Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
Thesis 2017:90

Rapeseed co-products in pig diets – Effects on nutrient and energy digestibility and metabolism

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Marta Pérez de Nanclares Fernández

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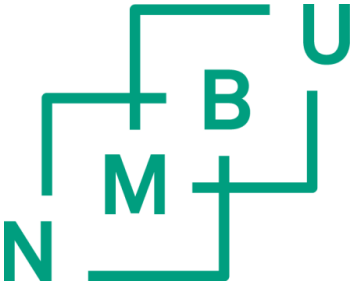

Thesis number 2017:90
ISSN 1894-6402
ISBN 978-82-575-1478-5

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Acknowledgements

The work presented in this thesis was carried out at the Department of Animal and Aquacultural Sciences of the Norwegian University of Life Sciences (NMBU), Ås, Norway. The PhD grant and research work were funded by NMBU and the FeedMileage project (Norwegian Research Council, Oslo, Norway; grant no. 233685/E50).

I would like to begin by expressing my sincere gratitude to my supervisors Nils Petter, Liv Torunn, Anne-Helene, and especially Margareth, with a big THANK YOU (notice the capital letters). Thank you for welcoming me into the group and giving me the opportunity to learn from your great knowledge and experience. For your time, enthusiasm, advice, guidance, and endless patience (you need a lot of patience to deal with a stubborn, picky Spaniard!). For planting the seed of scientific thinking in my brain. For allowing space for mistakes and falls and for giving me a hand to get up again when things were getting "less easy". For "pushing" and "shaking" me a bit here and there, to help me grow both professionally, and personally. And thank you for grabbing my feet to bring me down to earth when I was "flying up" into stress clouds, especially during the experiments and writing process.

I would also like to thank:

All of my co-authors for the enriching collaboration and their great work.

All of my colleagues for walking with me until the completion of my PhD. Thanks Jon for all your patience, advice, and good discussions, scientific and not that scientific ;). Thanks Leidy and Felipe for your loud voices and "latinness". And thank you Hanne for being my "confidant" during all this time, for LISTENING to my complaints, jokes, brain storms, laughter, crying… I am super happy you landed down in the basement with me and I feel extremely lucky to have had you as my office mate! I am sure you will be writing some acknowledging words yourself very soon ☺. And thank you for letting me turn on the heater in the office at all times ;).

The people at IHA for all their smiles during these years.

Norway for welcoming me and for becoming my second home (not that you had another option though ;)). For witnessing my "maturation" as a human being (*Homo sapiens*, Paulo esto va por tí). For making me discover, love, and appreciate nature. For giving me endless

and gorgeous “friluftsliv” experiences. For DNT. And for continuously reminding me that my thermal comfort only starts at 25 °C.

All my friends for lighting my life in Norway and making my Viking adventure very, very enjoyable. Thanks for the great fun. Without you, I would not have survived here for more than 2 weeks! Very special thanks to Paulo, Dadou, Stefano, Arnaud, Marta, and Joaquín, for becoming my second family away from home, you have made my life in “frozen land” much warmer. Thank you, my friends, for reading my acknowledgements, you do not need to keep reading ;)

Tina, thank you for ALWAYS listening and being there for me, for tolerating all the “pig talking” and even joining my “feces collection”, for all the sharing and complicity, and for your enduring encouragement, motivation, and support in all of my pursuits, even when the wind was not with us ☺.

The best for last, my greatest thanks go to my incredibly awesome parents. I will never be able to thank you enough for all the sacrifices you made so I could be where, what, and who I am today. Your unconditional love for me, advice, wisdom, and never ending support are just amazing and I could not have accomplished all of this work without that. I take this opportunity to thank you for bringing me to this World, and for the AMAZING job you have done as parents.

And…I will thank the rest later with “a couple” of beers!

Ås, October 2017

Marta Pérez de Nanclares Fernández

Table of contents

1. Abbreviations

The main abbreviations used throughout the present work are listed below. The rest of the abbreviations are described in the text.

AA	Amino acids
ADF	Acid detergent fiber
AID	Apparent ileal digestibility
ANF	Antinutritional factors
ATTD	Apparent total tract digestibility
CP	Crude protein
DE	Digestible/digested energy
DF	Dietary fiber
DM	Dry matter
DN	Digested nitrogen
FE	Feed efficiency
GABA	Gamma-Aminobutyric acid
GE	Gross energy
GIT	Gastrointestinal tract
HE	Heat production
IAA_{end}	Ileal endogenous amino acid losses
IN	Ingested nitrogen
ME	Metabolizable energy
N	Nitrogen
NE	Net energy
NDF	Neutral detergent fiber
RE	Retained energy
RN	Retained nitrogen
RS	Rapeseed
RSM	Rapeseed meal
SBM	Soybean meal
SID	Standardized ileal digestibility
TID	True ileal digestibility

2. Summary

The European pig industry is heavily dependent on imported protein feedstuffs, particularly soybean meal (**SBM**). Increased and more efficient use of locally produced protein sources, such as rapeseed (**RS**), could alleviate the dependency on imports and improve the sustainability and self-sufficiency of pig production in Europe. Because feed represents up to 70% of the total cost for pig producers, small improvements in feed efficiency (**FE**) when using rapeseed meal (**RSM**) could enhance production profitability. Digestive and metabolic efficiencies are key factors affecting the net FE in pigs. Alternative protein feedstuffs, including RSM, are typically more fibrous compared with SBM, and can contain other anti-nutritional factors that could compromise FE.

Two experiments were thus conducted to assess the effects of replacing SBM with RS co-products on energy and nutrient digestibility and metabolism in pigs. The present thesis includes three papers, Papers I and II are based on data collected from Experiment 1 while Paper III is based on data collected from Experiment 2.

In Experiment 1, 40 Norwegian Landrace young pigs were fed either a SBM-based diet or a test diet (**RSF**) where SBM and wheat were partially replaced with high-fiber RS co-products (20% of a coarse fraction from air-classified RSM, and 4% of pure RS hulls). Paper I evaluated the effects on apparent nutrient and energy digestibility and investigated potential biological mechanisms associated with differences in digestibility. Feeding the RSF diet increased the thyroid to body weight ratio. Apparent ileal (**AID**) and total tract (**ATTD**) digestibility of energy and most nutrients, including amino acids (**AA**), monosaccharides, and phosphorus, were reduced by the RSF diet. There was a considerable variation in nutrient digestibility among individual pigs within both dietary groups. The reduction in digestibility by the RSF diet was associated with digestive enzyme activity, as the reduced AID of crude protein (**CP**) and AA coincided with reduced trypsin activity and the unaffected AID of starch coincided with similar amylase and maltase activities in the jejunum of the pigs.

