds. To date, effects of processing conditions on nutrient profile of expellers have been little investigated. Some authors found that processing can affect the content of glucosinolates (Newkirk and Classen, 2002; Seneviratne *et al.*, 2011) and of amino acids in the residual expellers (Seneviratne *et al.*, 2011), whereas, to our knowledge, no information are available about possible effects on fatty acid profile of these by-products. In the current work, the fatty acid profile of canola expellers appeared in line with literature (on average: C18:1, 55.5 %; C18:2, 20.3 %; C18:3, 6.3 %). Deng and Scarth (1998) reported that the conventional canola oil contains about 6% of SFA, 55-60% of oleic acid, 20-26% of linoleic acid, and 8-10% of α-linolenic acid. During the storage, peroxides values decreased notably ($P<0.01$), irrespective from temperature and time (Table 4). In the current work, peroxide values provided by canola expellers averaged 4.22 and 4.11 mEq/kg fat before and after storage, respectively (Table 4). Vazquez-Aňon and Jenkins (2007) observed that peroxide values similar to those found in this experiment (3.5 mEq/kg fat) did not impair rumen fermentation and microbial activity. The same authors hypothesized that peroxide values of 215 mEq/kg fat could be sufficient to affect negatively the rumen function. On this basis, it could be concluded that canola expellers showed a good oxidative stability at all temperatures and times of storage. This pattern is also supported by the responses of Kreis test, that resulted negative for all samples (data not shown). In this experiment, the permanence of a good oxidative stability was presumably favoured by moderate temperatures used. To this regard, Koski *et al.* (2002) found that rapeseed oil stored at 70°C reached peroxide values of 70 mEq/kg fat within only 4 days. Naczk *et al.* (1998) indicated that rapeseed and canola oil, compared to other oilseeds, have the greatest content of phenolic compounds with antioxidant properties (i.e. sinapic acid and tocopherols), and this argument was supported by several studies (Bandoniene *et al.*, 2000; Koski *et al.*, 2002; Amarowicz *et al.*, 2003; Shen *et al.*, 2012). The presence of these compounds makes rapeseed and canola oil more persistent

to the occurrence of oxidative process (Naczk *et al.*, 1998). In the current work, canola expellers were not characterized for phenols, but it could be supposed that these substances, being heat-stable (Shen *et al.*, 2012), contributed to preserve the oxidative stability of expellers during the storage at different conditions.

Conclusions

Under different temperatures (12, 24 and 36°C) and times of storage (10, 20 or 30 d), canola expellers showed to maintain a good oxidative stability, as evidenced by low peroxide values (< 10 mEq/kg fat) and negative response for Kreis test. From these preliminary results it could be hypothesized that the storage of by-products derived from the productive cycle of biofuel is possible. Further, canola expellers tested in this experiment, despite great oil residual (17-19% EE on DM basis), could be potentially used as animal feeds, as the level of peroxides was definitely under the threshold supposed to impair rumen function (> 200 mEq of peroxides/kg fat). However, the latter hypothesis should be validated *in vivo*.

References

Amarowicz, R., Raab, B., Shahidi, F., 2003. Antioxidant activity of phenolic fractions of rapeseed. J. Food Lipids 10:51-62.

AOAC, 2003. Official methods of analysis. 17th ed. (2th revision) Association of Official Agricultural Chemists, AOAC International, Gaithersburg, MD, USA.

Bandoniene, D., Pukalskas, A., Venskutonis, P.R., Gruzdiene, D., 2000. Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. Food Res. Int. 33:785-791.

Baquero G., Esteban, B., Rius, A., Riba, J.R., Puig, R., 2010. Small-scale production of straight vegetable oil from rapeseed and its use as biofuel in the Spanish territory. Energ. Policy 38:189-196.

Deng, X., Scarth, R., 1998. Temperature effects on fatty acid composition during development of low-linolenic oilseed pape (*Brassica napus* L.). J. Am. Oil. Chem. Soc. 75:759-766.

Esteban, B., Baquerio, G., Puig, R., Riba, J.R., Rius, A., 2011. Is it environmentally advantageous to use vegetable oil directly as biofuel instead of converting it to biodiesel? Biomass Bioenerg. 35:1317-1328.

Folch, J., Lees, M., Sloane-Stanely, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–507.

Glencross, B., Hawkins, W., Curnow, J., 2004. Nutritional assessment of Australian canola meals. II. Evaluation of the influence of the canola oil extraction method on the protein value of canola meals fed to the red seabream (*Pagrus auratus*, Paulin). Aquacult. Res. 35:25-34.

Harris, P., James, A.J.,. 1969. Effect of low temperature on fatty acid biosynthesis in seeds. Biochim. Biophys. Acta 187:13-18.

Koski, A., Psomiadou, E., Tsimidou, M., Hopia, A., Kefalas, P., Wähälä, K., Heinonen, M., 2002. Oxidative stability and minor constituents of virgin olive oil and cold pressed rapeseed oil. Eur. Food Res. Technol. 214:294-298.

Läubli, M.W., Bruttel, P.A., 1986. Determination of the oxidative stability of fats and oils: comparison between the active oxygen method (AOCS Cd 12-57) and the Rancimat method. J. Am. Oil Chem. Soc. 63: 792-795.

Leming, R., Lember, A., 2005. Chemical composition of expeller extracted and cold-pressed canola meal. Agraarteadus 16:103-109.

Matthäus, B., 2012. Oil technology. In: Technological Innovations In Major World Oil Crops, Vol . 2. Perspectives. Ed. S.K. Gupta, Jammu, India.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.

Naczk, M., Amarowicz, R., Sullivan, A., Shahidi, F., 1998. Current research developments on polyphenols of rapeseed/canola: A review. Food Chem. 62:489-502.

National Research Council (NRC), 2001. Nutrient Requirements of Dairy Cattle, 7th revised ed. National Academy Press, Washington, DC, USA.

Newkirk, R.W., Classen, H.L., 2002. The effects of toasting canola meal on body weight, feed conversion efficiency and mortality in broiler chickens. Poult. Sci. 81:815-825.

NGD_ Norme Grassi e Derivati, III edizione 1976 e III supplemento 1989. Ed. Stazione Sperimentale degli Oli e dei Grassi, Milano, Italy, Methods NGD C56-79.

O'Brien, R., 2008. Fats and oils formulating and processing for applications. Third Edition: Formulating and processing for applications. CRC Press, Schulenburg, Texas, USA.

SAS® User's Guide, 2005. 5th ed. SAS Instit., Inc., Cary, NC, USA.

Seneviratne, R.W., Beltranena, E., Goonewardene, L.A., Zijlstra, R.T., 2011. Effect of crude glycerol combined with solvent-extracted or expeller-pressed canola meal on growth performance and diet nutrient digestibility of weaned pigs. Anim. Feed Sci. Technol. 170:105-110.

Shen, Z., Wijesundera, C., Ye, J.H., 2012. Effect of seed heat-treatment on the oxidative stability of canola oil body emulsions. Food. Nutr. Sci. 3:981-990.

Spragg, J., Mailer, R., 2007. Canola meal value chain quality improvement. A final report prepared for AOF and Pork CRC. JCS Solutions Pty Ltd., Victoria, Australia.

Thacker, P.A., Petri, D., 2009. The effects of canola or mustard biodiesel press cake on nutrient digestibility and performance of broiler chickens. Asian-Aust. J. Anim. Sci. 22:1531-1539.

Vázquez-Añón, M., Jenkins, T., 2007. Effects of feeding oxidized fat with or without dietary antioxidants on nutrient digestibility, microbial nitrogen, and fatty acid metabolism. J. Dairy Sci. 90:4361-4367.

Weigal, J., 1991. Oilseed meals and the effect of processing on their subsequent quality. In: Recent Advances in Animal Nutrition in Australia. Ed. D. J. Farrell, University of New England, Armidale, Australia.

Table 1. Chemical composition of canola seeds and canola expellers before storage (t = 0) in the three pressing sessions.

Item	Canola seeds			Canola expellers		
	Pressing session			Pressing session		
	I	II	II	I	II	III
Dry matter %	94.1	93.8	94.1	92.4	92.2	92.5
Ether extract % DM	42.2	46.3	44.7	17.5	19.1	18.7
Crude protein % DM	20.6	20.3	20.5	30.7	30.6	30.9
Neutral detergent fibre % DM	27.6	25.8	26.9	21.1	19.8	21.4
Ash % DM	4.3	4.2	4.2	6.3	6.2	6.4
Non-structural carboidrates %DM	5.3	3.4	3.7	24.4	24.3	22.6

Table 2. Effect of feed and pressing session on fatty acid profile of canola seeds and canola expellers before storage (t = 0) in the three pressing sessions.

Fatty acids, %	Canola seeds			Canola expellers			SEM	*P* value	
	Pressing session			Pressing session				Feed	Pressing session
	I	II	III	I	II	III			
C14:0	0.09	0.09	0.09	0.07	0.11	0.12	0.005	0.18	0.06
C16:0	7.63	7.69	7.38	8.10	8.23	8.37	0.045	0.57	0.93
C18:0	1.09	1.09	1.09	1.50	1.10	1.09	0.045	0.07	0.06
SFA	10.3	10.3	10.1	11.3	12.1	11.3	0.221	<0.01	<0.05
C18:1	59.9	59.4	59.5	56.1	56.1	56.0	0.532	<0.01	0.10
MUFA	64.5	64.1	64.4	62.0	62.1	62.0	0.341	<0.01	0.17
C18:2	19.1	19.3	19.3	20.4	19.4	20.4	0.163	<0.01	0.26
C18:3 n-3	6.2	6.1	6.2	6.0	6.0	6.0	0.010	0.61	0.07
PUFA	25.3	25.6	25.5	26.7	25.7	26.6	0.167	<0.01	0.21
Ω-6	19.0	19.2	19.2	20.8	19.6	20.8	0.215	<0.01	0.19
Ω-3	6.1	6.0	6.1	6.1	6.1	6.1	0.010	0.45	0.06
SFA/UFA	0.11	0.12	0.11	0.13	0.14	0.13	0.002	<0.01	<0.05
MUFA/PUFA	2.55	2.51	2.52	2.33	2.41	2.33	0.027	<0.01	0.40
Ω-6/Ω-3	3.14	3.17	3.10	3.38	3.20	3.38	0.034	<0.01	0.28
Peroxides meqO_2/kg Fat	1.29	1.47	2.05	7.13	4.55	3.24	0.618	<0.01	0.21

Table 3. LS means: effect of pressing session, storage temperature, storage lenght and their interactions on chemical composition of canola expellers after storage at different temperatures and times.

	Dry matter	Ether extract	Crude protein	Neutral detergent fibre	Ash	Non-structural carboidrates
Pressing session, P						
I	92.8	18.2	30.9	21.4	6.21	23.23
II	92.8	18.8	30.8	20.5	6.21	23.60
III	92.8	19.4	31.2	21.1	6.41	21.88
Storage temperature, T						
12°C	92.1	18.8	30.8	21.3	6.29	22.86
24°C	92.3	19.2	31.0	21.2	6.31	22.33
36°C	94.0	18.5	31.1	20.6	6.30	23.52
Storage lenght, L						
10 d	92.5	19.0	31.0	20.9	6.29	22.85
20 d	92.8	18.3	31.0	20.9	6.31	23.52
30 d	93.0	19.1	31.0	21.2	6.31	22.34
SEM	0.162	0.245	0.062	0.130	0.016	
P value						
P	0.913	0.189	0.072	0.018	<0.001	0.080
T	<0.001	0.507	0.175	0.041	0.680	0.270
L	<0.001	0.383	0.974	0.340	0.681	0.280
P × T	0.211	0.271	0.561	0.144	0.197	0.440
P × L	0.198	0.497	0.400	0.320	0.321	0.846
T × L	<0.001	0.266	0.733	0.382	0.402	0.444

Table 4. Effect of pressing session, storage time, storage temperature and their interactions on fatty acid profile of canola expellers after storage at different temperatures and times.

	Fatty acids, %														
	C14:0	C16:0	C18:0	SFA	C18:1	MUFA	C18:2	C18:3	PUFA	Ω-6	Ω-3	SFA/UFA	MUFA/PUFA	Ω-6/Ω-3	Peroxides
Pressing session (PS)															
I	0.07	7.4	1.4	11.1	54.7	61.8	20.4	6.4	27.1	20.6	6.5	0.12	2.28	3.18	4.99
II	0.18	7.5	1.0	11.1	55.9	62.1	20.2	6.2	26.7	20.4	6.4	0.12	2.32	3.20	4.31
III	0.11	8.0	1.2	11.1	56.0	62.0	20.4	6.2	26.8	20.6	6.3	0.12	2.31	3.30	3.38
Storage temperature (T)															
12°C	0.07	7.8	1.2	10.8	56.2	62.2	20.4	6.2	26.9	20.6	6.3	0.12	2.31	3.26	5.69
24°C	0.20	7.7	1.1	11.0	55.7	62.0	20.5	6.3	26.9	20.7	6.4	0.12	2.30	3.24	4.21
36°C	0.08	7.4	1.2	11.4	54.8	61.7	20.2	6.3	26.8	20.4	6.4	0.13	2.30	3.18	2.79
Storage time (ST)															
10 d	0.10	7.9	1.1	11.0	55.4	61.9	20.0	6.2	27.0	20.7	6.3	0.12	2.28	3.30	4.12
20 d	0.10	7.9	1.2	10.4	55.7	62.3	20.7	6.3	27.2	20.5	6.5	0.12	2.30	3.18	4.17
30 d	0.18	7.3	1.2	11.3	55.0	61.7	20.4	6.5	26.9	20.5	6.6	0.13	2.29	3.11	3.66
SEM	0.032	0.21	0.044	0.215	0.286	0.163	0.070	0.067	0.096	0.073	0.065	0.003	0.008	0.031	0.374
P value															
PS	0.60	<0.05	<0.05	0.99	0.16	0.81	0.08	<0.01	0.20	0.09	0.35	0.99	<0.05	0.25	<0.01
T	0.38	0.34	0.41	0.59	0.20	0.53	<0.01	0.07	0.88	<0.01	0.78	0.58	0.62	0.52	<0.01
ST	0.89	0.14	0.86	0.38	0.64	0.66	<0.01	<0.05	<0.01	<0.05	0.10	0.40	<0.01	0.08	0.06
PS × T	0.62	0.28	0.49	0.32	0.30	0.26	0.36	0.24	0.32	0.61	0.44	0.32	0.14	0.36	0.30
PS × ST	0.61	<0.01	0.53	0.80	0.44	0.87	<0.01	0.53	0.56	<0.01	0.59	0.69	0.75	0.50	<0.01
T × ST	0.51	0.16	0.30	0.67	0.43	0.67	<0.01	<0.01	0.15	<0.01	0.92	0.77	0.06	0.65	0.06

Chapter 4.

(Second Contribute)

Nitrogen level of *medium* influences *in vitro* gas production of high-protein feeds

M. Guadagnin[a,*], F. Tagliapietra[b], M. Cattani, L. Bailoni[a]

[a]*Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Viale dell'Università 16, 35020 Legnaro (PD). Italy*

[b]*Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy*

* Corresponding author. Tel: -39 049 8272777; Fax: +39 049 8272633; EM: matteo.guadagnin@unipd.it

Abstract

Aim of this work was to evaluate the effects of a N-rich (MS) and a N-free (NF) medium on *in vitro* gas production (GP) of high-protein feeds. Samples (0.55 g) of soybean meal (SBM) and whole soybean seed (WSS), and canola expeller (CE) were placed in three replicates into bottles (310 ml) with 25 ml of rumen fluid and 50 ml of a N-rich medium, or with 5 ml of rumen fluid and 95 ml of a N-free medium, and incubated for 72 h. Each bottle was equipped with a pressure detector and a mechanical valve that was set to release gas at fixed pressure (3.4 kPa). Feeds were also analysed in triplicate for proximate composition and for aNDF(dNDF) and true DM (TDMd) digestibility at 48 h. Experimental data were analysed by ANOVA, considering the effects of medium and feed as sources of variation. Compared to the two soybeans, CE showed lower values of dNDF and TDMd ($P < 0.01$). The NF medium provided lower GP within the first 24 h of incubation compared to MS, whereas the two media did not differ for GP at 48 and 72 h of incubation. With respect to MS, the NF medium showed to reduce fermentation rate at early phases of incubation, by delaying the times required to produce half of asymptotic GP (T½) and to reach the maximum rate of GP (T_{max}), and by decreasing the maximum rate of GP (R_{max}) ($P < 0.01$ for all parameters). The feeds did not differ for GP within the first 12 h of incubation, whereas CE showed lower GP from 24 h to the end of incubation with respect to the two soybeans ($P < 0.01$). However, CE was more rapidly fermented at early phases of incubation, as evidenced by the lower values of T½ and T_{max} and by greater values of R_{max} compared to SBM and WSS ($P < 0.01$ for all parameters). In conclusion, the N level of medium seems to affect kinetics of GP, but not the total GP provided by high-protein feeds. Compared to soybeans, CE showed a greater fermentation rate at early phases of incubation, and this pattern could be attributed to a greater degradability of protein fraction.

Keywords: N-rich and N-free medium, *in vitro* gas production, high-protein feeds

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, digestible aNDF; EE, ether extract; GP, gas production; $lignin_{(sa)}$, lignin determined by solubilization of cellulose with sulphuric acid; TDMd, true DM digestibility.