Paper II evaluated metabolic effects of pigs fed the two diets described in Experiment 1 through metabolomics analyses of digesta, liver, and serum samples. Analysis of digesta samples identified sinapine, sinapic acid, and gluconapin as exposure markers to the RSF diet. Lower concentrations of total free AA were found in the digesta from duodenum and jejunum of the RSF pigs while higher concentrations were observed in the ileal digesta of these pigs.

Feeding the RSF diet increased the concentration of γ-aminobutyric acid (**GABA**), a non-proteinogenic AA, along the entire gastrointestinal tract. Concentrations of microbial metabolites, namely short chain fatty acids and secondary bile acids, were similar between the two dietary groups. Analysis of liver samples showed increased concentrations of GABA and reduced concentrations of intermediate metabolites of the urea cycle (arginine, citrulline, and ornithine) in pigs fed RSF. Hepatic free serine and glycine, important intermediate metabolites in one-carbon metabolism were decreased and increased by the RSF diet, respectively. In addition, a decrease in ascorbic acid level (an antioxidant) and increased concentrations of various oxidative stress and lipid peroxidation markers, including oxidized thiol metabolites, pyroglutamate, and butanal, were observed in the liver of pigs fed the RSF diet. Analysis of serum samples revealed greater concentrations of total free AA, butanal, and 2-oxoglutaric acid in pigs fed the RSF diet.

In Experiment 2, 32 crossbred young pigs (Danish Landrace/Yorkshire × Duroc) were fed either a SBM-based control diet or test diets where SBM and wheat were partially replaced with 10, 20, and 30% of RSM for three weeks. Paper III evaluated the effects of this dietary change on nutrient and energy digestibility, nitrogen (**N**) retention, energy metabolism, and substrate oxidation based on data from N balance and respiration experiments. Increasing inclusion of RSM in the diet linearly reduced the ATTD of energy and most nutrients, including most of the fiber components and their monomeric residues. Rapeseed meal inclusion did not affect the efficiency of utilization of digested N (**DN**) for retention or total N excretion, while it induced a shift in N excretion from urine to feces. Despite enlarging the liver, increasing inclusion of RSM in the diet did not affect total heat production or the efficiency of utilization of metabolizable energy (**ME**) for energy retention.

Overall, it can be concluded that partially replacing SBM with up to 30% of RS co-products reduced the digestive efficiency of young pigs, but did not compromise overall protein and energy metabolism or efficiency of utilization of DN or ME for retention. However, inclusion of RS co-products enlarged the thyroid and livers of the pigs, affected protein metabolism at a molecular level, and induced a change in the redox and oxidative stress status of the pigs.

3. Sammendrag

Svineproduksjonen i Europa er i dag svært avhengig av importerte proteinråvarer, særlig soyamel (**SBM**). Økt og mer effektiv bruk av lokalproduserte proteinkilder, som rapsfrø (**RS**), kan redusere avhengigheten av importerte råvarer, og øke bærekraften og selvforsyningen av europeisk svineproduksjon. Fôret representerer opptil 70% av den totale kostnaden i svineproduksjonen, og dermed kan små forbedringer av fôreffektiviteten (**FE**) ved bruk av rapsfrømel (**RSM**) bidra til økt lønnsomhet for produsentene. Fordøyelighet og metabolisme er sentrale faktorer som påvirker omsetningen av næringstoffer hos gris. Alternative proteinfôrmidler, som RSM, er typisk mer fiberrike sammenlignet med SBM, og kan også inneholde andre anti-næringsstoffer som kan redusere fôreffektiviteten.

Det ble derfor gjennomført to forsøk for å vurdere effekten av å erstatte SBM med co-produkter av rapsfrø, på fordøyelighet og metabolisme av energi og næringsstoffer hos gris. Disse forsøkene er samlet i tre artikler i denne avhandlingen. Artikkel I og II er basert fra data fra det første forsøket, mens artikkel III er basert på forsøk nummer to.

I forsøk en ble 40 unggris av Norsk Landsvin fôret med enten en SBM-basert fôrblanding eller en forsøksfôrblanding (**RSF**), der SMB og hvete delvis ble erstattet med høyt fiber RS-co-produkter (20% av en grov-fraksjon fra luftkondisjonert RSM, og 4% skall fra RS). Artikkel I beskriver effekt på apparent fordøyelighet av energi og næringsstoffer, og tar for seg potensielle biologiske mekanismer knyttet til forskjeller i fordøyelighet. Grisene som ble fôret med RSF-fôret hadde økt vekt av skjoldbrukskjertelen sett i forhold til kroppsvekten. Apparent ileal (**AID**) and total (**ATTD**) fordøyelighet av energi og de fleste næringsstoffene, inkludert aminosyrer (**AA**), monosakkarider og fosfor, var redusert med RSF-fôret. Det var en betydelig variasjon i fordøyelighet av næringsstoffer blant enkeltgriser innen begge fôr-gruppene. Reduksjonen i fordøyelighet med RFS-fôret ble knyttet til aktiviteten av fordøyelses-enzymer i jejunum, da redusert AID av råprotein (**CP**) og AA sammenfalt med redusert trypsin-aktivitet, og AID av stivelse, som ikke ble påvirket, sammenfalt med tilsvarende amylase- og maltase-aktiviteter.

Artikkel II omhandler metabolske effekter hos griser som fikk de samme forsøksfôrene som er beskrevet i artikkel I, ved metabolomic-analyser av innhold fra tarmkanalen (digesta), lever- og serum-prøver. Analyser av digesta-prøver identifiserte sinapin, sinapinsyre og gluconapin som eksponeringsmarkører for RSM-fôret. Det ble funnet lavere konsentrasjoner

av totalt frie AA i digesta fra duodenum og jejunum hos RSF-grisene, mens konsentrasjonen var høyere i ileal digesta fra de samme grisene. RSF-fôret økte konsentrasjonen av γ-aminosmørsyre (**GABA**), en ikke-protein AA, langs hele tarmkanalen. Konsentrasjonene av mikrobielle metabolitter, nemlig kortkjedede fettsyrer og sekundære gallesyrer, var like mellom de to fôr-gruppene. Analyse av leverprøver viste økt konsentrasjon av GABA og redusert konsentrasjon av intermediære metabolitter fra urea-syklusen (arginin citrullin og ornitin) hos griser som fikk RSF-fôret Hepatisk fri serin og glycin, som er viktige intermediære metabolitter i en-karbon omsetningen, ble henholdsvis redusert og økt med RSF-fôret. I tillegg ble det observert en reduksjon i askorbinsyre-nivå (en antioksidant), og økte konsentrasjoner av ulike oksidative stress-markører og lipid peroxidase-markører (inkludert oksiderte thiol-metabolitter, pyroglutamat og butanal), i lever hos grisene som fikk RSF-fôret. Analyse av serum-prøver viste høyere konsentrasjoner av totalt frie AA, butanal og 2-oxoglutarsyre hos griser som fikk RSF-fôret.

I forsøk to ble det benyttet 32 krysnings-griser (Dansk Landsvin/Yorkshire x Duroc), som ble fôret med enten et SBM-basert kontrollfôr eller et testfôr der SBM og hvete ble gradvis erstattet med 10, 20 eller 30% RSM i en forsøksperiode på tre uker. Artikkel III beskriver effektene disse endringene i fôret hadde på fordøyelighet av energi, nitrogen (N)-retensjon, energi-metabolisme, og substrat-oksidasjon basert på data fra N-balanse og respirasjons-forsøk. Økende innblanding av RSM i fôret resulterte i en lineær reduksjon av ATTD for energi og de fleste næringsstoffene, medregnet de fleste fiber-komponentene og deres monomere endeprodukter. Bruk av RSM i fôret påvirket ikke effektiviteten i utnyttelse av fordøyd N (**DN**) til retensjon eller total N-utskillelse, mens det indikerte et skifte i N-utskillelsen fra urin til avføring (feces). Til tross for at økende innblanding av RSM i fôret forstørret leveren, ble ikke total varmeproduksjon eller utnyttelse av omsettelig energi (**ME**) til energi-retensjon påvirket

Samlet sett kan det konkluderes med at en gradvis erstatning av SBM med opptil 30% av RS co-produkter reduserte effektivitet av fordøyelsen hos unge griser, mens protein- og energi-metabolismen, eller effektiviteten i utnyttelsen av DN eller ME til retensjon, ikke ble påvirket. Bruk av RS-co-produkter førte til økt vekt av skjoldbruskkjertel og lever hos grisene, påvirket proteinmetabolismen på et molekylært nivå, og førte til en endring av grisenes redox-status og den oksidative stress-statusen.

4. List of publications

The present thesis is based on the papers listed below. The papers will be referred to by their roman numbers throughout the thesis.

Paper I: **Pérez de Nanclares, M**., Trudeau, M.P., Hansen, J.Ø., Mydland, L.T., Urriola, P.E., Shurson, G.C., Piercey Åkesson, C., Kjos, N.P., Arntzen, M.Ø. and Øverland, M. 2017. High-fiber rapeseed co-product diet for Norwegian Landrace pigs: Effect on digestibility. Livestock Science. 203, 1-9. Doi. Org/10.1016/j.livsci.2017.06.008.

Paper II: Chen, C., **Pérez de Nanclares, M**., Kurtz, J.F., Trudeau, M.P., Wang, L., Yao, D., Saqui-Salces, M., Urriola, P.E., Mydland, L.T., Shurson, G.C. and Øverland, M. 2017. Identification of redox imbalance as a prominent metabolic response elicited by rapeseed feeding in swine metabolome. Journal of Animal Science. (Submitted August 2017).

Paper III: **Pérez de Nanclares, M**., Marcussen, C., Tauson, A-H., Hansen, J.Ø., Kjos, N.P., Mydland, L.T., Bach Knudsen, K.E. and Øverland, M. 2017. Increasing levels of rapeseed meal in diets for pigs: Effects on protein and energy metabolism. Animal (Submitted August 2017).

5. General introduction

5.1. Contextual framework

5.1.1. Global trends and challenges

Following the common saying, "an image is worth more than a thousand words", I will start introducing the background for the work of this thesis with a series of graphical illustrations (Figures 1 and 2).

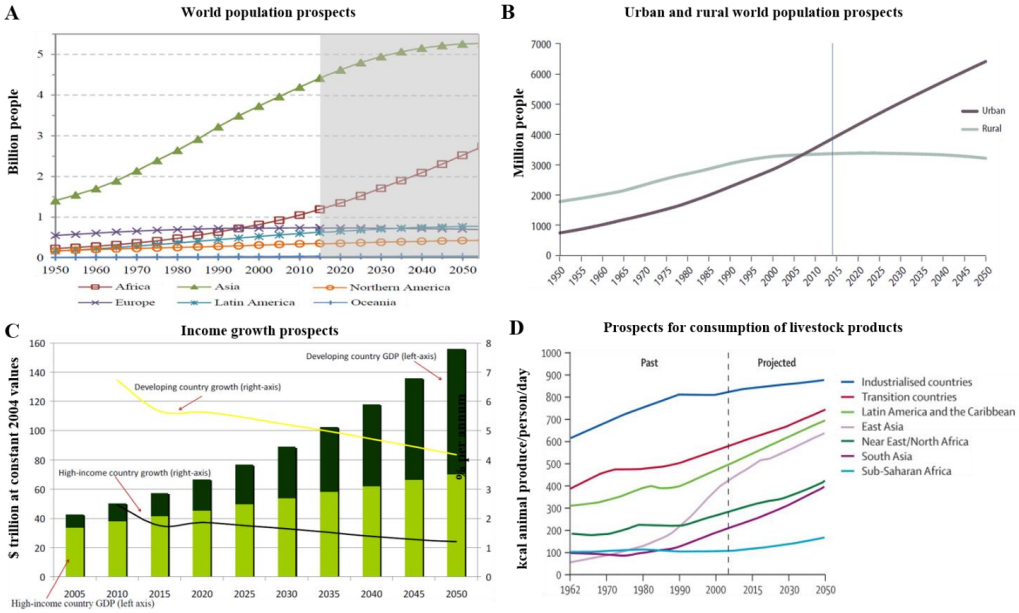

Figure 1. Global trends: a) Projection of the world's population growth from 1950 to 2050, source: UNPD, 2017. B) Projection of the urban and rural world population, source: UNPD, 2014. C) Projection of the world's income growth, GDP = Gross Domestic Product, source: van der Mensbrugghe *et al.*, 2009. D) Trends in consumption of livestock products per person (milk, eggs, and dairy products, excluding butter), source: McMichael *et al*., 2007.

The world is experiencing an increase in the demand for livestock products (Thornton, 2010). Figure 1 shows some of the main socio-economical factors driving this growing demand: A) Human *population is growing* and it is estimated to reach nearly 10 billion by 2050, with most of the increase taking place in the developing countries (UNPD, 2017). B) The world population is undergoing a rapid *urbanization* process, with one third of the global population expected to live in cities by 2050 (UNPD, 2014). The urbanization process is accompanied by changes in lifestyle and consumption patterns. C) A global *economic growth* rate of 2.9%

per year is expected between 2005 and 2050, with 1.6% for developed countries and 5.2% for developing countries (van der Mensbrugghe *et al.*, 2009). In this regard, it is known that as income grows, so does the demand for livestock products (Steinfeld *et al.*, 2006). The combination of the two latter factors is leading to a transformation of the diet in the developing countries towards a more western type livestock-based diet (Steinfeld *et al.*, 2006). Altogether, these trends are leading to an increase in overall demand for food, and particularly for animal products (Figure 1D).