1. Introduction

In vitro techniques are largely employed to evaluate energy value of ruminant feeds, because of higher labor efficiency and reduced costs compared to *in vivo* and *in situ* methods (Lopez, 2005). Most of these techniques entail to analyse feed samples using incubation media exceeding in N and lacking in energy, which becomes the limiting factor for microbial growth, in order to characterize feeds as energy sources (Goering and Van Soest, 1970; Menke and Steingass, 1988; Theodorou et al., 1994). However, when feeds are evaluated for their N contribution, incubations should be conducted using media low in N or N-free, to make the dietary N the only N source available for microbial growth and to limit confounding effects related to the presence of N in the buffer (Grings et al., 2005). To date, the effects of N level in the incubation media on rumen fermentation have been little investigated. Wood et al. (1998) incubated wheat straw using a N-low and a N-rich medium and attributed the differences in GP to the different availability of N. Other authors (Grings et al., 2005) measured *in vitro* GP of four silage-based diets using N-low or N-rich media, but did not observe significant differences among the two media. Cone et al. (2005; 2009) evaluated protein fermentation of fifteen ingredients using a N-free medium where the dietary N was the only N source for microbial growth. To achieve a N-free medium, these

authors used a N-free buffer and treated rumen fluid with a mixture of simple sugars, so that rumen microbes consumed all N present in the rumen fluid.

To date, soybean meal is the most used protein supplement in animal feeding, because of its balanced amino acid profile and widespread availability (Gatlin et al., 2007). However, the increasing market price of soybean seed and meal has been recently forcing farmers to consider the use of alternative protein sources in ruminant diets. Canola represents the trademark of a particular rapeseed with low content of erucic acid (< 5% by weight, for EU) and glucosinolates (< 30 mmol/kg DM, for EU). In the last years an increasing number of farms has been equipping with facilities for mechanical extraction of oil from canola seeds. After the extraction, the oil is usually converted in biodiesel and utilized as fuel for tractors, whereas the resulting by-products (canola press cakes) can be included in ruminant diets as protein and fat sources. This productive cycle offers economical and environmental advantages, as it contributes to reduce the need for carburant fossil in agricultural industry and to recycle the resulting expellers as animal feeds (Baquero *et al.*, 2010; Esteban *et al.*, 2011). However, to date, information about fermentation characteristics of canola expeller are still limited.

Aim of the current study was to compare the *in vitro* GP values provided by high-protein feeds (soybean meal, whole soybean seed and canola expeller) using a N-rich and a N-free medium.

2. Materials and methods

2.1 Feeds

Canola expellers (CE) were obtained from cold-pressing of canola seeds. Canola seeds were cultivated and harvested at the pilot farm of the Veneto Agricultura association (Caorle, Venezia, Italy). Canola seeds were single-crushed at moderate temperatures (60°C) using two presses

equipped with a rotating screw shaft within an horizontal barrel (Mailca Srl, Modena, Italy). Whole soybean seed (WSS) and soybean meal (SBM) were commercial feeds used at the experimental farm of the University of Padova (Legnaro, Padova, Italy). Prior to incubation, about 1 kg of each feed was ground by a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, Germany) with a screen size of 1 mm. Thus, 15 samples were randomly prepared for each feed: 12 were used for the incubation run and the remaining 3 for chemical analyses.

2.2 Incubation procedures

A single incubation run was carried out with 3 sets of 12 bottles. A first set of 12 bottles (3 feeds × 4 replications) was filled with 0.550 ± 0.0010 g of feed sample, 25 ml of rumen fluid and 50 ml of the Goering and Van Soest (1970) medium (ratio 1:2 v/v) and incubated for 48 h to determine aNDF (aNDFd) and true DM (TDMd) degradability. Other 24 bottles (3 feeds × 4 replications × 2 buffers) were incubated for 72 h to assess GP kinetics: 12 bottles were filled with 0.550 ± 0.0010 g of feed sample, 25 ml of rumen fluid and 50 ml of the MS medium (ratio 1:2 v/v; Menke and Steingass, 1988), whereas the remaining 12 bottles were filled with 0.550 ± 0.0010 g of feed sample, 5 ml of rumen fluid and 95 ml of the NF medium (ratio 1:19 v/v) as described by Cone et al. (2009). For each medium, four bottles were included as blanks (bottles with only buffer and rumen fluid, without feed sample). The resulting experimental design was: 3 buffers 3 feeds 4 replications, plus 12 blanks, for a total of 48 bottles incubated.

The NF medium was prepared according to Menke and Steingass (1988), except for the buffer mineral solution that was N-free, as it contained only the sodium bicarbonate (39.25 g/l solution) and not the ammonium bicarbonate. Then, the NF medium was mixed with rumen fluid in a ratio

of 19:1, to limit the N contribution of rumen fluid (Cone et al., 2009). After that, according to the procedure of Cone et al. (2009), the buffered rumen fluid was incubated at 39 ± 0.5°C with 10 g/l of simple sugars (glucose, 3.33 g/l; xylose, 3.33 g/l and soluble starch (Merck 1252, Merck, Darmstadt, Germany), 3.33 g/l) to consume all N supplied by rumen fluid. In this way, during the incubation rumen microorganisms used only the N provided by feed for their growth (Cone et al., 2005, 2009). Over the entire pre-incubation GP was monitored, and fermentations were stopped when GP ceased definitively (about after 4 h). At the beginning of incubation pH of MS and NF media were 6.85 and 6.80, respectively. Rumen fluid was collected 3 h after morning feeding from 3 dry Holstein-Friesian cows housed at the Experimental Farm of the University of Padova, and fed hay *ad libitum* and 2 kg/d of concentrate (50% of corn meal, 25% of barley meal, and 25% of soybean meal). The collection was performed by a suction technique, using a vacuum pump made up of a glass container connected to a semi-flexible oro-ruminal probe equipped with a steel strainer on the top (Tagliapietra et al., 2012). The pH of the rumen fluid was measured immediately after collection and only fluids with pH lower than 6.8 were used for *in vitro* tests. Rumen fluid was stored into two thermal flasks preheated to 39 ± 0.5°C and transferred to the laboratory. At the laboratory, rumen fluid was filtered through 3 layers of cheesecloth to eliminate feed particles and mixed with the media. All these operations were performed under anaerobic conditions by flushing continuously with CO_2. Afterwards, all bottles were placed into a ventilated incubator (39.0 ± 0.5°C). During the incubation the bottles were not stirred. At the end of incubation pH values of MS and NF media were 6.80 and 7.00, respectively.

2.3 Gas Production

A fully automated GP technique (Ankom^{RF} Gas Production System, Ankom Technology®, NY, USA) made up of 48 glass bottles (310 ml) equipped with pressure detectors (pressure range: −69, +3447 kPa; resolution: 0.27 kPa; accuracy: ±0.1% of measured value) and wireless connected to a computer was used. During the incubation the pressure changes in the headspace of bottles, measured as difference with respect to atmospheric pressure at the beginning of incubation (Po), were transmitted via a radio frequency at a fixed interval time (1 min) and recorded in a database. Gas accumulated in the headspace of bottles was automatically released by an open-closed valve when a pressure variation of +3.4 kPa was reached. This threshold pressure, previously used by Tagliapietra et al. (2010), is similar to that used with other automated systems (+4.5 kPa; Calabrò et al., 2005). Pressure values of gas were cumulated and converted in terms of volume (GP, ml/g DM) using the ideal gas law:

$$GP = (\Delta P/Po) \times Vo \quad (1)$$

where: ΔP is the cumulative pressure change (kPa) in the bottle; Vo is the headspace volume (ml) of bottle and Po is atmospheric pressure (kPa) measured at the beginning of the incubation.

Curves of GP were fitted using model of Groot et al. (1996):

$$GP_t = A/[1 + (T½/t)^c] \quad (2)$$

$$T_{max} = T½ \times ((c - 1)/(c + 1))^{1/c} \quad (3)$$

$$R_{max} = (c \times T_{max}^{c-1})/ (T½^c + T_{max}^c) \quad (4)$$

In the equations (2), (3), and (4), A is the asymptotic GP (ml/g DM), T½ is the time (h) at which half of A is produced; t is the observation time (h); c is a constant representing the sharpness of

curve profile; T_{max} is the time (h) at which the maximum gas production rate is obtained; R_{max} is the maximum gas production rate (ml/h).

2.4. Chemical Analyses

Chemical analyses were carried out in the same laboratory by the same technician. At the end of incubation (48 h with GV buffer, and 72 h with MS and NF buffer), all fermentation fluids were analysed for pH. All feeds were analysed in triplicate for dry matter (DM; # 934.01; AOAC, 2003), crude protein (CP; # 976.05; AOAC, 2003), ether extract (EE; # 920.29; AOAC, 2003), ash (# 942.05; AOAC, 2003), starch (# 948.02; AOAC, 2003), and neutral detergent fibre (aNDF) (Mertens, 2002). The aNDF fraction, inclusive of residual ash, was determined with α-amylase and sodium sulphite using the Ankom^{220} Fibre Analyzer (Ankom Technology®, Macedon, NY, USA). The contents of acid detergent fibre (ADF), expressed as inclusive of ash, and sulphuric acid lignin ($lignin_{(sa)}$) were determined sequentially after aNDF determination (Robertson and Van Soest, 1981). At the end of incubation all residuals fermentation fluids were transferred into crucibles (30 ml por. 2, Robu Glasfilter-Geräte GMBH®, Hattert, Germany) and treated as indicated for aNDF analysis (Mertens, 2002) with a Fibertech Analyzer (VELP® Scientifica, Milano, Italy). The proximate composition of feeds is reported in Table 1.

2.6. Computation of TDMd

The digestible aNDF (dNDF, g/kg NDF) and the true DM digestibility (TDMd, g/kg DM) were computed according to Goering and Van Soest (1970):

$$dNDFd = [(aNDF_{feed} - aNDF_{res}) / aNDF_{feed}] \quad (5)$$

$$TDMd = 1000 \times [(DM_{feed} - aNDF_{res}) / DM_{feed}] \quad (6)$$

where $aNDF_{feed}$ is the aNDF content of the feed, $aNDF_{res}$ is the residual aNDF measured at 48 h of fermentation, DM_{feed} is the amount of DM incubated; all these amounts were expressed in grams of DM.

2.5. Statistical analysis

Experimental data were subjected to analysis of variance using the general linear model procedure (PROC GLM) of SAS (2005). The model considered the effect of medium, feed, and their interaction as sources of variation: As the effect due to the interaction medium × feed was never significant, this source of variation was excluded from the model.

3. Results

3.1 Chemical composition of feeds

The chemical composition of feeds is reported in Table 1. Compared to SBM and WSS, CE showed lower CP content and greater fibrous fraction (aNDF and ADF). The lipid content was similar among CE and WSS (199 and 202 g EE/kg DM for CE and WSS, respectively), whereas, as expected, SBM showed the lowest EE content (20 g EE/kg DM).

3.2 Degradability values and kinetics of GP

The values of aNDF (dNDF) and true DM digestibility (TDMd) are reported in Table 2. Compared to WSS and SBM, CE showed lower values both of dNDF and TDMd ($P < 0.01$), whereas no differences were detected among the two soybeans. For all feeds the values of dNDF resulted more variable compared to the corresponding TDMd (SEM = 0.95 and 0.01 for dNDF and TDMd, respectively). Gas production provided by the two buffers and by the three feeds at

various times of incubation and kinetic parameters of GP are highlighted in Table 2. Compared to MS, the NF buffer provided lower GP within the first 24 h of incubation ($P < 0.01$), whereas the two media did not differ for GP measured at 48 and 72 h of incubation. Further, the NF medium showed to slow down fermentation process compared to MS, as evidenced by greater values of T½ and T_{max} and lower values of R_{max} ($P < 0.01$ for all parameters). This pattern is also clearly evidenced by Figure 1, that shows the entire curves of GP provided by the two media. With both the media, the three feeds showed similar GP at 6 and 12 h of incubation, whereas CE provided lower values of GP compared to the two soybeans at 24 h until the end of incubation ($P < 0.01$). The GP kinetics provided by the three feeds when incubated with the MS and the NF medium are graphically reported in Figure 2a and 3a, respectively. However, CE was more rapidly fermented during the first phases of incubation, as clearly evidenced by the lower values of T½ and T_{max} and by greater values of R_{max} with respect to the two soybeans ($P < 0.01$ for all parameters). The kinetics of GP, expressed as ml gas/g CP, provided by the three feeds when incubated with the MS (Figure 2b) and the NF medium (Figure 3b) are reported. compared t

4. Discussion

4.1 Comparison among buffers

In the current experiment two different buffers were tested: one N-rich (MS) and the other one N-free (NF). Compared to MS, the NF medium showed to slow down fermentation process at the beginning of incubation, delaying for all feeds the time at which half of asymptotic GP was obtained (T½) and the time at which the maximum GP rate was reached (T_{max}). This pattern could be due to a range of different factors. The first factor could be related to the lower proportion of rumen fluid in the NF medium compared to MS (1:19 and 1:2 of ratio between rumen fluid and buffer for NF and MS, respectively). Rymer et al. (1999) examined three

different proportions between rumen fluid and buffer (50, 150 and 300 ml of rumen fluid/l of total fermentation fluid) and observed that the initial GP rate increased at increasing ratios, although all differences disappeared at T½ and T_{max}. Similarly, Pell and Schofield (1993) observed that, when the proportion of rumen fluid was increased to a level of 2.0 ml/10 ml of fermentation fluid, the GP rate increased and the lag time was reduced. On this basis, the same authors recommended a minimum concentration of 20 ml of rumen fluid/100 ml of fermentation fluid, to reduce the risk of limiting GP. The second factor could be that the NF medium provided only energy as support of microbial growth. It is recognized that microbial growth in the rumen is optimized by a balanced and simultaneous availability of energy and N sources, and that an imbalance between these two sources causes a lower efficiency of rumen fermentation (Sinclair et al., 1993). However, Russell and Strobel (1993) argued that an excess of energy is more deleterious for microbes compared to an excess of N, as the excess of ATP deriving from excess of energy is toxic for rumen microorganisms. Thus, it can be hypothesized that rumen microbes were more damaged by the lack of N in the NF medium than by the lack of energy in the MS medium. The third factor could be that the NF medium, prior to be incubated, was treated with a mixture of simple sugars, to consume all N present in the rumen fluid. It is largely recognized that composition of rumen fluid, expressed as kind and number of microorganisms, is primarily influenced by composition of diet fed to animals (Mould et al., 2005), and this pattern is more evident for high-concentrate diets (Bryant and Burkey, 1953; Maki and Foster, 1957). On this basis, it could be hypothesized that the pre-incubation with simple sugars may have altered the composition of rumen population, thus the resulting microbial pattern at the beginning of incubation could have not been optimal for fermentation of high-protein feeds as those analysed in the current work.

However, differences in GP among the two media disappeared at 48 and 72 h of incubation. The similar GP values at latter phases of incubation could be attributed to the comparable content of bicarbonate in the two media. Bicarbonate is commonly included in all media, in order to better simulate saliva production and rumen conditions (Rymer et al., 2005). However, the bicarbonate content of the medium may influence GP, as bicarbonate ions react with volatile fatty acids and generate gas (CO_2). This proportion of gas is defined as "indirect gas", since it is released by the medium and not from feed fermentation. Rymer et al. (1998) observed that the media of Goering and Van Soest (1970), Steingass (1983), Theodorou (1993), and Huntington et al. (1998) differed for total GP and attributed this result to the different amount of bicarbonate in the four buffers. However, Omed et al. (1998) argued that bicarbonate could be completely replaced by phosphate in the buffer solution, even if rumen microbes require both bicarbonate and phosphate to grow (Williams, 1998). This replacement could permit to limit shortcomings related with the production of "indirect gas", since the phosphate does not release CO_2 (Blümmel et al., 1999). However, Kennedy et al. (2000) argued that also phosphate components could alter GP kinetics, through a slight increment of fiber degradability.

4.2 Comparison between feeds

In the current experiment the proximate composition of the two soybeans (SBM and WSS) resulted close to tabled data (INRA, 1988; NRC, 2001). Canola expellers showed an EE content in line with findings of Leming and Lember (2005), and intermediate between 13% DM reported by Spragg and Mailer (2007) and 27% DM observed by Thacker and Petri (2009). To this regard, several authors indicated that the EE content of expellers is mostly influenced by the number of pressings (Weigal, 1991; Glencross et al., 2004). When the oil is mechanically extracted, seeds are usually subjected to single or double-pressing; however, the double-pressing is preferable as

it ensures a higher efficiency of oil recover (Matthäus, 2012). In the present experiment, canola seeds were pressed only once, and this could explain the great oil content of expellers. Further, canola seeds were processed at moderate temperatures without the application of external heat ("cold-pressing" at 60°C), but the only heat was that produced by crushing of seeds. Differently, both soybeans were subjected to toasting at 100°C for 30 min, to deactivate anti-nutritional factors present in the seeds (i.e. trypsin inhibitors). Heat-treatment (at temperatures ≥ 100°C) was found to reduce the rumen degradability of fibre (McKinnon and Walker, 2009) and protein (Mustafa et al., 1999; Kass et al., 2006). The reduced dNDF was attributed to a possible binding effect exerted by heat on structural carbohydrates (McKinnon and Walker, 2009). However, since canola seeds were pressed at moderate temperatures, in our case lower values of dNDF obtained for CE compared to the two soybeans were presumably related to great ADL content of CE. With regard to the protein fraction, heat treatment (i.e. toasting) is purposely adopted to reduce protein degradability in the rumen, increasing the proportion of bypass protein and the intestinal availability of essential AAs as lysine and methionine. As mentioned above, both the soybeans were toasted, and this could explain the lower degradability showed by these two feeds compared to CE, especially at first phases of incubation. Therefore, values and kinetics of GP evidenced by the three feeds were not only related to their proximate composition, but also to processing conditions, that modified chemical and physical properties of the three substrates. However, values of GP reported in this study should be considered in relative terms, as possible underestimations cannot be excluded. Indeed, all tested feeds were characterized by a notable protein content. To this regard, Cone and Van Gelder (1999) observed that each point percentage of protein caused a reduction in GP of 2.48 ml, and attributed these results to the fact that protein fermentation leads to the formation of ammonia that inhibits the release of CO_2 ("indirect gas")

from the fermentation fluid. Recently these findings were supported by Tagliapietra et al. (2011), which noted that high-protein feeds (> 16% CP) produced smaller amounts of gas compared to other categories of feeds.