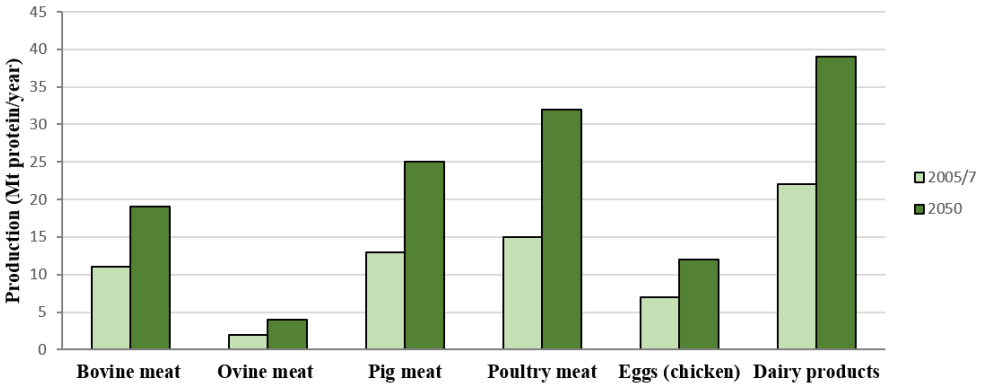

Figure 2. Projected change in global protein production for major livestock commodities. Based on data from Alexandratos and Bruinsma, 2012.

Livestock production is growing rapidly, especially in developing countries, and is expected to continue expanding (Figure 2) to meet the forecast increase in animal protein demand (Alexandratos and Bruinsma, 2012). Fast-growing and feed efficient species, such as pigs, will likely play an important role in the future animal protein supply. Expansion of livestock production can be accomplished by increases in number of animals and/or productivity (Alexandratos and Bruinsma, 2012). Both livestock population and productivity have increased markedly since the 1960s. However, the development of new tools and techniques in the fields of genomics, metagenomics, transcriptomics, proteomics, and metabolomics, provides potential for increasing productivity further (Thornton, 2010). The livestock sector will play a crucial role in ensuring future food security, but it is expected to minimize its contribution to several concerns. Among the livestock-associated concerns are: environmental deterioration (via waste disposal, chemical pollution, deforestation, etc.), food-feed-bioenergy competition for natural resources (particularly land and water), health concerns (foodborne diseases transmitted by animal origin foods, zoonotic and human

diseases emerging from livestock, etc.), climate change (through greenhouse gas emissions), and social, cultural, and ethical concerns (FAO, 2017).

Following what seems like a "chain reaction" illustrated in Figures 1 and 2, and as feed comprises a major input to livestock systems, a consequent boost in the demand for animal feeds is also expected (Lee *et al.*, 2016). The feed manufacturing industry will also need to keep up with the growing demand for protein-based animal feeds while facing several challenges, including food and feed safety concerns, development of antibiotic resistance, chemical contamination of feeds, use of genetically modified (**GM**) crops, etc. (FAO-IFIF, 2010). These problems and the tremendous demand for animal protein have brought attention to the sources of feed protein and their quality, safety, and suitability for future sustainable supply.

The livestock and feed manufacturing industries are very closely linked and they share one of the major challenges facing the world today: meeting the growing demand for high-quality food and feed in a sustainable manner, meaning in an *economically* viable and *environmentally* and *socially* responsible way. This will require joint efforts from the two sectors. In addition, and concerning the contribution from animal science research to this challenge, an interdisciplinary approach that implements new knowledge and technologies from the fields of genetics, nutrition, and health seems appropriate and promising. Strategies to simultaneously improve productivity and sustainability include: i) to diversify feed sources, especially protein sources, making a better use of crop/industry by-products, organic waste, and alternative crops as animal feed, and reducing reliance on feed crops; ii) to bring about *better use of local resources*; iii) to investigate new *traits related to feed efficiency* (**FE**) *and robustness* against these dietary changes; and iv) to develop new phenotypes with improved sustainable animal productivity that can ultimately be integrated into breeding programs (FAO, 2017; Thornton, 2010).

5.1.2. Protein sources in animal feed

Protein constitutes the second main nutrient component in pig feed after energy, and so dietary protein contributes greatly to the feed cost (Boggess *et al.*, 2008). The suitability of a protein feedstuff depends not only on its protein concentration, but also on its content of the essential amino acids (**EAA**) required by the animal, on how digestible the protein and amino acids (**AA**) are, as well as the content of toxic substances associated with it. Currently, plant and

animal products are the most commonly used sources for protein in animal feeds, with the major part of the animals' protein requirement being supplied by plant protein (Beski *et al*., 2015). Compared with animal proteins, plant protein sources are generally cheaper but they are more unbalanced and poor in certain EAA and their use may be limited by the presence of fiber and other antinutritional factors (**ANF**). The most common protein sources used in animal feed are presented in Table 1. The three "big sources" of high-quality protein are soybean meal (**SBM**), animal by-products, and fish meal (Boland *et al*., 2013). In addition to providing quality protein, fish meal is a good source of calcium and phosphorus (**P**). However, there are some concerns related to the use of fish meal, including price volatility, availability, variable protein quantity and quality among different sources, contamination by pollutants and heavy metals, and associated over-fishing and irresponsible use of marine resources (FAO, 2004). In slaughter pigs, there is also concern about "fishy" taste and smell of the meat.

Table 1. Major protein sources used in the formulation of feeds for animals and their crude protein (CP) content and apparent ileal digestibility (AID). Source: Boland *et al*., 2013.

Protein source	**Usage[1] (M tonnes)**	**CP content[2] (% as is)**	**AID of CP[2, 3] (%)**
Oil meals	316		
Soybean meal		45-49	85
Rapeseed meal		33-39	70
Sunflower meal		34-38	77
Cottonseed meal		30-42	78
Animal by-products	10		
Meat meal		45-58	57-72
Feather meal		83	65
Blood meal		93	87
Fish meal	7	55-71	83
Pulses			
Peas		21	74

[1] FAO, 2004
[2] CBV, 2007
[3] Values relate to pigs

Animal by-products derive mainly from poultry and poultry processing, fish and fish processing, milk and dairy processing, and meat packing and rendering processes (Denton *et al.*, 2005). Among these by-products, meat meal, bone meal, meat and bone meal (**MBM**), feather meal, and blood meal have all been used in pig diets (Denton *et al.*, 2005). They provide protein and additional nutrients, are a great complement to grain and plant protein for

animal feed, and they represent an alternative for by-product disposal while adding value to livestock production. However, food and feed safety concerns have recently been raised, especially after the mad cow disease outbreak in UK in 1986, which led to the ban of MBM protein from all farm animal feed in the European Union (**EU**) in 2001 (FAO, 2004). As a result, MBM was mostly replaced by SBM, and so European animal feed manufacturing became largely based on plant protein, especially on SBM.

Soybean meal is the most widely used protein source in animal nutrition, accounting for about 75% of all protein used in animal feed worldwide (FAO, 2004). Most of this SBM is used to feed pigs and poultry (Stein *et al*., 2013). In fact, SBM is often referred to as the "gold standard" because other protein sources are usually compared to it (Cromwell, 2008). The popularity of SBM is attributable not only to its high protein content (about 44 to 49%), but this protein is also highly digestible (see Table 1 for digestibility in pigs) and has a well-balanced AA composition (Cromwell, 2008). Soy protein is rich in lysine, threonine, and tryptophan, which are limiting AA for pigs and are deficient in corn and other cereals commonly fed to pigs (Stein *et al*., 2013). Therefore, SBM is a great complement to cereals for feed formulation. Soybean meal supplies 60% of the protein feedstuffs used for animal feed in the EU (Figure 3). However, the EU's self-sufficiency for this product is only 3% (Table 2), being the second largest soy-importing region after China (Kroes and Kuepper, 2015).

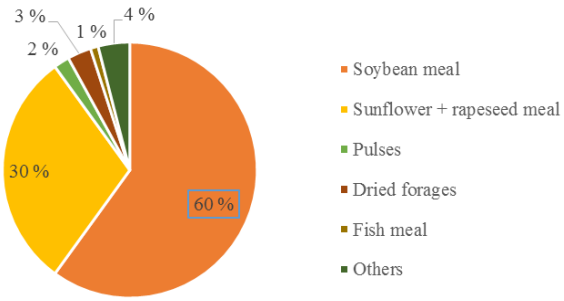

Figure 3. Protein rich feed materials used for animal feeding in the European Union in 2013/14. Adapted from: Bouxin, 2015.

Table 2. European Union (EU) balance sheet for protein rich feed materials in 2013/14. Source: Bouxin, 2015.

	EU production[1]	EU consumption[2]	Self-sufficiency (%)
Soybeans/meal	497	14752	3
Rape and sunflower seeds/meals	5680	7379	77
Pulses	417	393	106
Dried forages	613	570	108
Miscellaneous	786	1276	62
Subtotal	7993	24370	33
Fishmeal	270	351	77
Total	8263	24721	33

[1] EU production from EU seeds (in 1000 tons)
[2] Including consumption by the pet industry and on-farm uses (in 1000 tons)

Over 80% of the soy in the world is produced by the United States, Brazil, and Argentina combined (Kroes and Kuepper, 2015). The facts that more than half of this crop is GM and that the EU applies a zero-tolerance policy for non-approved GM material result in higher prices for the approved varieties and the non-GM SBM for the EU (Coma, 2010). The heavy dependency on imports exposes the EU to trade instability, availability problems, and price volatility for SBM (de Visser *et al*., 2014). In addition, the expansion of soybean production, largely incentivized by the massive demand of SBM from the livestock sector, necessitates more water, land, chemicals, and energy. This entails a series of environmental and social impacts, including deforestation, greenhouse gas emissions, pollution, transport distance and carbon footprint, degradation of local habitats and biodiversity, land use conflicts and land grabbing, displacement of small producers, labor issues, inequality in local populations, and social tension (Coma, 2010; Stiles, 2016).

The reliance of the livestock sector on SBM for animal feed compromises the criteria for meeting the increasing demand for livestock products in a *sustainable* manner: economically, environmentally, and socially friendly. Therefore, there is great interest in establishing secure, cost-effective, and high-quality sources of protein for animal feed, alternative to SBM. Possible means to achieve this include *enhancing the use of existing local protein resources* (motivation for this thesis), and/or developing suitable and competent novel protein sources (out of the scope of this thesis). Regarding existing local resources and with focus in Europe, the use of unconventional protein by-products from the food and biofuel industries, especially sunflower meal and rapeseed meal (**RSM**), is growing because of better price and availability compared to SBM (Florou-Paneri *et al*., 2014). Other alternative local feedstuffs that can be used in pig feed include cottonseed meal, flaxseed meal, peas, beans, lupines, and alfalfa.

However, oil meal crops (cottonseed, sunflower, and rapeseed) seem to be more competitive than starch crops (lupines, peas, beans) and alfalfa, mainly due to the higher value of oil vs. starch (de Visser *et al*., 2014). This is in favor of the raw material chosen for the work of this thesis, which is RSM. An important contribution to future protein supply for animal feed will need to come from novel protein sources. Likely sources include yet unexploited plant sources, algae, insects, and microorganisms such as bacteria, fungi, and yeast (van der Spiegel *et al.*, 2013; Matassa *et al*., 2016). However, this deviates from the scope of the thesis and will not be discussed further.

5.2. Rapeseed meal as an alternative protein source to soybean meal

Rapeseed (**RS**), an oilseed crop from the Brassicaceae family (mainly *Brasssica napus* in Europe), is grown for the production of vegetable oil for human consumption, animal feed, and biodiesel. Rapeseed can be cultivated in cold and dry climates, where soybean do not thrive (USDA-ERS, 2017). The major RS producers are the EU, Canada, China, and India (Carré and Pouzet, 2014). Although RS is the second oilseed worldwide after soybean, it constitutes the major oilseed for European agriculture (Figure 4), who has witnessed a dramatic increase in RS production in recent years, mainly driven by the expansion of the biofuel industry (Carré and Pouzet, 2014). This directly translates into an increased availability of RSM, the by-product of RS oil extraction, which has awakened the interest of nutritionists and feed producers.

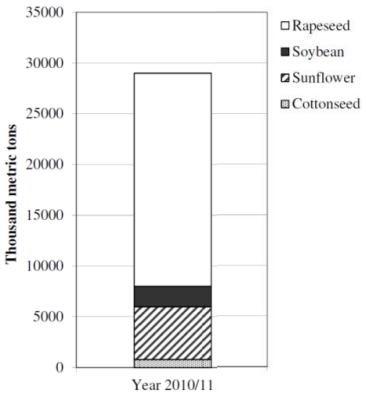

Figure 4. Oilseed production in the European Union (27 countries) for the year 2010/2011. Adapted from Florou-Paneri *et al*., 2014.