5. Conclusions

The level of N in the medium strongly influenced GP kinetics at early phases of incubation, without any effect on total GP provided by the three substrates. In particular, the absence of N in the medium seems to impair microbial activity and growth during the first 24 h of incubation. Compared to the two soybeans, CE showed a lower *in vitro* degradability and GP but a greater fermentation rate at early phases of incubation, and this pattern could be attributed to a higher degradability of protein fraction.

Acknowledgments

This study was made possible by a grant from the Veneto Agricoltura (Veneto Region Agency),Viale dell'Università, 14, 35020 Legnaro (PD), "BIOSIRE: biofuels and electric propulsion creating sustainable transport in tourism resorts" funded by the Community program Intelligent Energy for Europe (IEE).

References

AOAC. 2003. Official Methods of Analysis, 17th ed., 2nd rev. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Blümmel, M., Aiple, K.P., Steingass, H., Becker, K., 1999a. A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality. J. Anim. Physiol. Anim. Nutr. 81, 157-167.

Bryant, M.P., Burkey, L.A., 1953. Number and some predominate groups of bacteria in the rumens of cows fed different diets. J. Dairy Sci. 36, 218–224.

Calabrò S., Lopez, S., Piccolo, V., Dijkstra, J., Dhanoa, M.S., France, J., 2005. Comparative analysis of gas production profiles obtained with buffalo and sheep ruminal fluid as the source of inoculum. Anim. Feed Sci. Technol. 123-124, 51-65

Cone, J. W., and A. H. Van Gelder. 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76:251-264.

Cone, J.W., Rodrigues, M.A.M., Guedes, C.M., Blok, M.C., 2009. Comparison of protein fermentation characteristics in rumen fluid determined with the gas production technique and the nylon bag technique. Anim. Feed Sci. Technol. 153, 28-38

Cone, J.W., Van Gelder, A.H., Bachmann, H., 2005. Influence of inoculum source on gas production profiles. Anim. Feed Sci. Technol. 99, 221–231.

DePeters, E.J., Fadel, J.G., Arosemena, a., 1997. Digestion kinetics of neutral detergent fiber and chemical composition within some selected by-product feedstuffs. Anim. Feed Sci. Technol. 67, 127-140

Esteban, B., Baquero, G., Puig, R., Riba, J., Rius, A., 2011. Is it environmentally advantageous to use vegetable oil directly as biofuel instead of converting it to biodiesel?. Biomass Bioenergy. 35, 1317-1328

Glencross, B., W. Hawkins, and J. Curnow. 2004a. Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (*Pagrus auratus*, Paulin). Aquacult. Res. 35:15-24.

Glencross, B., W. Hawkins, and J. Curnow. 2004b. Nutritional assessment of Australian canola meals. II. Evaluation of the influence of the canola oil extraction method on the protein value of canola meals fed to the red seabream (*Pagrus auratus*, Paulin). Aquacult. Res. 35:25-34.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

Huntington, J.A., Rymer, C., Givens, D.I., 1998. The effect of host diet on the gas production profile of hay and high-temperature dried grass. Anim. Sci. 67, 59–64.

Kaldmae, H., Leming, R., Kass, M., Lember, A., Tölp, S., Kärt, O., 2010. Chemical composition and nutritional value of heat-treated and cold-pressed rapeseed cake. Veterinarija ir Zootechnika. 49

Kaldmäe, H., R., M. Leming, A. Kass, A. Lember, S. Tölp, and O. Kärt. 2010. Chemical composition and nutritional value of heat-treated and cold-pressed rapeseed cake. Vet. Med. Zoot. 49:55-60.

Kennedy, P.M., Lowry, J.B., Conlan, L.L., 2000. Phosphate rather than surfactant accounts for the main contribution to enhanced fibre digestibility resulting from treatment with boiling neutral detergent. Anim. Feed Sci. Technol. 86, 177–190.

Klein-Hessling, H. 2007. Value canola meal before using it. World Poult. 23:24-28.

Koenig, K. M., and K. A. Beauchemin. 2005. Barley versus protein-supplemented corn-based diets for feedlot cattle evaluated using the NRC and CNCPS beef models. Can. J. Anim. Sci. 85:377-388.

Leming, R., and A. Lember. 2005. Chemical composition of expeller-extracted and cold-pressed canola meal. Agraarteadus 16:103-109.

Maki, L.R., Foster, E.M., 1957. Effect of roughage in the bovine ration on types of bacteria in the rumen. J. Dairy Sci. 40, 905–913.

Mazurkiewicz, J., A. Przybyl, G. Czyzak-Runowska, and A. Lyczynski. 2011. Cold-pressed rapeseed cake as a component of the diet of common carp (*Cyprinus Carpio* L.): effects on growth, nutrient utilization, body composition and meat quality. Aquacult. Nutr. 17:387-394.

Mckinnon, J. J., and A. M. Walker. 2009. Comparison of canola and mustard presscake from biodiesel production as protein sources for growing cattle. Can. J. Anim. Sci. 89:401-408.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Int. 85:1217-1240.

Mould, F., Kliem, K., Morgan, R., Mauricio, R., 2005. In vitro microbial inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123-124, 31-50

Mould, F.L., Kliem, K.E., Morgan, R., Mauricio, R.M., 2005. *In vitro* microbial inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123-124, 31-50.

Mustafa, A.F., Christensen, D.A., Mckinnon, J.J., Newkirk, R., 1999. Effects of stage of processing of canola seed on chemical composition and in vitro protein degradability of canola meal and intermediate products. Agriculture

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National academy of Sciences, Washington, DC.

Omed, H.M., Axford, R.F.E., Chamberlain, A.G., Givens, D.I., 1989. A comparison of three laboratory techniques for the estimation of the digestibility of feedstuffs for ruminants. J. Agric. Sci. Camb. 113, 35–39.

Pell, A.N., Schofield, P., 1993. Computerized monitoring of gas production to measure forage digestion *in vitro*. J. Dairy Sci. 76, 1063–1073.

Petit, H. V., and D. M. Veira. 1994. Effect of post-weaning protein supplementation of beef steers fed grass silage on performance during the finishing phase, and carcass quality. Can. J. Anim. Sci. 74:699-701.

Robertson, J.B., Van Soest, P.J., 1981. The detergent system of analysis and its application to human foods. In: James, W.P.T., Theander, O. (Eds.), The Analysis of Dietary Fiber in Food. Marcel Dekker Inc., New York, NY, USA, pp. 123–158.

Russell, J.B., Strobel, H.J., 1993. Microbial energetics. In: Forbes, J.M., France, J. (Eds.), Quantitative Aspects of Ruminant Digestion and Metabolism. CABI, Wallingford, UK, pp. 165–186.

Rymer, C., Huntington, J.A., Givens, D.I., 1999. Effects of inoculum preparation method and concentration, method of inoculation and pre-soaking the substrate on the gas production profile of high temperature dried grass. Anim. Feed Sci. Technol. 78, 199–213.

Rymer, C., Huntington, J.a., Williams, B.a., Givens, D.I., 2005. In vitro cumulative gas production techniques: History, methodological considerations and challenges. Anim. Feed Sci. Technol. 123-124, 9-30

Rymer, C., Moss, A.R., Deaville, E.R., Givens, D.I., 1998b. Factors affecting the amount of indirect gas produced by the *in vitro* gas production technique. In: Deaville, E.R., Owen, E., Adesogen, A.T., Rymer, C., Huntington, J.A., Lawrence, T.L.J. (Eds.), In Vitro Techniques for Measuring Nutrient Supply to Ruminants. BSAS, Edinburgh, UK, pp. 89–91, BSAS Occ. Publ. No. 22.

SAS® User's Guide: Statistics, 5th ed. 2005. SAS Institute, Cary, NC, USA.

Seneviratne, R. W., E. Beltranena, L. A. Goonewardene, and R. T. Zijlstra. 2011a. Effect of crude glycerol combined with solvent-extracted or expeller-pressed canola meal on growth performance and diet nutrient digestibility of weaned pigs. Anim. Feed Sci. Technol. 170:105-110.

Seneviratne, R. W., E. Beltranena, R. W. Newkirk, L. A. Goonewardene, and R. T. Zijlstra. 2011b. Processing conditions affect nutrient digestibility of cold-pressed canola cake for grower pigs. J. Anim. Sci. 89:2452-2461.

Seneviratne, R. W., M. G. Young, E. Beltranena, L. A. Goonewardene, R. W. Newkirk, and R. T. Zijlstra. 2010. The nutritional value of expeller-pressed canola meal for grower-finisher pigs. J. Anim. Sci. 88:2073-2083.

Spragg, J., and R. Mailer. 2007. Canola meal value chain quality improvement. Canola meal value chain quality improvement. A final report for AOF and Pork CRC. Project 1B-103-0506.

Steingass, H., 1983. Bestimmung des energetischen Futterwertes von wirtschaftseigenen Futtermitteln aus der Gasbildung bei der Pansenfermentation in vitro. PhD Thesis, University of Hohenheim, Hohenheim, Germany.

Tagliapietra, F., Cattani, M., Bailoni, L., Schiavon, S., 2010. In vitro rumen fermentation: effect of headspace pressure on the gas production kinetics of corn meal and meadow hay. Anim. Feed Sci. Technol. 158, 197-201

Tagliapietra, F., Cattani, M., Hansen, H.H., Hindrichsen, I.K., Bailoni, L., Schiavon, S., 2011. Metabolizable energy content of feeds based on 24 or 48h *in situ* NDF digestibility and on *in vitro* 24h gas production methods. Anim. Feed Sci. Technol. 170, 182-191

Tagliapietra, F., Cattani, M., Hindrichsen, I.K., Hansen, H.H., Colombini, S., Bailoni, L., Stefano Schiavon, 2012. True dry matter digestibility of feeds evaluated *in situ* with different bags and *in vitro* using rumen fluid collected from intact donor cows. Anim. Prod. Sci. 338-346

Thacker, P. A., and D. Petri. 2009. The effects of canola or mustard biodiesel press cake on nutrient digestibility and performance of broiler chickens. Asian-Aust. J. Anim. Sci. 22:1531-1539.

Theodorou, M.K., 1993. A new laboratory procedure for determining the fermentation kinetics of ruminant feeds. Ciencia e investigacion agraria 20, 332–344.

Weigal, J. 1991. Oilseed meals and the effect of processing on their subsequent quality. Page 317 in Recent Advances in Animal Nutrition in Australia. Ed. D. J. Farrell, University of New England, Armidale.

Williams, B.A., 1998. Poster discussion session report. In: Deaville, E.R., Owen, E., Adesogen, A.T., Rymer, C., Huntington, J.A., Lawrence, T.L.J. (Eds.), In Vitro Techniques for Measuring Nutrient Supply to Ruminants. BSAS, Edinburgh, UK, pp. 282–284, BSAS Occ. Publ. No. 22.

Wood, C.D., Murray, A.H., Moss, A.R., Givens, D.I., 1998. Use of the gas production technique to investigate responses of supplementing lowquality forages: 1. In vitro interactions. In:

Deaville, E.R., Owen, E., Adesogen, A.T., Rymer, C., Huntington, J.A., Lawrence, T.L.J. (Eds.), In Vitro Techniques for Measuring Nutrient Supply to Ruminants. BSAS, Edinburgh, UK, pp. 92–94, BSAS Occ. Publ. No. 22.

Woyengo, T. A., E. Kiarie, and C. M. Nyachoti. 2010. Energy and amino acid utilization in expeller-extracted canola meal fed to growing pigs. J. Anim. Sci. 88:1433-1441.

Table 1. Dry matter (DM, g/kg) and chemical composition (g/kg DM) of feeds (n = 3)

Feed	Soybean meal	Whole soybean seed	Canola expeller
DM	874	888	895
aNDF	153	135	291
ADF	74	62	202
$Lignin_{(sa)}$	4	12	75
CP	483	385	287
EE	20	202	199
Ash	63	52	66
NSC[a]	281	226	157
Starch	38	42	129

[a]Calculated as NSC = 100 – (CP – Ash – EE – aNDF)

Table 2. *In vitro* NDF (NFDd) and true dry matter (TDMd) degradability of feeds (n = 3) measured at 48 h of incubation.

	Feed			SEM
	Soybean meal	Whole soybean seed	Canola expeller	
NDFd[a] (g/kg aNDF)	910[A]	916[A]	681[B]	0.95
TDMd[b] (g/kg DM)	981[A]	981[A]	829[B]	0.01

[a] NDF degradability at 48h of incubation.

[b] True dry matter digestibility at 48 h of incubation.

Table 3. Effect of medium and feed on gas production (ml/g DM) and kinetic parameters of gas production

	Medium[a]		Feed			*P* value		SEM
	N-rich (MS)	N-free (NF)	Soybean meal	Whole soybean seed	Canola expeller	Medium	Feed	
Gas production, ml/g DM								
6 h	69±8.8	35±4.1	51±18.0	49±14.9	56±22.2	<0.01	0.11	4.2
12 h	111±7.9	80±8.9	99±18.7	94±15.6	92±18.0	<0.01	0.35	3.8
24 h	148±15.1	131±16.8	155±12.1	139±12.6	124±11.8	<0.01	<0.01	3.8
48 h	171±21.8	162±25.5	195±8.3	165±11.7	142±10.4	0.06	<0.01	5.2
72 h	180±24.2	171±28.8	207±7.8	172±11.7	147±10.9	0.09	<0.01	5.8
Kinetic parameters of gas production								
A[b], ml/g DM	192±28.0	181±32.9	224±7.6	182±12.6	154±14.2	<0.05	<0.01	6.7
R_{max}[c], ml/h	12.8±2.87	6.8±0.74	9.5±2.50	8.7±2.02	11.3±5.08	<0.01	<0.05	0.84
T½[d], h	9.3±2.24	13.6±2.29	13.8±2.55	11.5±1.98	9.1±2.68	<0.01	<0.01	0.76
T_{max}[e], h	4.1±2.18	11.5±1.57	9.2±4.47	8.6±3.32	5.6±4.39	<0.01	<0.01	1.00
c[f]	1.32±0.162	1.75±0.073	1.51±0.210	1.62±0.202	1.48±0.332	<0.01	0.09	0.056

[a] MS = N-rich medium (Menke and Steingass, 1988); NF = N-free medium

[b] A = asymptotic gas production.

[c] R_{max} = maximum rate of gas production (ml gas/h).

[d] T½ = time at which half of asymptotic gas production is reached (h).

[e] T_{max} = time at which the maximum rate of gas production is observed (h).

[f] c = constant representing the sharpness of the curve.

Figure 3. Kinetics of gas production of N-rich (Menke and Steingass, 1988; dotted line) and N-free medium (solid line).

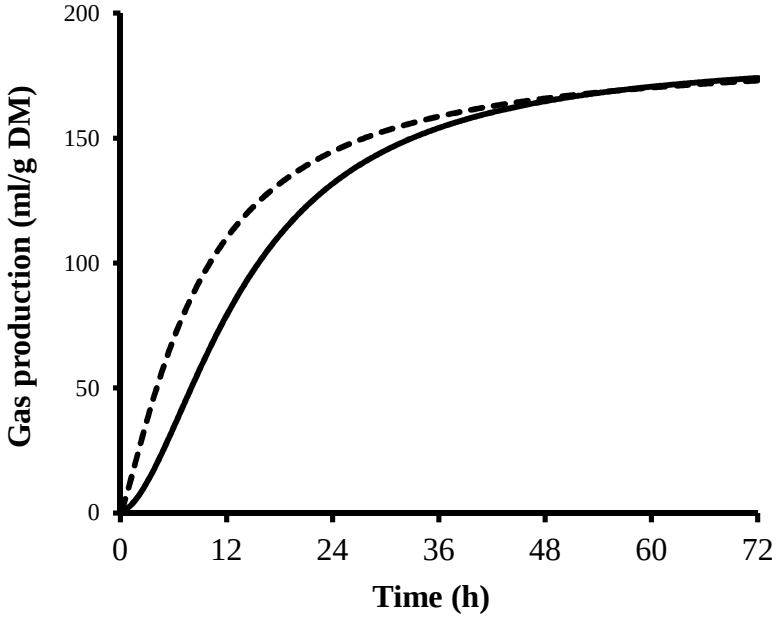


Figure 4 . Kinetics (ml/g DM) and variation (ml/g CP) of gas production of soybean meal (SBM; dotted line), whole soybean seed (WSS; dash dotted line), and canola expeller (CE; solid line) incubated with the N-rich medium (Menke and Steingass, 1988).