There are three main ways of extracting oil from RS (cold-pressed, expeller-pressed, and solvent extraction), the latter one being the most common and efficient (Leming and Lember, 2005; Spragg and Mailer, 2007). The by-product from the other two methods is commonly known as RS cake and is richer in oil and lower in protein than RSM. The oil extraction method, as well as the seed variety, growing and harvesting conditions, and manufacturing process will affect the nutritional value of the resulting RSM and, therefore, its suitability as a feed ingredient (Bell, 1993; Newkirk, 2011). It was realized that traditional RS varieties contained high levels of hazardous components that limited the consumption of RS products, including erucic acid in the oil (heart damaging effects for humans) and glucosinolates in the meal (unpalatable and harmful to livestock). To address these issues, conventional plant breeding programs were initiated to develop new varieties with low levels of erucic acid (< 2%) in the oil and low levels of glucosinolates in the meal (< 30 μmol/g), which are known as "canola" in North America, and as "double-zero" RS in Europe (Newkirk, 2009). The meal from these modern RS varieties is superior for animal feed than that of the traditional ones because they have been selected for improved protein and AA composition, and low levels of glucosinolates and erucic acid (Thomas, 2005). The existence of "double-zero" varieties and the growing availability of RSM from biofuel plants increase the opportunities for feeding RSM to livestock (Torres-Pitarch *et al*., 2014). Regarding its nutritional value, RSM has a relatively high protein content (about 34 to 38%) and a well-balanced AA profile (Maison, 2013). When compared to SBM, it contains less lysine but higher amounts of sulfur AA, i.e., methionine and cysteine (Newkirk *et al.*, 2003), thus these meals complement each other when used together in pig diets. However, the protein and AA in RSM are less digestible than in SBM, with true ileal digestibility (**TID**) values for pigs being generally 10% lower (Newkirk, 2009). As SBM is the "golden standard", comparing the nutrient composition of RSM vs. SBM seemed appropriate (Table 3). Table 3 shows that RSM has greater concentrations of ether extract but also contains more neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), and crude fiber (**CF**) than SBM, which results in decreased digestible and net energy (**DE** and **NE**, respectively) in pig diets (Montoya and Leterme, 2010). The low energy digestibility of RSM is one the main factors that limit its nutritive value. Rapeseed meal is a rich source of minerals and vitamins (Newkirk, 2009). Compared to SBM, it has higher amounts of available Ca, Mg, and P (NRC, 2012), which reduces the need for supplementing inorganic P in pig diets. Rapeseed meal is also richer in most of the B-vitamins than SBM (Bell, 1993).

Table 3. Main nutrient composition of de-hulled soybean meal (SBM), canola meal (CM), and "double-zero" rapeseed meal (00-RSM). The three meals are solvent-extracted products.

	SBM[1]	CM[1]	00-RSM[2]
Dry matter, %	90.0	91.3	88.7
Digestible energy, MJ/kg	15.1	13.4	11.6
Metabolizable energy, MJ/kg	13.8	12.6	10.6
Net energy, MJ/kg	8.8	8.0	6.3
Crude protein, %	47.7	37.5	33.7
Essential amino acids, %			
Arg	3.5	2.3	2
His	1.3	1.1	0.9
Ile	2.1	1.4	1.4
Leu	3.6	2.5	2.3
Lys	3	2.1	1.8
Met	0.7	0.7	0.7
Phe	2.4	1.5	1.3
Tyr	1.6	1.1	1
Thr	1.9	1.6	1.5
Trp	0.7	0.4	0.4
Val	2.2	1.8	1.7
Cys	0.7	0.9	0.8
Ether extract, %	1.5	3.2	2.3
Neutral detergent fiber, %	8.2	22.6	28.3
Acid detergent fiber, %	5.3	15.4	19.6
Crude fiber, %	3.9	10.5	12.4
Calcium, %	0.3	0.7	0.8
Total phosphorus, %	0.7	1.1	1.1

[1] NRC, 2012.
[2] Sauvant *et al*., 2004.

The availability, cost-effectivity, and nutritional value of RSM give it great potential to replace significant proportions of SBM in pig diets (Weightman *et al*., 2014). In fact, together with sunflower meal, it is already the second most used protein feedstuff in Europe after SBM (Bouxin, 2015). However, the use of RSM in pig diets has been associated with reduced feed intake, growth rate, and nutrient utilization (Landero *et al*., 2011; Seneviratne *et al*., 2011; Torres-Pitarch *et al*., 2014). These negative effects have been attributed to the high fiber content and other ANF in RSM, including glucosinolates, tannins, sinapine, etc. (Mejicanos *et al*., 2016).

Taken together, an increased and more efficient use of RSM would enhance sustainability and self-sufficiency of the European pig industry because: i) it is a by-product from a "home-grown" crop, so transport costs and associated carbon footprint would be reduced, in addition to adding value to the European RS production; ii) the EU is the leading producer of RS,

therefore, exposure to availability and trade instability concerns would be reduced; iii) environmental and social impacts associated to soybean production would be avoided. However, the presence of fiber and ANF can compromise the FE and performance of the pigs, which could affect the profitability of pig production.

5.3. Challenges with rapeseed meal in pig diets

Rapeseed meal has a high fiber content and other ANF that currently limit its efficient utilization in diets for monogastric animals, including pigs.

5.3.1. Fiber

The ADF, NDF, and CF contents in SBM and RSM are shown in Table 3 and a detailed composition of the non-starch polysaccharides (**NSP**), lignin, and fiber in soybean and RS meals and hulls is presented in Table 4. In contrast to soybeans, the RS hull fraction represents a high proportion of the whole seed and it stays with the meal after processing (Newkirk, 2009). The hull fraction represents about 30% of the RSM, and over 70% of the fiber in RS is concentrated in the hulls, constituting the main reservoir for NSP and lignin (Carré *et al.*, 2016). This explains the considerably higher fiber content in RSM compared to SBM. Dietary fiber (**DF**) is an integral component of pig diets, as it is present in almost all plant-based ingredients, especially in the co-products derived from oil and biofuel production which are increasingly used in diets for pigs (Kerr and Shurson, 2013). In fact, a minimum level of DF is required in order to maintain normal physiological gut function (Wenk, 2001). Moreover, in his review, Lindberg (2014) reported several positive effects of DF such as increasing satiety, stimulating gut health, affecting behavior, and overall improving animal well-being. However, high content of DF is associated with impaired nutrient utilization and reduced NE values in the diet (Noblet and Le Goff, 2001), and increased viscera weight in pigs (Jørgensen *et al.*, 1996). The negative relation between fiber level and nutrient and energy digestibility has been extensively reported in pigs (e.g. Jørgensen *et al*., 1996, Jørgensen *et al*., 2007; Len *et al*., 2009a,b). The magnitude of such impacts will be determined by the fiber's physicochemical properties, especially solubility and fermentability, and therefore may differ among fiber sources (Lindberg, 2014). In addition, effects of DF on pig performance also depend on the animal age and breed. In this regard, an increased capacity to digest fibrous feedstuffs by increasing age and body weight (**BW**) has been shown and attributed to a more

developed and larger gastro-intestinal tract (**GIT**), lower feed intake/kg BW, slower digesta transit time, and a higher cellulolytic activity than in younger pigs (Jørgensen *et al*., 2007; Shi and Noblet, 1993). Similarly, pig breeds indigenous to South East Asia have been shown to digest fiber better than western breeds genetically improved based on a high growth performance (Len *et al*., 2009a, 2009b; Urriola and Stein, 2012). The higher fiber digestive capacity of indigenous pigs is mainly explained by a longer mean retention time of digesta in the GIT, which results in prolonged contact between the digesta and the digestive enzymes, epithelial absorptive surface, as well as the gut microbiota (Lindberg, 2014).