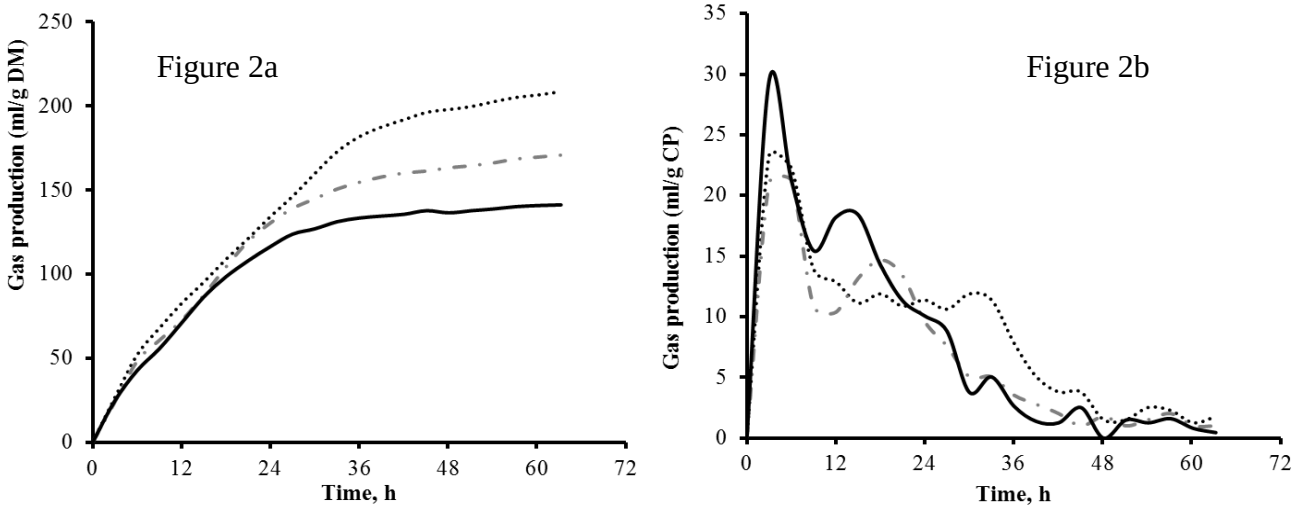
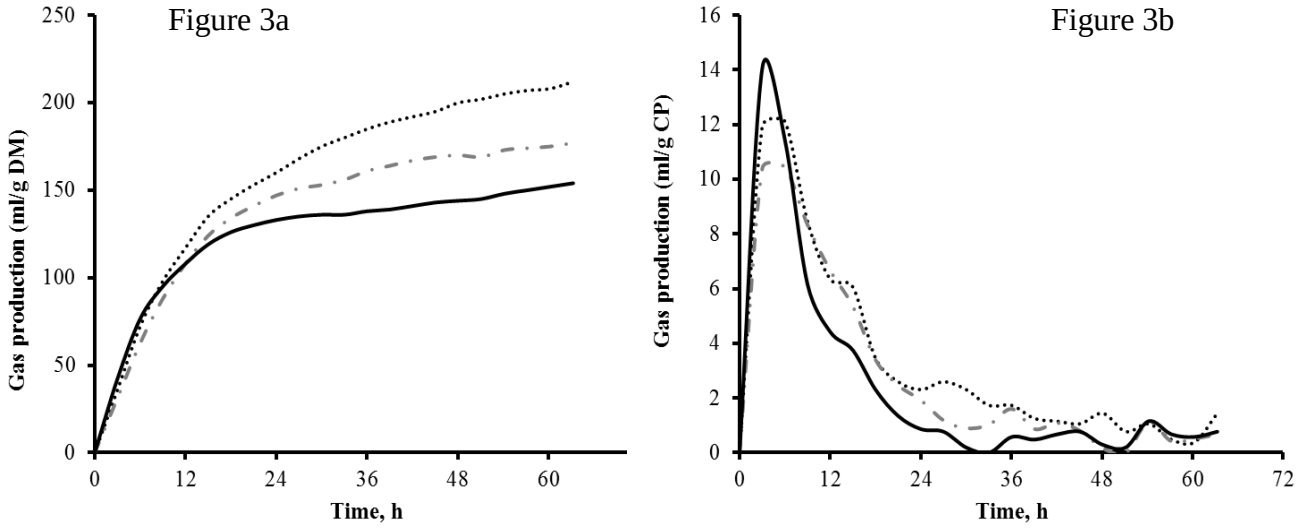


Figure 5. Kinetics (ml/g DM) and variation ml/g CP) of gas production of soybean meal (SBM; dotted line), whole soybean seed (WSS; dash dotted line), and canola expeller (CE; solid line), incubated with the N-free medium.



Chapter 5.

(Third contribute)

In vitro rumen fermentation and microbial yield of diets containing whole soybean seed and canola expeller at two different inclusion levels

M. Guadagnin[1], F. Tagliapietra[2*], M. Cattani[2], S. Schiavon[2], A. Belanche[3], C.J. Newbold[3], L. Bailoni[1]

[1]Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy;

[2]Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy;

[3]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, SY23 3AL, United Kingdom

[*]*Corresponding author:* F. Tagliapietra

franco.tagliapietra@unipd.it

Tel. +39 0498272622

Fax +39 0498272633

ABSTRACT

Aim of the current experiment was to compare *in vitro* four diets for beef cattle that combined two different protein sources; toasted whole soybean seed (WSS) and cold-pressed canola expeller (CE) (38.5 *vs.* 28.7 % CP and 20.2 *vs.* 19.9 % EE on DM basis, for WSS and CE, respectively) at high (14% on DM basis) and low inclusion level (7% on DM basis). All diets (20 ± 0.5 g; ground at 1 mm) were incubated at 39°C in 4 replicates into separated nylon bags (110×60 mm, pore size 100 µm) using Rusitec fermenters (16 fermentation vessels in total). Firstly, diets were incubated for 10 d to stabilize microbial population in the RUSITEC (adaptation period). Then, diets were incubated for 7 d (experimental period) to evaluate degradability, gas (GP), volatile fatty acids (VFA) and ammonia production, and N balance. Moreover, microbial N outflow was estimated using ^{15}N as microbial marker. ANOVA was performed to evaluate the effects of supplement, inclusion level and their interaction. Compared to WSS, CE provided greater values of aNDF degradability ($P<0.01$), produced less acetate and propionate ($P<0.001$) but more butyrate and branched-chain VFA, thus the resulting total VFA production was similar for the two supplements. With regard to nitrogen balance, CE showed greater ^{15}N enrichment in the non-ammonia N ($P<0.01$) and nominally lower values of microbial N derived from ammonia compared to WSS ($P=0.057$). Degradability, GP, acetate and total VFA production were comparable for the two inclusion levels. At low inclusion level the propionate production was greater, the (acetate + butyrate)/propionate ratio and branched-chain VFA were lower ($P<0.001$). At low inclusion level, the ^{15}N enrichments for ammonia N, non-ammonia N and total bacteria N were also greater than those observed at high inclusion level ($P<0.001$). Conversely, no differences were found for microbial N and efficiency of microbial N production among the two inclusion levels. In conclusion, the two supplements showed different fermentation properties likely due to differences in their chemical composition and processing method. To this end, the manipulation of dietary protein level seemed to lead primarily to a variation of bypass protein, without effects on the synthesis of microbial N.

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; CP, crude protein; DM, dry matter; DMD, DM digestibility; dNDF, digestible aNDF; E-NAN_{TD}, non ammonia nitrogen of total digesta ^{15} N enrichment; E-TB, total bacteria ^{15} N enrichment; E-NH_3, ammonia nitrogen ^{15} N enrichment; EE, ether extract; EMS, efficiency of microbial synthesis; ENU, efficiency of nitrogen utilization; GP, gas production; lignin($_{sa}$), lignin determined by solubilization of cellulose with sulphuric acid; MN, microbial nitrogen; NADR, nitrogen apparent digestibility in the rumen; NAN, non-ammonia nitrogen; N-NH_3, ammonia N; OM, organic matter; OMAD, OM apparent digestibility; TOMd, true OM digestibility; VFA, volatile fatty acids.

Key words: Canola expeller, Rusitec, Microbial growth, Rumen degradability.

1. Introduction

In the last years constraints introduced by Nitrate Directive (European Commission, 1991) and increasing market price of soybean are forcing farmers to consider the use of low protein diets and alternative protein sources. Currently, rapeseed (*Brassica Napus*) is largely used in animal feeding and represents the second most widely traded protein supplement after soybean seed (*Glycine max*). Canola meal, that is derived by the solvent-extraction of oil from a Canadian variety of rapeseed, has been favorably used as alternative to soybean in beef cattle (Petit and Veira, 1994; Koenig and Beauchemin, 2005), pig (Seneviratne et al., 2010, 2011a,b; Woyengo et al., 2010) and fish diets (Mazurkiewicz et al., 2011). On the opposite, there are limited information about canola expeller, the by-product obtained from the mechanical extraction of oil (Thacker and Petri, 2009; Kaldmäe et al., 2010). In the last years an increasing number of farms is equipping with small-scale facilities for mechanical extraction of oil from oleaginous seeds. After the extraction, the oil is usually utilized as fuel, whereas residual expellers are fed to animals as protein and fat sources. This productive cycle provides both economical and environmental advantages, as it contributes to reduce the need for carburant fossil in agricultural industry and to recycle the resulting expellers as animal feeds

(Baquero et al., 2010; Esteban et al., 2011). The mechanical extraction of oil is less efficient compared to the solvent-extraction, thus canola expellers contain a higher fat percentage (8-20% DM) than the corresponding meals (<5% DM). High fat percentage of canola expellers could inhibit fibre digestibility in ruminants and, further, make these products more prone to oxidation and less storable at farm level. To date, the nutritional and energy value of canola expellers are still poorly characterized, as well as their effects on rumen fermentation (Kaldmae et al., 2010). Therefore, the aim of the current experiment was to compare *in vitro* four diets for beef cattle containing whole soybean seed or canola expeller dosed at two inclusion levels, in terms of digestibility, gas production, end-products of fermentation, and nitrogen balance.

2. Material and methods

2.1 Equipment

All procedures were carried out in accordance with protocols approved by the Aberystwyth University Local Ethical Review Committee. The study was conducted using two rumen simulation techniques (RUSITEC) made up of 8 fermentation vessels each (Czerkawski & Breckenridge, 1977). Each fermentation vessel had a nominal volume of 700 ml. Rumen solid contents for incubation were collected before morning feeding from 4 dry ruminally-fistulated Holstein-Friesian cows fed a mixed diet (70% roughages and 30% concentrates) at maintenance level. After the collection, rumen fluid was transferred to the laboratory within 30 min. An artificial saliva (McDougall, 1948) was continuously infused by an automatic pump (205U, Watson-Marlow Ltd., Falmouth, Cornwall, UK) in the system (dilution rate: 27.5 ml/h) and was replaced daily to avoid microbial contamination.

2.2 Diets

Four iso-lipid and isonitrogenous diets, composed by feed ingredients commonly included in diets for beef cattle in farms of Northern Italy, were incubated (Table 1). Diets were supplemented with whole soybean seeds (WSS) or canola expeller (CE) at a high (14.0 % DM) or low (7.0 % DM) inclusion level. Different amounts of soybean meal were also included to achieve two dietary

protein levels: low (10.9% CP) and high (14.7% CP). Whole soybean seeds were collected from the experimental farm of the University of Padova (Legnaro, Padova, Italy). Canola expeller was derived by a canola variety (Excalibur; Dekalb, Monsanto Agricoltura Spa, Lodi, Italy) that was grown and harvested in a pilot farm located in Valle Vecchia (Caorle, Venezia, Italy). Canola seeds were crushed at moderate temperatures (60°C) using two single-screw presses equipped with a rotating screw shaft within an horizontal barrel (Mailca Srl, Modena, Italy). The two presses had an input flow rate of 120 kg/h of seeds, with a production of 40 kg/h of oil and 80 kg/h of expellers. Prior to incubation, all diets were ground by a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, Germany) with a screen size of 1 mm.

2.3 Incubation procedures

The whole incubation lasted 17 d (10 d for adaptation and 7 d for sample collection). The adaptation period was used to check the correct working of the system and to stabilize microbial population. On day 1, each fermentation vessel was filled with 300 ml of rumen fluid, 300 ml of artificial saliva, and two nylon bags (110x60 mm, pore size 100 µm), one containing rumen solid contents (60 ± 0.5 g) as starter for fermentation activity, and the other one containing the diet (20 ± 0.5 g DM). The diets were randomly assigned to each fermentation vessel and incubated in 4 replicates at 39 ± 0.5°C. On day 2, nylon bag containing rumen solid contents was removed and replaced with a nylon bag containing the diet. From day 3 to the end of experiment (day 17), one of the two nylon bags present in the same vessel was alternatively replaced, to give a 48-h incubation time to each nylon bag. During the bag replacement process, fermenters were continuously flushed with CO_2 to maintain anaerobic conditions. During the incubation, nylon bags were gently agitated to simulate the rumen movement. On day 11, a dose of 3.15 mg of N^{15} (95% enriched $(^{15}NH_4)_2SO_4$) was added into each fermentation vessel to label instantaneously the NH_3-N pool. Then a solution of $(^{15}NH_4)_2SO_4$ was added to the artificial saliva at a rate of 3.70 mg/l, to label microbial protein with ^{15}N, and to estimate microbial synthesis. During days 11, 12 and 13, nylon bags were collected after 48 h of incubation, washed twice with artificial saliva and then washed in the cold rinse cycle

(20 min) of a washing machine to remove the attached bacteria. Residuals were analyzed for DM, N, and residual aNDF to evaluate the corresponding digestibility. The same days, vessel fluids were analysed for pH. Moreover, an aliquot (4 ml) of vessel fluid was collected from each fermentation vessel and diluted with 1 ml deproteinizing solution (10% metaphosphoric acid and 0.06% crotonic acid, w/v) to determine VFA profile. Another aliquot (1 ml) of rumen fluid was collected from each vessel and diluted with 0.6 ml of 25% trichloroacetic acid to determine NH_3 concentration. On days 14, 15 and 16, vessel outflows were collected and saturated with 10 ml/d of $HgCl_2$ to stop fermentation process. Residuals into nylon bags, after 48 h of incubation, were collected for 3 days from each vessel and mixed with 250 ml of their correspondent fermentation fluids and homogenized using a Stomacher (260 rpm; 2 min), in order to reconstitute the total digesta (TD). An aliquot of TD (about 500 g) was wetted and adjusted with 1M-NaOH to pH 10, and dried at 90°C for 16 h to remove NH_3-N (Firkins et al., 1992), lyophilized, and stored at -20ºC until the determination of non ammonia nitrogen (NAN) and ^{15}N enrichment (E-NAN_{TD}). A second aliquot of TD (about 500 g) was used to isolate total bacteria (TB), following the procedure of Hristov et al. (2001). Briefly, the aliquot of TD was centrifuged at 500 *g* (5 min; 4ºC) to separate undigested feed particles and attached bacteria or protozoa. After that, the supernatant was centrifuged at 30,000 *g*, thus the pellet and the supernatant were separated. The pellet was analysed for N and ^{15}N enrichment (E-N_{TB}). An aliquot of supernatant (about 200 g) was acidified with 3 ml H_2SO_4, and stored at -20ºC until determination of NH_3-N and ^{15}N enrichment (E-NH_3). For the determination of NH_3-N pool, an aliquot (12 ml) of supernatant was diluted with 8 ml of NaOH (10M) and NH_3-N was evaporated over 7 d at room temperature and absorbed onto 5 mm glass filter disks impregnated with 15 µl of 2.5M $KHSO_4$ (Brooks et al., 1989). Disks were dried for 24 h in a desiccators using H_2SO_4 and were analysed for ^{15}N. During the whole experimental period, fermentation gases were measured.

2.4 Analytical procedures

All analytical procedures were carried out in the same laboratory by the same technician. All feeds were analysed in triplicate for dry matter (DM; # 934.01; AOAC, 2003), crude protein (CP; # 976.05; AOAC, 2003), ether extract (EE; # 920.29; AOAC, 2003), ash (# 942.05; AOAC, 2003), starch (# 948.02; AOAC, 2003), and neutral detergent fibre (aNDF) (Mertens, 2002). The aNDF fraction, inclusive of residual ash, was determined with α-amylase and sodium sulphite using the Ankom^{220} Fibre Analyzer (Ankom Technology®, Macedon, NY, USA). Acid detergent fibre (ADF), expressed as inclusive of residual ash, and sulphuric acid lignin ($lignin_{(sa)}$) contents were determined sequentially after aNDF determination (Van Soest et al., 1991). The VFA profile of fermentation fluids was determined using the Varian CP-8400 Auto-sampler gas-chromatograph (Agilent Technologies Inc., Palo Alto, USA) equipped with a HP-FFAP column (Agilent Technologies Inc., Palo Alto, USA). Ammonia content was determined by a colorimetric method (Weatherburn, 1967). Total N and ^{15}N enrichment of NAN_{TD} ($E\text{-}NAN_{TD}$) of TB (E-TB) and of $N\text{-}NH_3$ ($E\text{-}NH_3$) were established using a N analyzer connected to a 20-20 mass spectrophotometer (ANCA/SL, PDZ Europe Ltd., Crewe, Cheshire, UK). Total gas production (GP) was measured using a drum-type gas meter (Series TG1, Ritter Apparatebau GmbH, Bochum, Germany), and was expressed at normal atmospheric conditions (1 atm, 20ºC).

2.5 Computations

The DM digestibility (DMD), the OM apparent digestibility (OMAD) in the rumen, the nitrogen apparent digestibility in the rumen (NADR), the true OM digestibility (TOMd) and aNDF digestibility (dNDF) measured at 48 h of incubation were computed as follows (Goering and Van Soest, 1970):

$$DMD = (DM_{feed} - DM_{res}) / DM_{feed};$$

$$OMAD = (OM_{feed} - OM_{res})/OM_{feed}$$

$$NADR = (N_{feed} - N_{res})/N_{feed}$$

$$TOMd = (OM_{feed} - aNDF_{res}) / OM_{feed};$$

$$dNDF = (aNDF_{feed} - aNDF_{res}) / aNDF_{feed}$$

where: DM_{feed}, OM_{feed}, N_{feed} and $aNDF_{feed}$, , are the amounts of DM, OM, N and aNDF incubated respectively; DM_{res}, OM_{res}, N_{res} and $aNDF_{res}$ are the amounts of DM, OM, N and aNDF measured on the residuals after 48h of incubation.

The proportion of NAN_{TD} of microbial origin (MN%) was estimated by dividing the ^{15}N enrichment (atom % excess) from the proportion of NAN of digesta from each vessel by the enrichment of TB pellets ($MN\% = E\text{-}NAN_{TD}/E\text{-}N_{TB}$) (Carro and Miller, 1999). Daily MN flow was estimated from total NAN multiplied for the proportion attributed to the microbes (MN flow = NAN/MN%, mg/d). The proportion of microbial N derived from NH_3-N was estimated dividing ^{15}N enrichment of TB by the enrichment of NH_3-N ($E\text{-}N_{TB}/E\text{-}NH_3$, %). The efficiency of microbial synthesis (EMS) was estimated as the ratio of daily amount of MN flow and OM apparently digested in the rumen (g MN/kg OMAD). The daily efficiency of microbial nitrogen utilization (ENU) computed as gram of microbial N/available N ×100; in RUSITEC, available N was computed as dietary N – undegraded N (MN/[N intake – NAN+MN) as described by Bach et al. (2005).