Table 4. Nonstarch polysaccharide, lignin, and fiber composition (% of dry matter) of soybean and rapeseed meals and hulls. Source: Bach Knudsen, 2014.

	Soybean		Rapeseed	
Item[1]	Meal	Hulls	Meal	Hulls
NSP				
Cellulose	5.9	32.2	5.2	10.8
NCP	15.1 (6.2)[2]	32.2 (12.6)	16.8 (5.5)	24.5 (7.3)
Rhamnose	0.2 (0.1)	0.6 (0.3)	0.3 (0.1)	0.8
Arabinose	2.6 (0.9)	4.4 (1.3)	4.3 (1.2)	7.1
Xylose	1.7 (0.2)	8.0 (0.3)	1.7 (0.4)	1.8
Mannose	1.3 (0.4)	5.0 (2.1)	0.5 (0.1)	0.7
Galactose	4.2 (1.7)	2.5 (1.3)	1.8 (0.6)	2.7
Glucose	0.6 (0.5)	1.0 (0.7)	2.1 (0.9)	1.5
Uronic acids	4.5 (2.4)	10.7 (6.6)	6.1 (2.2)	9.5
Total NSP	21.0	64.5	22.0	35.3
Klason lignin	1.8	2.1	13.3	26.2
Dietary fiber	22.8	66.6	35.4	61.5
Soluble NSP, %	27.2	18.9	15.5	11.9

[1] NSP, nonstarch polysaccharides; NCP, noncellulosic polysaccharides.
[2] Values in parenthesis are soluble components.

Rapeseed meal has a moderate ADF content and a relatively low level of NDF, therefore a relatively low NDF to ADF ratio (Table 3), and is rich in NSP (Table 4). However, the fermentability rate of the NSP from RSM is lower than those from SBM (58 vs. 84%, Pustjens, 2013). In addition, compared to SBM, RSM contains higher levels of lignin due to the high degree of lignification of the RS hulls (Table 4). Lignin is highly resistant to degradation and can reduce the digestive processes considerably (Wenk, 2001). The complex, rigid, and lignified fiber matrix in RSM also results in binding and entrapment of nutrients (Pustjens *et al.*, 2013). Altogether, these factors indicate that the fiber fraction in RSM is more insoluble, indigestible, and difficult to degrade/ferment than the fiber in SBM. Such type of fiber can

cause specific negative effects on the digestive and absorption processes in the animals (Wenk, 2001). Replacement of SBM with up to 25% of RS co-products reduced nutrient and energy digestibility in pigs and this effect was attributed to the higher fiber content (Landero *et al.*, 2011; 2012; Sanjayan *et al*., 2014). Previous research showed that the meal from "yellow" rapeseed (*B. campestris* yellow) has lower fiber content (9.7 vs. 17.0% ADF and 15.9 vs. 23.6% NDF) than the meal from "black-coated" rapeseed (*B. napus* black) (Mejicanos *et al.*, 2016). In addition, Hansen *et al*. (2017) demonstrated the possibility of obtaining RSM fractions with reduced fiber content through milling, sieving, and air-classification techniques. The authors attributed the reduction in fiber content to the removal of a large part of the hull fraction and showed an improved apparent total tract digestibility (**ATTD**) of crude protein (**CP**) and lysine in minks fed the "low-fiber fraction" of RSM compared to minks fed the parent RSM. This is in agreement with an older experiment where apparent ileal digestibility (**AID**) of AA was higher in pigs fed de-hulled RSM compared to pigs fed the parent RSM (Grala *et al*., 1998). However, such processing methods are not yet economically and practically viable and, therefore, not adopted at a commercial scale. Further breeding and processing efforts are needed to reduce fiber content and increase fiber degradability and nutrient accessibility in RSM. In this sense, recent work by Pustjens *et al*. (2014) reported that the remaining recalcitrant fibers detected in feces of pigs fed RSM diets had a great amount of alkali-labile bonds and suggested that alkaline pretreatment of RSM might improve fermentation of RS NSP in pigs. Alternatively, genetic selection of "indigenous-like" pigs with greater ability to digest recalcitrant and insoluble fiber could be another approach to achieve an increased and more efficient use of RSM in pig diets. This thesis represents a pilot step towards the second approach.

5.3.2. Glucosinolates

Glucosinolates are secondary metabolites characteristic to some plants of the order Brassicales, including the genus *Brassica*, where rapeseed belongs (Khajali and Slominski, 2012). Glucosinolates constitute a biochemical defense mechanism for the plants against herbivory (War *et al*., 2012). Upon tissue damage, glucosinolates are hydrolyzed by endogenous enzymes (myrosinases) into thiocyanates, isothiocyantes, nitriles, and other breakdown products, which represent the "defense-active" components (Ahuja *et al.*, 2010). For simplification, the chemistry of these compounds is not discussed, but it is well described by Fahey *et al*. (2001), where many other relevant references are given. Among the wide

variety of identified glucosinolates (over 120) only 30 have been detected in RS, with three of them present in considerably higher concentrations: progoitrin, gluconapin, and glucobrassicanapin (Fahey *et al*., 2001). Concentrations and composition of glucosinolates in RSM vary among different seed varieties and origin (Tripathi and Mishra, 2007). As mentioned in the previous section, in recognition of adverse effects of glucosinolates on humans and farm animals, RS varieties with low levels of glucosinolates (< 30 μmol/g vs. ≈130 μmol/g in traditional varieties) were developed through plant breeding (Newkirk, 2009). The toxicity of glucosinolates for animals seems to be associated with their breakdown products rather than with the intact glucosinolates (Bell, 1993). Similarly to what happens during tissue damage from herbivory, hydrolysis of glucosinolates into its breakdown products during RSM processing and ingestion/digestion (by myrosinase activity, heat, low pH, GIT properties, digesta transit time, microbial-myrosinase activity, etc.) can cause harmful effects on the animals and this is why glucosinolates are considered ANF. Bell (1993), Campbell and Schöne (1998), and EFSA (2008), among others, refer to the deleterious effects associated with the breakdown products from glucosinolates. These include: reduced diet palatability and subsequent impairment of feed intake (due to their bitter taste), impaired liver and kidney function, inflammation and local necrosis in the mucosa of the GIT, interference with iodine uptake and synthesis of thyroid hormones (T_3 and T_4), eventually leading to hypothyroidism and enlargement of thyroid gland. Effects on thyroid function affect the metabolism of almost all tissues, including the reproductive organs, which can lead to reduced fertility. Subsequently, animal growth and performance can be compromised by glucosinolates in the diet.