2.6 Statistical Analysis

All data were analyzed by PROC MIXED for repeated measures of SAS (2005). The model accounted for the diet [4 levels, for each combination of supplement (CE or WSS) and inclusion level (low or high)], the inclusion level and their interaction as fixed effects, and the vessel nested within the diet as random effect. Contrasts were carried out between: i) the two inclusion levels (low and high); ii) the two supplements (CE and WSS). Treatment effects were accepted as significant with a *P* value of 0.05 or less.

3. Results

3.1 Chemical composition of supplements

Compared to WSS, CE showed lower CP (287 and 385 g/kg DM, for CE and WSS, respectively) a similar EE content (199 and 202 g/kg DM, in the same order) and a greater fibrous fraction (291 and 135 g aNDF/kg DM, in the same order), with a greater proportion of $lignin_{(sa)}$ (75 and 12 g/kg

DM, in the same order). Metabolizable energy of CE estimated according to NRC (1996) resulted lower than that of WSS (3.30 and 3.40 Mcal/kg DM, for CE and WSS, respectively).

3.2 VFA profile

The VFA proportions in fermentation fluids are reported in Table 3. The inclusion level did not change acetate and total VFA production. At high inclusion level, propionate was lower, whereas both butyrate and (acetate + butyrate)/propionate ratio were higher, as well as branched-chain VFA (P<0.001 for all variables).

With respect to WSS, CE produced less acetate and propionate (P<0.001 for both), but more butyrate and branched-chain VFA (P<0.001 for both), thus total VFA production did not differ among the diets with different supplement feed. Compared to WSS, CE showed a higher (acetate + butyrate)/propionate ratio (P<0.001).

3.3 Digestibility and gas production values

The digestibility and GP values are reported in Table 4. The inclusion level did not affect any parameter, with the exception of NADR, that resulted higher (P<0.01) at low inclusion level. A nominally lower GP was observed for the two diets at high inclusion level (P=0.069). Compared to WSS, CE provided a higher NADR and dNDF (P<0.01) and nominally higher values of TOMd (P=0.055). No differences were detected for other variables among the two supplements. Similarly, no significant differences were observed for interaction between supplement and inclusion level.

3.4 Nitrogen balance

The data of N balance are reported in Table 5. At high inclusion level, the values of E-NAN, E-TB, and E-NH_3 resulted greater (P<0.001) than those observed at low inclusion level. The daily flow of non ammonia nitrogen (NAN) was higher (P=0.005) in diets at high inclusion level and only nominally higher (P=0.086) in WSS. Conversely, the values of MN flow and EMS did not differ in the two inclusion levels. The MN/NAN ratio was higher in CE compared to WSS, whereas no differences were observed among the two inclusion levels. The efficiency of nitrogen utilization (ENU) was different (P=0.001) only among inclusion levels, with the lower values obtained for the

high level (on average 92.9 and 96.2%, for high and low inclusion level, respectively). The two supplements did not differ for any variable, except for values of E-NAN, that resulted greater for CE compared to WSS (P<0.01). Moreover, the proportion of MN produced from N-NH_3 was nominally (P=0.057) greater for WSS than for CE.

4. Discussion

4.1 Effects of inclusion level

The four diets incubated in this experiment were almost isoenergetic, but were formulated using two different levels of CE or WSS. Therefore, the content of some nutrients (in particular CP and EE) was notably different among diets. When CE and WSS were included at low level (7.0% DM), the starch content of corresponding diets was 35.8% DM, the protein content was 10.9% DM, and the lipid content was 4.6% DM. Conversely, when the amounts of CE and WSS were doubled (14.0% DM), the dietary starch decreased (32.0% DM), whereas the protein (14.7% DM) and the lipid content (5.7% DM) increased. Small differences were also present for aNDF content, that was 33.6 and 32.1% DM when CE was added at low and high level, respectively, and 32.8 and 30.5% DM when WSS was added at low and high level, respectively. As expected, the different chemical composition led to differences for main end-products of fermentation (gas, VFA, and ammonia). In diets with low level of supplemented feeds, propionate production resulted greater, as result of higher starch content. Conversely, in diets with high level of WSS and CE, yield of branched-chain VFA was greater, as result of higher CP content (Hungate, 1966). High protein content could also explain the nominally lower GP values observed at high inclusion level. To this regard, some authors (Cone and Van Gelder, 1999) found that casein produced only 32% of gas evolved from fermentation of carbohydrates. More recently, Tagliapietra et al. (2011), from the incubation of 11 different feeds *in vitro*, noted that feeds with a CP content >16% DM produced lower amounts of gas. The same authors also noted that GP values provided by high-protein feeds were poorly correlated with the corresponding digestibility measures. To this regard, Chenost et al. (2001)

argued that GP measures should be corrected for CP content of feeds for a direct comparison with digestibility values.

This experiment was conducted using an artificial saliva (McDougall, 1948) that does not supply N, so that rumen fluid and diets represented the only N sources for microorganisms. On this basis, it can be supposed that the N supplied by the diet was the only source available for microbial protein synthesis. As stated by Cone et al. (2005), these conditions are optimal to evaluate the dietary contribution in terms of N and to detect differences among CP levels, as was the case here. In the current experiment, the amount of NAN, computed as the sum of microbial N and bypass N, was 246 and 168 mg/d for high and low inclusion level, respectively. This difference is imputable to the different amount of bypass N, as the amount of microbial N resulted comparable at the two inclusion levels (on average 100 mg/d). Thus, for high-protein diets, the resulting amount of bypass N (146 mg/d) corresponded to the gap between the amount of protein supplied by the two levels. From these results, there is evidence that the use of higher protein level led only to an increased bypass of N, without improving the rumen synthesis of microbial N. This pattern is supported by literature, as several studies (Windschitl and Stern, 1988; Calsamiglia et al., 1995; Cunningham et al., 1996) observed that the rumen synthesis of microbial N is primarily influenced by protein degradability, rather than by protein content of the diet. Further, Cecava et al. (1990) noted that the supplementation of low degradable protein led to a change of microbial N flow and efficiency of microbial synthesis, and attributed this result to a different availability of ammonia N, AA, and peptides. Since the synthesis of microbial N was comparable for the two protein levels, values of ENU and ratio between MN and NAN resulted higher in low protein diets. Similar results have been recently reported by Belanche et al. (2012). To this regard, Bach et al. (2008) stated that low-protein diets could have a higher efficiency of microbial synthesis compared to high-protein diets because of a more balanced ratio between energy and N sources. In particular, it can be hypothesized that, in low-protein diets, microbes channeled a greater proportion of CP toward microbial protein synthesis, reducing the amount used as source of energy. The lower efficiency of

microbial synthesis observed for high-protein diets was also confirmed by high level of ammonia in rumen fluids. Further, as ammonia concentration of rumen fluid is inversely related to the values of ENU (Bach et al., 2005), ENU resulted lower in high-protein diets compared to those with low protein level. However, when microbial N was referred to the amount of OM degraded (OMAD), no differences were detected among the protein levels, as evidenced by the comparable EMS values (from 13 to 21 g of microbial N/kg OMAD). These values appeared in line with other studies performed with RUSITEC (Lee et al., 2003; Martinez et al., 2010).

4.2 Effects of supplements

Canola meal derived from the chemical extraction of oil, has been extensively tested as possible alternative to soybean meal in diets for beef cattle (Petit and Veira, 1994; Koenig and Beauchemin, 2005), pig (Seneviratne et al., 2010, 2011a,b; Woyengo et al., 2010) and fish (Mazurkiewicz et al., 2011). On the opposite, little information is available about the validity to canola expeller, the residual of mechanical extraction of oil from canola seeds. The lower employment of canola expeller in animal nutrition could be due to the fact that this feed, compared to the corresponding meal, is plagued by a more variable chemical composition and, hence, nutritional value. To this regard, it has been largely documented that the chemical and physical characteristics of canola expeller are strongly influenced by processing conditions, as the number of pressing and the temperature at which the oil extraction is performed (Weigal, 1991; Glencross et al., 2004a,b). The lipid content of CE used in the current study was greater compared to others (McKinnon and Walker, 2009; Seneviratne et al., 2010; Woyengo et al., 2010; Seneviratne et al., 2011a,b), which reported a lipid content included between 10.2 and 13.3 % EE on DM basis, and this result could be attributed to the fact that in this study canola seeds were pressed only once and thus a lower recovery of oil was achieved. However, a wide range of different lipid contents (from 10.0 to 19.0 % EE on DM basis) is reported for canola expeller (Leming and Lember, 2005; Spragg and Mailer, 2007), as result of different processing conditions. To this regard, Kaldmäe et al. (2010) found that the lipid content of cold-pressed CE varied extremely (15.8±4.3 % EE on DM basis). The CE used

in the current experiment was single-pressed, and no external heat was applied during oil extraction, but the only heat was that generated from the crushing of seeds. The choice of cold-pressing was due to the fact that several studies (Mustafa et al., 1999; Kass et al., 2005; Klein-Hessling, 2007; Spragg and Mailer, 2007) noted that degradability and digestibility of some AA (i.e. lysine) decreased after heat treatment (Van Soest, 1994). Some authors (McKinnon and Walker, 2009) have recently observed that also aNDF degradability was reduced by heat treatment, probably because the process caused the binding of some structural carbohydrates.

In the current study, the two supplements enhanced a comparable microbial protein synthesis, as clearly shown by MN flow and EMS values. However, the differences obtained for NAN ($P<0.086$) and for microbial N derived from ammonia ($P<0.057$) and the higher N degradability of CE, suggest that rumen microorganisms used differently the N supplied by CE and WSS. In particular, results seem to evidence that, especially in the case of CE, microbes produced a greater proportion of microbial N using non ammonia N (i.e. AA and peptides) and, to a lesser extent, ammonia N. To this regard, some authors (Salter et al., 1979; Wallace, 1997) noted that the proportion of microbial N derived from ammonia depends on the availability of N sources. In particular, they estimated that the minimum contribution to microbial N from ammonia was 26 percent when high amounts of peptides and AA were available, with a potential maximum of 100 percent when ammonia was the sole N source. According to this, Carro and Miller (1999) sustained that the most part of rumen microorganisms uses ammonia N as main N source for protein synthesis, but however when sources of non-ammonia N and energy are not limiting, as was likely the case here, microbes can increase the utilization of non-ammonia N and reduce that of NH_3.

5. Conclusions

Results of the present *in vitro* experiment seem to confirm that the manipulation of dietary protein level (from 10.9 to 14.7% CP) leads primarily to an increment of bypass N, without any improvement in the synthesis of microbial N.

The two supplements showed different fermentative properties, both due to intrinsic (proximate composition) and extrinsic factors (processing method). Compared to whole soybean seed, canola expellers showed a greater protein degradability, and promoted a comparable microbial N synthesis. When incubated with canola expeller, microorganisms synthesized a greater proportion of microbial N from AA and peptides, using to a lesser extent ammonia N as substrate for protein synthesis. On this basis, canola expeller derived from cold-pressing of seeds could represent a valuable alternative to whole soybean seed as protein and fat supplement in ruminant diets. However, further research is needed to confirm this hypothesis.

References

AOAC, 2003. Official Methods of Analysis, 17th ed., 2nd rev. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Bach, A., Calsamiglia, S., Stern, M.D., 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88 Suppl 1, E9-21.

Bach, A., Ruiz Moreno, M., Thrune, M., Stern, M.D., 2008. Evaluation of the fermentation dynamics of soluble crude protein from three protein sources in continuous culture fermenters. J. Anim. Sci. 86, 1364-1371.

Baquero, G., Esteban, B., Rius, A., Riba, J.R., Puig, R., 2010. Small-scale production of straight vegetable oil from rapeseed and its use as biofuel in the Spanish territory. Energ Policy. 38, 189-196.

Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J., 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. J. Nutr. 142, 1684-1692.

Brooks, P.D., Stark, J.M., McInteer, B.B., Preston, T., 1989. Diffusion method to prepare soil extracts for automated n-15 analysis. Soil Sci. Soc. of America J. l 53, 1707-1711.

Calsamiglia, S., Stern, M.D., Firkins, J.L., 1995. Effects of protein source on nitrogen metabolism in continuous culture and intestinal digestion *in vitro*. J. Anim. Sci. 73, 1819-1827.

Carro, M.D., Miller, E.L., 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). Br. J. Nutr. 82, 149-157.

Cecava, M.J., Merchen, N.R., Berger, L.L., Fahey, G.C., 1990. Intestinal supply of amino acids in sheep fed alkaline hydrogen peroxide-treated wheat straw-based diets supplemented with soybean meal or combinations of corn gluten meal and blood meal. J. Anim. Sci. 68, 467-477.

Chenost, M., Aufrère, J., Macheboeuf, D., 2001. The gas-test technique as a tool for predicting the energetic value of forage plants. Anim. Res. 50, 349-364.

Cone, J.W., Jongbloed, A.W., Van Gelder, A.H., De Lange L., 2005. Estimation of protein fermentation in the large intestine of pigs using a gas production technique. Anim. Feed Sci. Technol. 123-124, 463-472.

Cone, J.W., Van Gelder, A.H., 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76, 251-264.

Cunningham, K.D., Cecava, M.J., Johnson, T.R., Ludden, P.A., 1996. Influence of source and amount of dietary protein on milk yield by cows in early lactation. J. Dairy Sci. 79, 620-630.

Czerkawski, J.W., Breckenridge, G., 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr. 38, 371-384.

Esteban, B., Baquero, G., Puig, R., Riba, J., Rius, A., 2011. Is it environmentally advantageous to use vegetable oil directly as biofuel instead of converting it to biodiesel?. Biomass Bioenerg 35, 1317-1328.

European Commission. 1991. Implementation of Nitrates Directive. http://ec.europa.eu/environment/water/water-nitrates/index_en.html Accessed Apr. 14, 2010.

Firkins, J.L., Berger, L.L., Merchen, N.R., Fahey, G.C. Jr., Mulvaney, R.L., 1992. Ruminal nitrogen metabolism in steers as affected by feed intake and dietary urea concentration. J. Dairy Sci. 70, 2302-2314.

Glencross, B., Hawkins, W., Curnow, J., 2004a. Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (*Pagrus auratus, Paulin*). Aquacult. Res. 35, 15-24.

Glencross, B., Hawkins, W., Curnow, J., 2004b. Nutritional assessment of Australian canola meals. II. Evaluation of the influence of the canola oil extraction method on the protein value of canola meals fed to the red seabream (*Pagrus auratus, Paulin*). Aquacult. Res. 35, 25-34.

Goering, H.K., Van Soest, P.J., 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

Hristov, A.N., Huhtanen, P., Rode, L.M, Acharya, S.N., McAllister, T.A., 2001. Comparison of the ruminal metabolism of nitrogen from 15N-labeled alfalfa preserved as hay or as silage. J. Dairy Sci. 84, 2738-2750.

Hungate, R.E., 1966. The rumen and its Microbes. Academic Press, New York, NY, USA.

Kaldmäe, H.R., Leming, M., Kass, A., Lember, A., Tölp, S., Kärt, O., 2010. Chemical composition and nutritional value of heat-treated and cold-pressed rapeseed cake. Vet. Med. Zoot. 49, 55-60.

Kass, M., Kaldmäe, H., Kärt, O., Ots, M., Olt. A., 2005. Effect of temperature on the quality of rapeseed cake protein. Proc. 11th Baltic Anim. Breed. Gen. Conf., Palanga, Lithuania.198-201.

Klein-Hessling, H., 2007. Value canola meal before using it. World Poult. 23, 24-28.

Koenig, K.M., Beauchemin, K.A., 2005. Barley versus protein-supplemented corn-based diets for feedlot cattle evaluated using the NRC and CNCPS beef models. Can. J. Anim. Sci. 85, 377-388.

Lee, M., Merry, R.J., Davies, D.R., Moorby, J.M., Humphreys, M.O., Theodorou, M.K., MacRae, J.C., Scollan, N.D., 2003. Effect of increasing availability of water-soluble carbohydrates on *in vitro* rumen fermentation. Anim. Feed Sci. Technol. 104, 59-70.

Leming, R., Lember, A., 2005. Chemical composition of expeller-extracted and cold-pressed canola meal. Agraarteadus 16, 103-109.

Martinez, M.E., Ranilla, M.J., Tejido, M.L., Ramos, S., Carro, M.D., 2010. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. J. Dairy Sci. 93, 3684-3698.

Mazurkiewicz, J., Przybyl, A., Czyzak-Runowska, G., Lyczynski, A., 2011. Cold-pressed rapeseed cake as a component of the diet of common carp (*Cyprinus Carpio L.*): effects on growth, nutrient utilization, body composition and meat quality. Aquacult. Nutr. 17, 387-394.

McDougall, E. I., 1948. Studies on ruminant saliva 1. The composition and output of sheep's saliva. Biochem. J. 43, 99-109.

McKinnon, J.J., Walker, A.M., 2009. Comparison of canola and mustard presscake from biodiesel production as protein sources for growing cattle. Can. J. Anim. Sci. 89, 401-408.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217-1240.

Mustafa, A.F., McKinnon, J.J., Christensen, D.A., 1999. Effect of moist heat treatment on *in vitro* degradability and ruminal escape protein and amino acids of mustard meal. Anim. Feed Sci. Technol. 76, 265-274.

NRC. 1996. Nutrient Requirements of Beef Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Petit, H.V., Veira, D.M., 1994. Effect of post-weaning protein supplementation of beef steers fed grass silage on performance during the finishing phase, and carcass quality. Can. J. Anim. Sci. 74, 699-701.

Salter, D.N., Daneshvar, K., Smith, R.H., 1979. The origin of nitrogen incorporated into compounds in the rumen bacteria of steers given protein- and urea-containing diets. Br. J. Nutr. 41, 197-209.

SAS® User's Guide: Statistics, 5th ed. 2005. SAS Institute, Cary, NC, USA.

Seneviratne, R.W., Beltranena, E., Goonewardene, L.A., Zijlstra, R.T., 2011. Effect of crude glycerol combined with solvent-extracted or expeller-pressed canola meal on growth performance and diet nutrient digestibility of weaned pigs. Anim. Feed Sci. Technol. 170, 105-110.

Seneviratne, R.W., Beltranena, E., Newkirk, R.W., Goonewardene, L.A., Zijlstra, R.T., 2011b. Processing conditions affect nutrient digestibility of cold-pressed canola cake for grower pigs. J. Anim. Sci. 89, 2452-2461.

Seneviratne, R.W., Young, M.G., Beltranena, E., Goonewardene, L.A., Newkirk, R.W., Zijlstra, R.T., 2010. The nutritional value of expeller-pressed canola meal for grower-finisher pigs. J. Anim. Sci. 88, 2073-2083.

Spragg, J., and R. Mailer. 2007. Canola meal value chain quality improvement. Canola meal value chain quality improvement. A final report for AOF and Pork CRC. Project 1B-103-0506.

Tagliapietra, F., Cattani, M., Hansen, H.H., Hindrichsen, I.K., Bailoni, L., Schiavon, S., 2011. Metabolizable energy content of feeds based on 24 or 48 h *in situ* NDF digestibility and on *in vitro* 24 h gas production methods. Anim. Feed Sci. Technol. 170, 182-191.

Thacker, P.A., Petri, D., 2009. The effects of canola or mustard biodiesel press cake on nutrient digestibility and performance of broiler chickens. Asian-Aust. J. Anim. Sci. 22, 1531-1539.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, third ed. Cornell University Press, Ithaca, NY, USA.

Van Soest, P.J, Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Wallace, R.J., 1997. Peptide metabolism and its efficiency in ruminant production. In: Rumen Microbes and Digestive Physiology in Ruminants (Onodera, R. et al., eds.). Japan Sci. Soc. Press, Tokyo S. Karger, Basel, pp. 95-105.

Weatherburn, M.W., 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971-974.

Weigal, J., 1991. Oilseed meals and the effect of processing on their subsequent quality. In: Recent Advances in Animal Nutrition in Australia. Ed. D.J. Farrell, University of New England, Armidale, pp. 317.

Windschitl, P.M., Stern, M.D., 1988. Evaluation of calcium lignosulfonate-treated soybean meal as a source of rumen protected protein for dairy cows. J. Dairy Sci. 71, 3310-3322.

Woyengo, T.A., Kiarie, E., Nyachoti, C.M., 2010. Energy and amino acid utilization in expeller-extracted canola meal fed to growing pigs. J. Anim. Sci. 88, 1433-1441.

Table 1. Chemical composition (g/kg DM), estimated feed ME according to NRC (2001; Mcal/kg DM), aNDF (dNDF; g/kg DM) and true DM digestibility (TDMd; g/kg DM) of whole soybean seed and canola expeller (n = 3 per feed)

Item	Whole soybean seed	Canola expeller
DM	888	895
CP	385	287
Starch	42	129
Ether extract	202	199
NDF	135	291
ADF	62	202
$Lignin_{(sa)}$	12	75
Ash	52	66
ME	3.40	3.30
dNDF	916	681
TDMd	981	829

Table 2. Ingredients (g/kg DM), chemical composition (% on DM basis), and estimated ME according to NRC (2001; Mcal/kg DM) of the four experimental diets.

	Inclusion level			
	High		Low	
Item	WSS[a]	CE[b]	WSS[a]	CE[b]
Ingredient				
Corn silage	330	316	376	369
Corn meal	290	278	331	324
Dry sugar beet pulp	105	100	119	117
Wheat bran	24	23	28	27
Wheat straw	40	39	46	45
Vitamin and minerals premix[c]	27	26	30	30
Soybean meal	44	78	-	18
Canola expeller	-	140	-	70
Whole soybean seed	140	-	70	-
Chemical composition				
DM	89.0	88.5	90.4	90.2
CP	14.7	14.7	10.9	10.9
Starch	32.0	32.0	35.8	35.8
Ether extract	5.69	5.71	4.56	4.61
aNDF	30.5	32.1	32.8	33.6
ADF	14.4	15.3	15.2	15.6
$Lignin_{(sa)}$	1.60	2.65	1.77	2.21
Ash	5.86	5.32	6.21	5.03
ME	2.57	2.49	2.49	2.47

[a] whole soybean seed

[b] canola expeller

[c] premix contained per kilogram of diet: calcium carbonate, grapes hulls, magnesium carbonate, sodium chloride, urea (4.3%), magnesium oxide, mono and di-calcium phosphate, wheat flour, saccharomyces cerevisiae 160 CFU; supplemented with: vitamin A 400,000 UI ; vitamin D3 40,000 UI; vitamin E 1000 mg; vitamin B1 80 mg; vitamin B12 0.40 mg; vitamin PP 4000 mg; choline chloride ruminally-protected 2000 mg; Mn 650 mg; Cu 100 mg; Zn 1500 mg; I 20 mg; Co 12 mg; Se 3 mg.

Table 3. Effects of two protein sources [whole soybean seed (WSS) and canola expeller (CE)] at two inclusion levels in the diet (high = 14% and low = 7% on DM) and their interaction on rumen fermentation pattern measured in the rumen simulation technique (RUSITEC) system.

	Inclusion level				SEM	*P*-value		
	High		Low			Supplement (S)	Inclusion (I)	S × I[c]
Item	WSS[a]	CE[b]	WSS[a]	CE[b]				
pH	6.74	6.74	6.76	6.75	0.021	0.849	0.201	0.849
NH_3-N, mmol/d	4.21	4.30	2.63	2.77	0.203	0.417	<0.001	0.417
VFA, mmol/d	30.0	27.9	28.8	28.0	1.19	0.102	0.562	0.102
Molar proportion, %								
Acetate	42.1	40.2	42.4	39.3	0.30	<0.001	0.150	<0.001
Propionate	29.5	26.7	32.4	32.0	0.34	<0.001	<0.001	<0.001
Butyrate	11.0	13.5	10.4	11.8	0.35	<0.001	<0.001	<0.001
Branched	17.3	19.6	14.9	16.9	0.25	<0.001	<0.001	<0.001
Ratio Acetate/propinate	1.43	1.51	1.31	1.23	0.022	0.706	<0.001	0.706
Ratio (Acetate+butyrate)/propionate	1.81	2.01	1.64	1.60	0.035	<0.001	<0.001	<0.001

[a] whole soybean seed
[b] canola expeller
[c] interaction supplement per inclusion level

Table 4. Effects of two protein sources [whole soybean seed (WSS) and canola expeller (CE)] at two inclusion levels in the diet (high = 14% and low = 7% on DM) and their interaction on digestibility values (g/kg DM; measured at 48 h of incubation), and gas production (l/d; measured at 24 h of incubation) in the rumen simulation technique (RUSITEC) system.

	Inclusion level				SEM	*P* value		
	High		Low			Supplement	Inclusion	S × I[c]
Item	WSS[a]	CE[b]	WSS[a]	CE[b]		(S)	level (I)	
DMD[d]	589	590	580	576	1.4	0.882	0.232	0.786
OMAD[e]	508	510	497	492	1.5	0.903	0.228	0.764
TOMd[f]	799	812	800	807	0.7	0.055	0.535	0.615
dNDF[g]	490	533	509	540	2.1	0.008	0.354	0.643
NADR[h]	402	466	436	485	1.4	<0.001	<0.001	0.068
GP[i]	1.28	1.33	1.41	1.39	0.072	0.571	0.069	0.714

[a] whole soybean seed
[b] canola expeller
[c] interaction supplement per inclusion level
[d] DM degradability at 48 h of incubation
[e] OM apparent digestibility at 48 h of incubation
[f] true OM digestibility at 48 h of incubation
[g] aNDF digestibility at 48 h of incubation
[h] nitrogen apparent digestibility in the rumen at 48 h of incubation
[i] gas production at 24 h of incubation

Table 5. Effects two protein sources [whole soybean seed (WSS) and canola expeller (CE)] at two inclusion levels in the diet (high = 14% and low = 7% on DM) and their interaction on ^{15}N enrichment of different N pools, microbial N flow and efficiency of microbial synthesis in the rumen simulation technique (RUSITEC) system.

	Inclusion level				SEM	*P* value		
	High		Low			Supplement	Inclusion	S × I[c]
Item	WSS[a]	CE[b]	WSS[a]	CE[b]				
[d]E-NAN_{TD}	0.21	0.24	0.10	0.15	0.015	0.005	<0.001	0.520
[e]E-TB	0.64	0.52	0.34	0.40	0.061	0.449	<0.001	0.050
[f]E-NH_3	1.80	1.85	1.29	1.50	0.099	0.088	<0.001	0.261
[g]NAN flow, mg/d	270	222	185	151	22.1	0.005	0.086	0.086
[h]MN flow, mg/d	97.5	111.2	98.9	100.8	27.19	0.693	0.820	0.765
[i]MN/NAN ratio, %	38.9	46.0	52.6	73.6	0.02	0.146	0.046	0.450
[l]ENU, %	91.9	93.9	95.6	96.9	1.34	0.383	0.001	0.579
[m]MN derived from N-NH_3, %	33.1	25.4	40.6	31.8	5.51	0.057	0.102	0.881
[n]EMS, g/kg	15.7	13.5	13.4	13.6	3.80	0.681	0.717	0.745

[a] whole soybean seed
[b] canola expeller
[c] interaction
[d] non ammonia nitrogen of total digesta ^{15}N enrichment (atoms % excess)
[e] total bacteria ^{15}N enrichment (atoms % excess)
[f] ammonia nitrogen ^{15}N enrichment (atoms % excess)
[g] non ammonia N outflow from the vessel
[h] microbial N outflow from the vessel
[i] computed as microbial N/non ammonia N
[l] efficiency of N utilization = g MN/g rumen-available N × 100
[m] MN derived from N-NH_3 = proportion of MN synthesized using NH_3 as a source of N (E-TB/ E-NH_3)
[n] efficiency of microbial synthesis (MN/OMAD)

Chapter 6.

(Fourth contribute)

Comparison among soybean seeds and rapeseed cake included in diets with two different CP levels using gas production technique

[1]Matteo Guadagnin, [2]Mirko Cattani, [2]Franco Tagliapietra [1]Lucia Bailoni,

[1]Department of Comparative Biomedicine and Food Nutrition, University of Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

[2]Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

Corresponding author: Dr. Matteo Guadagnin, Department of Comparative Biomedicine and Food Nutrition, University of Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy – Tel. +39.049.8272777

Abstract

This experiment aimed to evaluate effects of four diets containing two protein and fat sources (supplement) (whole soybean seed or canola expeller) included in two levels (high or low) on *vitro* gas production (GP) and some end-products of fermentation (volatile fatty acids and N-NH3). Diets were supplemented with whole soybean seeds (WSS) or canola expeller (CE) at two inclusion levels: high (14.0 % DM) or low (7.0 % DM). Different amounts of soybean meal were also added to the diets to achieve two increasing protein contents: low (10.9% CP) and high (14.7% CP). A preliminary incubation (72 h) was performed to determine t½, the time at which the diets produced half of asymptotic GP. From this incubation the values of t½ provided by the four diets resulted, on

average, 16 h. After that, a second incubation was carried out, using the same experimental design described above, and stopped in correspondence of t½ (16 h). Mean value of t½ was considered, instead of single t½ value provided by each diet. Rumen fluid was collected using an oral-esophageal probe from 3 dry Holstein-Friesian cows. After filling, all bottles were placed into a ventilated incubator set at 39.0 ± 0.1 °C. The chemical composition of the two supplements is given in Table 1. Compared to WSS, CE showed a lower CP (287 and 385 g/kg DM, for CE and WSS, respectively) and a comparable EE content (199 and 202 g/kg DM, in the same order), and a greater fraction of aNDF (291 and 135 g/kg DM, in the same order) with a higher degree of lignification (75 and 12 g $lignin_{(sa)}$/kg DM, in the same order). The ME estimated according to NRC (1996) resulted lower for CE compared to WSS (3.30 and 3.40 Mcal/kg DM, for CE and WSS, respectively).

The inclusion level did not show to influence GP values, except at 24 h of incubation, (P<0.05). High-inclusion diets provided greater GP rate, expressed as hourly GP per gram of DM, within the first 20 h of incubation; however, after this time and until about 40 h of incubation the pattern was opposite, with low-inclusion diets providing greater GP rate. The supplement affect GP values, except at 12 h of incubation, where CE provided greater amounts of gas compared to WSS (P<0.01). Both values of dNDF and TDMd were affected by inclusion level, supplement and their interaction. Values of GP measured at t½ were not influenced by inclusion level, supplement and their interaction. The two supplement did not differed in total gas production but the different gas production rate suggest that inclusion of CE in diet for ruminant can improve N utilization by rumen bacteria. The high lipid content of canola expellers could be a limit to its use in diets, anyway at level used in this experiment did not showed detrimental aspect for fermentations.

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; CE, diets supplemented with canola expellers; CP, crude protein; DM, dry matter; dNDF, digestible aNDF;

$N\text{-}NH_3$, ammonia N; TDMd, true DM digestibility; t½, time at which half of GP is obtained; VFA, volatile fatty acids; WSS, diets supplemented with whole soybean seeds;

Key words: Canola expeller, Gas production, Rumen degradability.

Introduction

In the last years constraints introduced by Nitrate Directive (European Commission, 1991) and increasing market price of soybean are forcing farmers to consider the use of low protein diets and alternative protein supplements (Xiccato et al., 2005; Yan et al., 2007). Canola represents the trademark of a particular variety of rapeseed (*Brassica Napus*) characterized by a content of erucic acid and glucosinolates lower than 5% by weight and 30 mmol/kg DM, respectively. In the last years an increasing number of farms has been equipping with small-sized facilities for mechanical extraction of oil from canola seeds. After extraction, the oil is converted into biodiesel and utilized as fuel for tractors, whereas the corresponding by-products (expellers) are included in ruminant diets as protein and fat sources. This productive cycle offers both economical and environmental advantages, at it allows to reduce the need for carburant fossil in agricultural sector and to recycle by-products of oil industry as animal feeds (Baquero et al., 2010; Esteban et al., 2011). However, to date, information about the nutritional and energy value of canola expellers are still limited, as well as their possible effect on rumen fermentation. Literature indicates that oil content of canola expellers is extremely variable, with a fat percentage ranging from 8 to almost 30% DM, as result of different processing conditions (Leming and Lember, 2005; Spragg and Mailer, 2007; Thacker and Petri, 2009). High fat percentage of expellers could impair rumen fermentation, by reducing activity and growth of rumen microorganisms (Jenkins, 1993).

This experiment aimed to evaluate effects of four diets containing two protein and fat sources (whole soybean seed or canola expeller) supplemented at two inclusion levels (high or low) on *vitro* gas production (GP) and some end-products of fermentation (volatile fatty acids and N-NH3)

measured after 72 h of incubation or at t½, that is the time at which microbial activity is supposed to be maximal.

Material and methods

2.1 Diets

Four iso-lipid and isonitrogenous diets, composed by feed ingredients commonly included in diets for beef cattle in farms of Northern Italy, were incubated (Table 1). Diets were supplemented with whole soybean seeds (WSS) or canola expeller (CE) at two inclusion levels: high (14.0 % DM) or low (7.0 % DM). Different amounts of soybean meal were also added to the diets to achieve two increasing protein contents: low (10.9% CP) and high (14.7% CP). Whole soybean seeds were collected from the experimental farm of the University of Padova (Legnaro, Padova, Italy). Canola expeller was derived by a canola variety (Excalibur; Dekalb, Monsanto Agricoltura Spa, Lodi, Italy) that was grown and harvested in a pilot farm located in Valle Vecchia (Caorle, Venezia, Italy). Canola seeds were single-crushed at moderate temperatures (60°C) using two single-screw presses equipped with a rotating screw shaft within an horizontal barrel (Mailca Srl, Modena, Italy). The input flow rate of the presses was 120 kg/h of seeds, with a hourly production of 40 kg of oil and 80 kg of expellers. Prior to incubation, all diets were ground by a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, Germany) with a screen size of 1 mm.

2.2 Experimental design and incubation procedures

A preliminary incubation (72 h) was performed to determine t½, the time at which the diets produced half of asymptotic GP (Blümmel et al., 1997). The four diets were incubated, in four independent replicates, into individual glass bottles with rumen fluid and buffer. Four bottles, without sample diet (containing only rumen fluid and buffer), were included as blanks, for a total of 20 bottles incubated. From this incubation the values of t½ provided by the four diets resulted, on average, 16 h. After that, a second incubation was carried out, using the same experimental design described above, and stopped in correspondence of t½ (16 h). In the current trial a mean value of t½ was considered, instead of single t½ value provided by each diet. This analytical procedure was

followed as the present experiment aimed to evaluate digestibility and fermentation products (gas, VFA, and N-NH3) at times included in the interval period characterized by greater GP (between 12 and 24 h of incubation, in the present case; see Table 3), when microbial activity is supposed to be maximal and, presumably, microbial lysis has not yet occurred. Further, the adoption of a sole t½ value allowed to simplify analytical procedures.

Rumen fluid was collected using an oral-esophageal probe 2 h before morning feeding from 3 dry Holstein-Friesian cows. The cows were housed at the experimental farm of the University of Padova and daily fed hay *ad libitum* and 2 kg of concentrate (500 g/kg corn meal, 250 g/kg barley meal, and 250 g/kg soybean meal). Rumen fluid was stored into thermal flasks preheated to 39 ± 0.5°C and immediately transferred to the laboratory, strained through 3 layers of cheesecloth to eliminate feed particles and mixed with the buffer solution in a 1 to 2 ratio under anaerobic conditions, by flushing with carbon dioxide (Menke and Steingass, 1988). The buffer solution was prepared according to Menke and Steingass (1988), heated in a water bath at 39°C and purged continuously with carbon dioxide for 30 min. Sodium sulphide (0.33 g/l solution) was added to the buffer solution as reducing agent (Menke and Steingass, 1988). These operations required less than 30 minutes. Each bottle (capacity: 280 ml) was filled with 0.500 ± 0.0010 g of sample diet and 75 ml of buffered rumen fluid (205 ml of resulting headspace volume), keeping headspace of bottles continuously flushed with carbon dioxide, to maintain anaerobic conditions. After filling, all bottles were placed into a ventilated incubator set at 39.0 ± 0.1 °C. The bottles were not shaken during the incubation.

2.3 Gas production

The wireless ANKOM^{RF} Gas Production System (Ankom Technology, Macedon, NY, USA) was used. This system is composed by 20 bottles (capacity: 280 ml) equipped with an ANKOM pressure sensor module (pressure range: –69 to +3447 kPa; resolution: 0.27 kPa; accuracy ±0.1% of measured values) including a microchip and a radio sender.

During the incubation the pressure changes in the headspace of bottles, measured as difference with respect to atmospheric pressure at the beginning of incubation (P0), were transmitted via a radio frequency to a computer at a fixed interval time (1 min) and recorded in a database. The gas accumulated in the headspace of each bottle was automatically released by an open-closed valve when a pressure variation of +3.4 kPa was reached (Tagliapietra et al., 2010). The cumulative gas pressure (ΔP) was calculated from pressure changes at various times and converted to units of volume (GP, ml) using the ideal gas law:

$$GP = (\Delta P/P0) \times Vo \quad (1)$$

where: ΔP is the cumulative pressure change (kPa) in the headspace of bottle; Vo is the headspace volume of bottle (205 ml), and P0 is atmospheric pressure read by the system at the beginning of incubation. The final GP measured for blanks (bottles containing only buffered rumen fluid) was subtracted from the final GP measured for other bottles (containing feed sample and buffered rumen fluid) to take into account for the baseline fermentation from buffered rumen fluid and expressed per g of DM incubated. The cumulative volumes were best fitted using the model proposed by Groot et al. (1996):

$$GP(t) = AGP/(1 + (t_{½}^{c}/t^{c}) \quad (2)$$

where: AGP is the asymptotic GP (ml/g of feed DM incubated), t½ is the time (h) at which half of the AGP is produced, and c is a constant representing the sharpness of the curve.

2.4 Chemical analysis

Samples of whole soybean seed, canola expeller, and diets were analysed in triplicate for dry matter (DM; # 934.01; AOAC, 2003), crude protein (CP; # 976.05; AOAC, 2003), ether extract (EE; # 920.29; AOAC, 2003), ash (# 942.05; AOAC, 2003), starch (# 948.02; AOAC, 2003). Neutral detergent fibre (aNDF), inclusive of residual ash, was determined with α-amylase and sodium sulphite, as suggested by Mertens (2002), using the Ankom^{220} Fibre Analyser (Ankom Technology, Macedon, NY, USA).

2.4 Degradability

At the end of the second incubation (16 h), residual fluids of fermentation were recovered from each bottle and transferred to weighed filter crucibles (30 ml, porosity 40-100 mm, Robu Glasfilter-Geräte GMBH, Hattert, Germany) and treated with a-amylase and sodium sulfite for aNDF analysis using a Fibertech Analyser (VELP Scientifica, Milan, Italy), following the procedure of Mertens (2002). After treatment, crucibles were dried at 60°C and weighed to determine the residual amount of NDF (aNDFres). Values of aNDF (dNDF) and true DM digestibility (TDMd) were computed according to Goering and Van Soest (1970).

2.5 Volatile fatty acids and ammonia N

At the end of the second incubation (t½), two aliquots (5 ml) of fermentation fluid were collected from each bottle. The aliquots were treated with two ml of metaphosphoric acid (25% w/v) and stored at -20°C until the analysis for ammonia N ($N\text{-}NH_3$) and VFA profile. The $N\text{-}NH_3$ content was determined using a potentiometer (Bench pH/ion meter, Oakton Instruments, Vernon Hills, USA) equipped with a specific electrode (pH meter BASIC 20, Crison Instruments, Alella, Spain), as indicated by Bailey (1980). Volatile fatty acid profile of buffered rumen fluid was determined using HPLC (Thermo-Finnigan, CA, USA) made up of a Spectra System P4000 solvent delivery system, a Spectra System AS3000 auto-sampler, a differential refractometer detector (Waters 410, Cary, NC, USA) and a 300 mm × 7.8 mm H_2SO_4 0.0025 N Bio-Rad stainless-steel column (Bio-Rad Laboratories, Richmond, CA, USA).

2.6 Statistical analysis

Data were analyzed using the general linear models procedure (PROC GLM) of SAS (2005). The model used was:

$$y_{ij} = \mu + D_i + \varepsilon_{ij}$$

where: y_{ij} = single observation, μ = overall mean, D_i = effect of diet (i = 4 levels) and ε_{ijk} = residual error term ~$N(0, \sigma^2_e)$. Orthogonal contrasts were carried out for inclusion level (high and low) and for supplement (whole soybean seed and canola expeller). Treatment effects were accepted as significant with a *P* value of 0.05 or less.

3. Results

The chemical composition of the two supplements is given in Table 1. Compared to WSS, CE showed a lower CP (287 and 385 g/kg DM, for CE and WSS, respectively) and a comparable EE content (199 and 202 g/kg DM, in the same order), and a greater fraction of aNDF (291 and 135 g/kg DM, in the same order) with a higher degree of lignification (75 and 12 g $lignin_{(sa)}$/kg DM, in the same order). The ME estimated according to NRC (1996) resulted lower for CE compared to WSS (3.30 and 3.40 Mcal/kg DM, for CE and WSS, respectively).

The values of GP at various incubation times and kinetic parameters obtained in the preliminary incubation (at 72 h) are shown in Table 3. The inclusion level did not show to influence GP values, except at 24 h of incubation, where high-inclusion diets provided greater GP compared to those at low-inclusion level ($P<0.05$). Further, two high-inclusion diets showed a significantly ($P<0.01$) lower t½. This pattern is also graphically represented in Figure 1. High-inclusion diets provided greater GP rate, expressed as hourly GP per gram of DM, within the first 20 h of incubation; however, after this time and until about 40 h of incubation the pattern was opposite, with low-inclusion diets providing greater GP rate. The supplement did not significantly affect GP values, except at 12 h of incubation, where CE provided greater amounts of gas compared to WSS ($P<0.01$). This pattern is graphically represented in Figure 2. When supplemented to low-inclusion diets, CE showed a greater GP rate within the first 10 h of incubation, compared to WSS. Accordingly, CE provided lower values of t½ with respect to WSS, irrespective from the inclusion level ($P<0.05$). Conversely, the interaction inclusion level × supplement was never significant.

The values of aNDF (dNDF) and true DM digestibility (TDMd), GP, and end-products of fermentation (VFA and N-NH3) achieved from the second incubation (at t½) are reported in Table 4. Both values of dNDF and TDMd were affected by inclusion level, supplement and their interaction. Values resulted, on average, greater for high-inclusion level than for low-inclusion level, and for CE than for WSS ($P<0.01$). Values of GP measured at t½ were not influenced by

inclusion level, supplement and their interaction. Similarly, also the ratio between the values of TDMd and GP did not differ significantly. The inclusion level did not exert effects on total VFA production, but changed VFA proportions. At high-inclusion level, the production of acetate resulted proportionally smaller compared to low-inclusion diets, whereas the opposite trend was observed for propionate (P<0.01). As consequence, the ratio between acetate and propionate resulted lower for two high-inclusion diets (P<0.01). Conversely, the supplement did not change neither VFA production or proportion. The interaction inclusion level × supplement resulted statistically significant only for acetate production (P<0.05). N-NH3 N (N-NH_3) concentrations measured at t½ were influenced by inclusion level, supplement and their interaction. Values resulted greater for high-inclusion level, in comparison with low, and for CE, in comparison with WSS (P<0.01).

4. Discussion

4.1 Effects of inclusion level

In the preliminary incubation (at 72 h) values of GP were influenced by inclusion level only at 24 h, resulting greater for high-inclusion diets. Accordingly, inclusion level did not affect GP when fermentations were stopped in correspondence of t½ (16 h). Cone and Van Gelder (1999), observed that the gas production is mainly influenced by protein content in the first part of incubation. Higher values of GP at 24 are confirmed by lower values of $t_{1/2}$ obtained, these results suggest that diets at high inclusion level are more quickly degradable than the diets at low level of inclusion as show as well from the gas production rate. In the incubation at 72 h, high-inclusion diets tended to produce greater amounts of gas, compared to low-inclusion ones, also at 12 h of incubation (P=0.09). From these differences it can be concluded that high-inclusion diets were more rapidly fermented within the first phases of incubation, even if the reason is not easily detectable. When fermentations were stopped at t½ (16 h), high-inclusion diets showed smaller values of dNDF compared to low-inclusion ones. This result could be likely attributed to greater lipid content of high-inclusion diets, that could have reduced activity and growth of rumen microorganisms, in particular of those

cellulosolytic, even if some rumen bacteria are able to protect themselves by toxic action of dietary lipids through the process of bio-hydrogenation (Maia et al., 2007). This hypothesis is also supported by the fact that, in the case of high-inclusion diets, a lower proportion of acetate, that is primarily produced by cellulosolytic bacteria, was yielded in favour of propionate production. To this regard, it is largely recognized that molar proportions of different VFA are strictly dependent on the type of fermented substrate (Beuvink and Spoelstra, 1992; Blümmel and Ørskov, 1993). However, the lipid content of high-inclusion diets (5.7% DM) was under the threshold (8-10% DM) that is supposed to gravely impair microbial activity and growth (Jenkins, 1993). On this basis, it should be supposed that microbial activity and growth were not particularly impaired by dietary lipids, also in the case of high-inclusion diets. This hypothesis is supported by values of TDMd, that resulted greater for high-inclusion diets, and by total VFA production, that was comparable for two inclusion levels. However, despite this latter similarity, yield of branched-chain VFA resulted greater in high-inclusion diets, as probable result of their greater CP content (Hungate, 1966).

Cone et al., (1997) argued that the most part of the protein is fermented during the first hours of incubation, together with the other soluble components. Furthermore Belanche et al. (2012) found that low protein level in the diets decreased OM mater digestibility. As expected values of N-NH3 were higher in high level diets due to the higher level of protein. The most part of rumen microbes uses as main sources of N, rumen N-NH3 but if other sources of non-N-NH3 are available, in condition of not limiting energy, as was likely the diets used in this experiment, bacterial can increase use of non-ammonia N (i.e. AA and peptides) and reduce the use of N-NH3 (Carro and Miller, 1999).

4.2 Effects of supplements

Two supplements mainly differed for starch, fibrous fraction (aNDF, ADF and $lignin_{(sa)}$), and CP content, whereas the EE content was comparable. Values of chemical composition resulted in line with tabled data (NRC, 1996). The EE content of canola expeller (199 g/kg DM) was in line with findings of Leming and Lember (2005), and intermediate between 13% DM reported by Spragg and

Mailer (2007) and 27% DM observed by Thacker and Petri (2009). Literature indicates that oil content of canola expellers is extremely variable, with a fat percentage ranging from 8 to almost 30% DM, as the mechanical extraction is a poorly standardized and efficient method (Leming and Lember, 2005; Thacker and Petri, 2009;). Anyway the lipid content of CE used in the current study compared to others had be considered greater, than those reported by the most part of studies where lipid content was included between 10.2 and 13.3 % EE on DM basis, (McKinnon and Walker, 2009; Seneviratne et al., 2010; Woyengo et al., 2010; Seneviratne et al., 2011a,b) probably because of canola seeds in our experiment were pressed only once leaving a higher oil residual. There is large evidence that the physical and chemical characteristics of canola expellers seem to be influenced by temperature at which the oil extraction is performed and by number of pressings (Weigal, 1991; Glencross *et al.*, 2004). In the current experiment canola seeds were pressed at moderate temperatures (cold-pressing at 60°C), and the only heat was that generated from the crushing of seeds. The choice of cold-pressing was due to the fact that heat treatment (i.e. at 100°C) is recognized to reduce notably protein and aNDF degradability (Mustafa et al., 1999; Kass et al., 2005; Klein-Hessling, 2007; Spragg and Mailer, 2007; McKinnon and Walker, 2009). Differently from CE, WSS used in the present experiment was subjected to toasting (at 100°C), as commonly practiced to deactivate anti-nutritional factors of soybean (i.e. trypsin inhibitors), and heat treatment could have presumably reduced rumen degradability of WSS protein fraction. On this basis, greater GP provided by diets supplemented with CE within the first hours of incubation at 72 h could be attributed to higher protein degradability of CE, as this supplement was not heat-treated. To this regard, Karlsson et al. (2009), using an innovative *in vitro* GP system, observed that rumen protein degradability of cold-pressed CE and soybean meal was comparable. The lower values of dNDF and TDMd provided by diets containing CE at t½ of incubation could be related to greater ADL content of this supplement, that could have reduced its rumen degradability.

5. Conclusions

In the current experiment inclusion level exerted only small effects on values of GP, both when fermentations were stopped at 72 h and at t½ (16 h). However, high-inclusion diets provided greater amounts of gas within the first phases of incubation (24 h), even if these effects were not detected at t½, the time at which microbial activity is supposed to be maximal and microbial lysis has not presumably yet occurred. When incubations were stopped at 72 h, diets supplemented with canola expeller showed greater values of GP at early phases of incubation (12 h) compared to those containing whole soybean seed. However, also in this case no differences were observed among two supplements in correspondence of t½.

Reduction of CP in diets for ruminant is a valid strategy to reduce N excretion, but reduction on N availability could limit rumen activity and degradability of feed. Using low nitrogen diets, is important supply the rumen with sources of N quickly degradable. Results of this experiment suggest the differences between inclusion levels did not depend only by the protein but also by the rate of fermentation of the other degradable components in the mixture of feedstuff.

The two supplement did not differed in total gas production but the different gas production rate suggest that inclusion of CE in diet for ruminant can improve N utilization by rumen bacteria for that is can be an alternative to WSS in diet at low CP content for ruminant. The high lipid content of canola expellers could be a limit to its use in diets, anyway at level used in this experiment did not showed detrimental aspect for fermentations.

6. References

AOAC, 2003. Official Methods of Analysis of the Association of Official Agricultural Chemists, 17th ed. (2th revision). AOAC International, Gaithersburg, MD, USA.

Baquero, G., Bernat, E., Antoni, R., Jordi-Roger, R., Rita, P., 2010. Small-scale production of straight vegetable oil from rapeseed and its use as biofuel in the Spanish territory. Energy Policy. 38, 189-196.

Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J., 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. J. Nutr. 142, 1684-1692.

Beuvink, J.M., Spoelstra, S.F., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Appl. Microbiol. Technol. 37, 505-509.

Blummel, M., Aiple, K.P, Steingabeta, H., Becker, K., 1999. A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality. J. Anim. Physiol. Anim. Nutr. 81, 157-167.

Blümmel, M., Ørskov, E.R., 1993. Comparison of gas production and nylon bag degradability of roughages in predicting feed intake in cattle. Anim. Feed Sci. Technol. 40, 109-119.

Carro, M.D., Miller, E.L., 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). Br. J. Nutr. 82, 149-157.

Cone, John W.,Antonie H.van Gelder Frank Driehuis, 1997. Description of gas production profiles with a three-phasic model. Anim. Feed Sci. Technol. 36, 493-445

Cone, J.W., Van Gelder, A.H., 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76, 251-264.

Glencross, B., Hawkins, W., Curnow, J., 2004a. Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (*Pagrus auratus, Paulin*). Aquacult. Res. 35, 15-24.

Glencross, B., Hawkins, W., Curnow, J., 2004b. Nutritional assessment of Australian canola meals. II. Evaluation of the influence of the canola oil extraction method on the protein value of canola meals fed to the red seabream (*Pagrus auratus, Paulin*). Aquacult. Res. 35, 25-34.

Hungate, R.E., 1966. The rumen and its Microbes. Academic Press, New York, NY, USA.

Jenkins, T.C., 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76, 3851-3863.

Kaldmäe, H.R., Leming, M., Kass, A., Lember, A., Tölp, S., Kärt, O., 2010. Chemical composition and nutritional value of heat-treated and cold-pressed rapeseed cake. Vet. Med. Zoot. 49, 55-60.

Karlsson, L., Hetta, M., Udén, P., Martinsson, K., 2009. New methodology for estimating rumen protein degradation using the in vitro gas production technique. Anim. Feed Sci. Technol. 153, 193-202

Kass, M., Kaldmäe, H., Kärt, O., Ots, M., Olt. A., 2005. Effect of temperature on the quality of rapeseed cake protein. Proc. 11th Baltic Anim. Breed. Gen. Conf., Palanga, Lithuania.198-201.

Klein-Hessling, H., 2007. Value canola meal before using it. World Poult. 23, 24-28.

Koenig, K.M., Beauchemin, K.A., 2005. Barley versus protein-supplemented corn-based diets for feedlot cattle evaluated using the NRC and CNCPS beef models. Can. J. Anim. Sci. 85, 377-388.

Leming, R., Lember, A., 2005. Chemical composition of expeller-extracted and cold-pressed canola meal. Agraarteadus 16, 103-109.

Maia, M.R.G., Chaudhary, L.C., Figueres, L., Wallace, R.J., 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Antonie Van Leeuwenhoek. 91, 303-314.

McKinnon, J.J., Walker, A.M., 2009. Comparison of canola and mustard presscake from biodiesel production as protein sources for growing cattle. Can. J. Anim. Sci. 89, 401-408.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of the digestibility and metabolizable energy contents of ruminant feedstuffs from the gas production when they are incubated with rumen liquor. J. Agric. Sci. 92, 499-503.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim. Res. Dev. 28, 7-55.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217-1240

Mustafa, A.F., McKinnon, J.J., Christensen, D.A., 1999. Effect of moist heat treatment on *in vitro* degradability and ruminal escape protein and amino acids of mustard meal. Anim. Feed Sci. Technol. 76, 265-274.

NRC. 1996. Nutrient Requirements of Beef Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Raun, N.S., Burroughs, W., 1962. Suction strainer technique in obtaining rumen fluid samples from intact lambs. J. Anim. Sci. 21, 454-457.

SAS® User's Guide, 2005. 5th ed. SAS Instit., Inc., Cary, NC, USA.

Seneviratne, R.W., Beltranena, E., Goonewardene, L.A., Zijlstra, R.T., 2011. Effect of crude glycerol combined with solvent-extracted or expeller-pressed canola meal on growth performance and diet nutrient digestibility of weaned pigs. Anim. Feed Sci. Technol. 170, 105-110.

Seneviratne, R.W., Beltranena, E., Newkirk, R.W., Goonewardene, L.A., Zijlstra, R.T., 2011b. Processing conditions affect nutrient digestibility of cold-pressed canola cake for grower pigs. J. Anim. Sci. 89, 2452-2461.

Seneviratne, R.W., Young, M.G., Beltranena, E., Goonewardene, L.A., Newkirk, R.W., Zijlstra, R.T., 2010. The nutritional value of expeller-pressed canola meal for grower-finisher pigs. J. Anim. Sci. 88, 2073-2083.

Spragg, J., and R. Mailer. 2007. Canola meal value chain quality improvement. Canola meal value chain quality improvement. A final report for AOF and Pork CRC. Project 1B-103-0506.

Thacker, P.A., Petri, D., 2009. The effects of canola or mustard biodiesel press cake on nutrient digestibility and performance of broiler chickens. Asian-Aust. J. Anim. Sci. 22, 1531-1539.

Tagliapietra, F., Cattani, M., Bailoni, L., Schiavon, S., 2010. In vitro rumen fermentation: Effect of headspace pressure on the gas production kinetics of corn meal and meadow hay. Anim. Feed Sci. Technol. 158, 197-201.

Van Nevel, C.J., Demeyer, D.I., 1988. Manipulation of rumen fermentation. In: The Rumen Microbial Ecosystem. : Ed. P.N. Hobson, Elsevier Applied Science, London, pp. 387-443.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, third ed. Cornell University Press, Ithaca, NY, USA.

Weigal, J., 1991. Oilseed meals and the effect of processing on their subsequent quality. In: Recent Advances in Animal Nutrition in Australia. Ed. D.J. Farrell, University of New England, Armidale, pp. 317.

Woyengo, T.A., Kiarie, E., Nyachoti, C.M., 2010. Energy and amino acid utilization in expeller-extracted canola meal fed to growing pigs. J. Anim. Sci. 88, 1433-1441.

Xiccato, G., Schiavon, S., Gallo, L., Bailoni, L., Bittante, G., 2005. Nitrogen excretion in dairy cow, beef and veal cattle, pig, and rabbit farms in Northern Italy. Ital. J. Anim. Sci. 4, 103-111.

Yan, T., Frost, J. P., Keaty, T. W. J., Agnew, R. E., Mayne, C. S., 2007. Prediction of nitrogen excretion in feces and urine of beef cattle offered diets containing grass silage. J. Anim. Sci. 85, 1982-1989.

Table 4. Dry matter (DM, g/kg), chemical composition (g/kg DM), aNDF (dNDF; g/kg DM) and true DM digestibility (TDMd; g/kg DM), and ME content (Mcal/kg DM) of whole soybean seed and canola expeller (n = 3 per feed).

	Whole soybean seed	Canola expeller
DM, g/kg DM	888	895
CP	385	287
Starch	42	129
Ether extract	202	199
aNDF	135	291
ADF	62	202
ADL	12	75
Ash	52	66
dNDF[a]	916	681
TDMd[b]	981	829
ME[c], Mcal/kg DM	3.40	3.30

[a]computed as aNDF - $aNDF_{res}$ / aNDF

[b]computed as (DM_{feed} – $aNDF_{res}$) / DM_{feed} (Goering and Van Soest, 1970)

[c]estimated according to NRC (2001)

Table 5. Ingredients (g/kg DM), chemical composition (% on DM basis), and ME content (Mcal/kg DM) of diets supplemented with high or low levels of whole soybean seed (WSS) and canola expeller (CE).

	Inclusion level			
	High		Low	
	WSS	CE	WSS	CE
Ingredients, g/kg DM				
Corn silage	330	316	376	369
Corn meal	290	278	331	324
Dry sugar beet pulp	105	100	119	117
Wheat bran	24	23	28	27
Wheat straw	40	39	46	45
Vitamin and minerals premix[a]	27	26	30	30
Soybean meal	44	78	-	18
Canola expeller	-	140	-	70
Whole soybean seed	140	-	70	-
Chemical composition, % on DM basis				
DM	89.0	88.5	90.4	90.2
CP	14.7	14.7	10.9	10.9
Starch	32.0	32.0	35.8	35.8
Ether extract	5.69	5.71	4.56	4.61
aNDF	30.5	32.1	32.8	33.6
ADF	14.4	15.3	15.2	15.6
ADL	1.60	2.65	1.77	2.21
Ash	5.86	5.32	6.21	5.03
ME[b], Mcal/kg DM	2.57	2.49	2.49	2.47

[a]Premix contained per kg of diet: calcium carbonate, grapes hulls, magnesium carbonate, sodium chloride, urea (4.3%), magnesium oxide, mono and di-calcium phosphate, wheat flour, saccharomyces cerevisiae 160 CFU; supplemented with: vitamin A 400,000 UI ; vitamin D3 40,000 UI; vitamin E 1000 mg; vitamin B1 80 mg; vitamin B12 0.40 mg; vitamin PP 4000 mg; choline chloride ruminally-protected 2000 mg; Mn 650 mg; Cu 100 mg; Zn 1500 mg; I 20 mg; Co 12 mg; Se 3 mg.
[b]estimated according to NRC (2001)

Table 6. Incubation at 72 h. Gas production (GP, ml/g DM) at various times of incubation and kinetic parameters of GP of diets supplemented with high or low inclusion level of whole soybean seed (WSS) and canola expeller (CE).

	Inclusion level				SEM	*P* value		
	High		Low					
	WSS	CE	WSS	CE		Inclusion level (I)	Supplement (S)	I × S
Gas production, ml/g DM								
at 6 h	49	54	45	51	2.6	0.34	0.11	0.66
at 12 h	105	118	97	110	3.9	0.09	<0.05	0.10
at 24 h	202	211	193	192	5.0	<0.05	0.50	0.46
at 48 h	272	269	273	265	5.8	0.86	0.48	0.77
at 72 h	292	288	292	287	6.2	0.99	0.41	0.90
Kinetic parameters of GP								
A[a], ml/g DM	313	299	321	320	7.3	0.15	0.47	0.50
T½[b], h	16.6	14.4	18.2	17.7	0.51	<0.01	<0.05	0.15
c[c]	1.63	1.47	1.61	1.60	0.045	0.22	0.09	0.12

[a]asymptotic GP

[b]time at which half of A is produced

[c]constant determining the sharpness of the curve

Table 7. Incubation at t½. Neutral detergent fibre (aNDF; g/kg DM) and true DM digestibility (TDMd; g/kg DM), gas production (GP, ml/g DM) and volatile fatty acids production (VFA, mmol/100 mmol), and ammonia N concentration (N-NH_3, mg/g DM) of diets supplemented with high or low inclusion level of whole soybean seed (WSS) and canola expeller (CE).

	Inclusion level				SEM	*P* value		
	High		Low			Inclusion (I)	Supplement (s)	I × S
	WSS	CE	WSS	CE				
dNDF[a], g/kg DM	193	253	205	240	2.3	<0.05	<0.01	<0.01
TDMd[b]	710	764	704	720	0.9	<0.01	<0.05	<0.05
GP, ml/g DM	145	151	137	137	13.0	0.16	0.67	0.72
TDMd/GP	5.01	5.08	5.18	5.19	0.49	0.61	0.89	0.92
Total VFA, mmol/100 mmol	2.73	2.84	2.56	2.57	0.591	0.14	0.70	0.72
Acetate	59.5	60.0	61.9	60.8	0.64	<0.01	0.30	<0.05
Propionate	27.5	27.6	26.2	27.0	0.42	<0.01	0.67	0.89
n-butyrate	10.6	10.1	9.87	10.4	0.62	0.50	0.94	0.12
Iso-butyrate	0.46	0.40	0.43	0.36	0.11	0.59	0.24	0.85
n-valerate	1.17	1.10	0.99	0.90	0.50	0.46	0.76	0.98
Iso-valerate	0.65	0.81	0.57	0.49	0.16	<0.05	0.61	0.17
Branched	2.39	2.14	1.92	2.06	0.69	0.24	0.77	0.70
Acetate/propionate	2.55	2.54	2.74	2.64	0.05	<0.01	0.05	0.08
N-NH_3, mg/g DM	1.95	2.44	1.08	1.16	0.013	<0.01	<0.05	<0.01

.

Figure 1. Gas production rate at high inclusion level (dotted line) and low inclusion level (solid line)

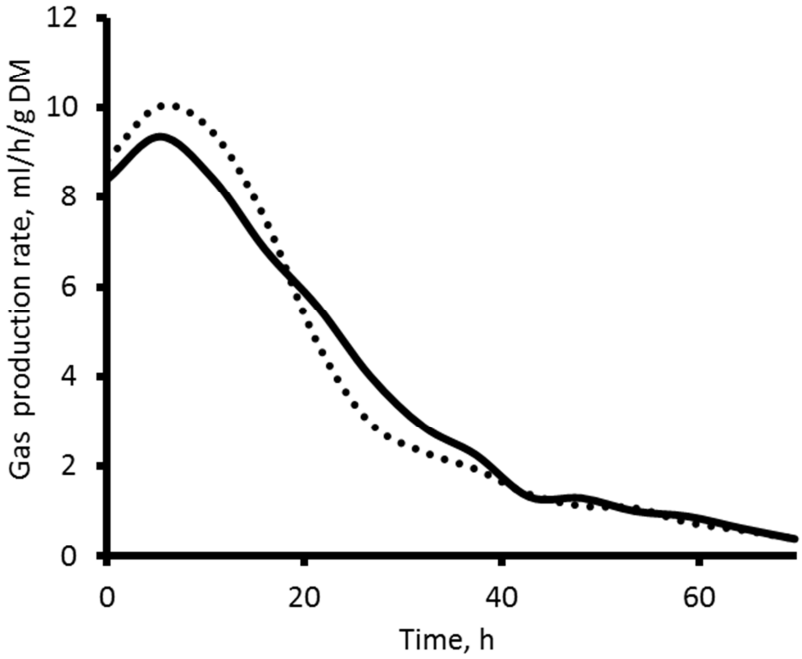
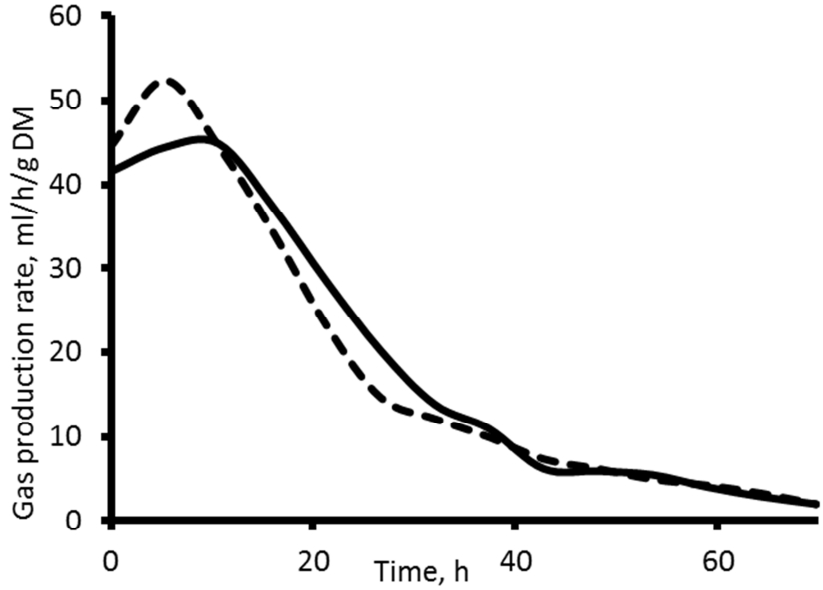


Figure 6. gas production rate for CE (dotted line) and WSS (solid line) at low inclusion level



Chapter 7

Conclusion

The main conclusions to be drawn from of this dissertation about CE are:

- Canola expellers extracted on the farm have high lipid content due to production by a mechanical system that is not very efficient at oil extraction. Due to this, it was proposed to detect its stability especially with relation to its lipids fraction. From the analyses CE showed high stability of lipid fraction with peroxide values under the threshold that is reported to impair rumen fermentation.
- High content of residual oil in canola expellers could inhibit rumen activity, therefore its inclusion in diets for ruminants must be considered to avoid inhibition of rumen activity.
- *In vitro* experiment showed CE had greater degradability compared to WSS and that it could be available for inclusion in low protein diets for ruminants.
- Diets containing CE showed a higher efficiency of nitrogen utilization and therefore a lower outflow of non-ammonia N from the fermenters, suggesting a lower amount of by-pass protein compared to WSS.
- Diets at low CP level reduced degradability and GP rate only in the first part of incubation, but total gas production did not differ.
- Inclusion of CE in diets at low CP content lead to a higher rate of degradation in the first hours of incubation compared to WSS.
- Modern techniques of animal feeding are designed (suggested by nitrate directive) to reduce N excretion by reducing the N content supplied by CP. Inclusion of CE in these diets can be a useful method to increase efficiency of nitrogen utilization due its higher degradability.
- Use of CE presents economic advantages for farmers (availability of protein animal sources and less dependence from fossil fuel) and reduces environmental pollution.

- Some disciplinaries (Parmigiano Reggiano cheese, Grana Padano cheese…) do not allow use of canola and its by-products for animal feeding.

Chapter 8

List of Publications

- L. Bailoni, Guadagnin M., Brigando A.,Tagliapietra F., Mantovani R. Estimation of in vitro digestibility of two commercial horses feeds using equine fecal inoculum in a Daisy II incubator; Atti del XII° Congresso internazionale 12° Convegno “Nuove acquisizioni in materia di Ippologia, New findings in equine practice” Druento (TO), 11-13 November 2010, pp 60-61.

- F. Tagliapietra, Guadagnin M., Cattani M., Schiavon S., Bailoni L. 2010. Associative effects of different feed combinations assessed by using a gas production system. Book of Abstracts of the 61st Annual Meeting of the European Association for Animal Production, Heraklion Greece, 23-27 August 2010, vol 16, 297.

- M. Guadagnin, Cattani M., Bondesan V., Bailoni L., Tagliapietra F., 2011. Comparison among soybean seeds and rapeseed cake included in diets with two different protein levels using gas production technique. EAAP 2011 Stavanger Norvey 29 agosto-2settembre 2011.

- M. Cattani, Tagliapietra F., Guadagnin M., Bailoni L. 2011. Repeatability of in situ and in vitro digestibility measured at 24 or 48 h of incubation. ASPA 2011. Cremona Italy. 7-10 giugno 2011.

- M. Guadagnin, Newbold C.J., Belanche A., Bondesan V., Bailoni L. 2011. Effects of soybean and rapeseed cake on in vitro rumen fermentation and microbial yield. ASPA 2011. Cremona Italy. 7-10 giugno 2011

- M. Guadagnin, Cattani M., Tagliapietra F., Schiavon S., Bailoni L. 2012. In vitro degradability and energy value of rapeseed cake produced on farm by cold extraction press. Book of Abstracts of the 63rd Annual Meeting of the European Federation of Animal Science, n. 18, Bratislava Slovakia, 27-31 August 2012, page 191.

- M.Guadagnin, Cattani M., Bailoni L.. 2012. Effect of pressing, storage temperature and storage time on chemical composition and fatty acid profile of canola expellers. Submitted to Italian Journal of Animal Science.

- M.Guadagnin, Tagliapietra F., Cattani M., Schiavon S., Belanche A., Newbold C.J., Bailoni L.. 2012. In vitro rumen fermentation and microbial yield of diets containing soybean seed and canola press cake at two different levels of inclusion. Submitted to Canadian Journal of Animal Science

- M. Guadagnin, Tagliapietra F., Cattani M., Bailoni L. 2012. Nitrogen level of medium influences in vitro gas production of high-protein feeds. Submitted to Animal Feed Science and Technology.

- M. Guadagnin, Tagliapietra F., Cattani M., Bailoni L. 2012. Comparison among soybean seeds and rapeseed cake included in diets with two different CP levels using gas production technique. Submitted to Animal Feed Science and Technology.

Acknowledgements

A special thanks to the following people:

Prof. Lucia Bailoni and Dr Franco Tagliapietra who guided me in this “adventure” transmitting to me their knowledge, and especially for their encouragement to do more every time.

Prof. Charles Jamie Newbold for the hospitality accorded and for his philanthropy that permitted me to live probably the best scientific experience of my life.

Dr. Mirko Cattani a valuable colleague and friend who did significantly helped me in the difficult moments during the whole PhD period.

Members of “ animal nutrition and wellness office” : Dr Giorgio Dalla Montà, Dr Annalisa Scollo, Dr. Giacomo Cesaro and Dr. Mirko Cattani for all funny and beautiful days spent together working hard.

PhD Students of PhD School in Animal and Food Science of the University of Padova for the wonderful working climate that they created and for made our PhD School the best of Padova.

All the technician of the ex-Department of Animal Science of the University of Padova, who provided me a friendly and collaborative climate.

Dr. Rosalba Moro for willingness to help me every time patiently and for the moral support and some loving earful.

All the “Team Newbold” of Aberystwyth University who give hospitality and made me feel a part of them.

Dr. Alejandro Belanche and Dr. Kostantinos Minas for all the time spent in lab to helping and teaching me.

Dr. Tina Blackmore and Mrs Hillary Worgan (and their horses Ruby and Bub) as well as for their invaluable aid also for their sympathy and the great friendship accorded me.

And finally, last but not least

Claudia, Mum and Dad for their support, understanding and encouragement to never give up.

Matteo