Harmful effects of glucosinolates are greater for monogastric animals (especially pigs) than for ruminants, and young animals are more sensitive to these compounds than adult animals (Tripathi and Mishra, 2007). The high sensitivity of younger animals may explain the reduced feed intake and growth observed in weaned pigs fed increasing levels of a RSM containing relatively low levels of glucosinolates (10.8 μmol/g, Landero *et al*., 2011). Contradictory results regarding effects of glucosinolates from RSM on pig organ weights and growth performance are found in the literature. While enlargement of thyroid, liver, and/or kidneys has been observed by some authors (Fandrejewski *et al*., 1994; Choi *et al*., 2015), others have reported decreased kidney (Parr *et al*., 2015) or unaffected liver and thyroid weights (Busato *et al*., 1991). Similarly, Mejicanos (2015) reported reduced FE in weaned pigs fed de-hulled RSM from *B. Juncea* and attributed it to higher amounts of gluconapin (10.1 μmol/g), while

the author observed improved FE when pigs were fed with RSM from *B. napus* black (2.1 μmol/g) compared to pigs fed *B. juncea* RSM or a SBM-control diet. Possible explanations for contradictory results are confounding effects from different criteria for diet formulation, previously based on CP and DE and not standardized ileal digestible (**SID**) AA or NE, and variation in animal age and RSM composition due to seed variety (recent vs. old cultivars) and origin (Mejicanos *et al*., 2016). Feeding diets containing 10 vs. 2 μmol glucosinolates/g to sows during late gestation and lactation reduced litter weight and induced hypothyroidism in the piglets (Schöne *et al*., 1997; Schöne *et al*., 2001). Feeding diets with less than 1 μmol glucosinolates/g did not have adverse effects in pigs, while levels above 1.34 μmol glucosinolates/g reduced feed intake and growth (Bowland, 1975). Iodine deficiency, increased T_3 and T_4 in serum, and thyroid hypertrophy in pigs was reported by Mawson (1994a,b) with levels between 9-10.1 μmol glucosinolates/g. Considering the previous results, Opalka *et al*. (2001) and Schöne *et al*. (2001) suggested a maximum total glucosinolate level below 2 μmol/g DM diet, provided iodine supplementation is above 1000 μg/kg. Therefore, glucosinolate concentration in RSM should be considered during feed formulation for pigs (Tripathi and Mishra, 2007). Processing methods of RSM such as heat treatment, water treatment, fermentation, microwaving, micronization, extrusion, etc., have been shown to reduce total glucosinolate content (reviewed by Tripathi and Mishra, 2007). Future research within processing and plant breeding could further reduce glucosinolate content in RSM and improve its nutritional value.

5.3.3. Other antinutritional factors

In addition to fiber and glucosinolates, there are other ANF that can affect the nutritional value of RSM, mainly sinapine, tannins, and phytic acid (Bell, 1993). Sinapine, the choline ester of sinapic acid, is the main phenolic ester in RS, occurring mainly in the hulls and therefore abundant in the meal (Nićiforović and Abramovič, 2014). Similar to glucosinolates, sinapine has a bitter taste that could contribute to the reduced palatability and feed intake when feeding RSM diets (Naczk *et al*., 1998). There is ongoing research to develop low-sinapine RS varieties with yellow hulls and lower fiber content (Mejicanos *et al.*, 2016). However, antioxidant and other health-beneficial properties have been attributed to sinapic acid and its derivatives, including sinapine, and therefore they have been suggested as a potential ingredient to be used in functional foods (Nićiforović and Abramovič, 2014). Tannins are polyphenolic compounds that also occur mainly in the hulls of RS, especially in

dark-colored hulls (Bell, 1993). Tannins have been reported to bind to proteins and digestive enzymes, especially proteases, resulting in reduced protein digestion (Khajali and Slominski, 2012). Addition of soluble tannins to broiler diets resulted in reduced growth (Leslie *et al*., 1976) and increased endogenous AA losses (Mansoori and Acamovic, 2007). However, over 70% of the tannins in RS are insoluble and might not greatly affect the nutritional value of RSM (Khajali and Slominski, 2012). Moreover, Biagi *et al*. (2010) showed that tannins had a positive effect on FE in weaned pigs, indicating that tannins may have beneficial effects. Together, the content of total phenolics in RSM is much higher than that found in meals from other oilseeds, being about 30 times higher than in SBM (Kozlowska *et al.*, 1990). Phytic acid is the main storage form of P in grains and seeds and it is considered an ANF because in addition to P, it binds to proteins and other minerals forming insoluble complexes and therefore reducing their bioavailability (Khajali and Slominski, 2012). In their review, Woyengo and Nyachoti (2013) report that dietary phytic acid reduces animal performance through decreased digestibility (due to binding with nutrients, digestive enzymes, or both) and increased endogenous losses, including AA and minerals (due to increased secretion of digestive juices).

5.4. Feed efficiency

Enhanced use of local protein resources to increase the sustainability and self-sufficiency of European pig production could be achieved: i) by improving the nutritional value of existing local protein sources through conventional and/or novel feed processing and plant breeding techniques, and/or ii) by improving the FE of the animals when they are fed diets based on such local feedstuffs.

Improvements in FE when feeding local high-fiber diets are crucial for a more efficient, sustainable, and environmentally friendly pork production, as feed costs constitute the largest variable expense for pig producers (Seneviratne *et al*., 2011) and selection for feed-efficient animals may reduce nitrogen (**N**) and P emissions (Shirali *et al*., 2012; Saintilan *et al*., 2013). However, the biological basis for variation in FE is not completely understood. There are many factors affecting the FE of an animal, making it an extremely complex trait to study. In fact, finding a good definition of FE is a complicated task in itself. A traditional definition of FE used by scientists is BW gain per unit of feed or energy consumed, i.e., FE is commonly expressed based on a ratio of feed or energy intake and growth achieved (Patience *et al*.,

2015). Because the ultimate goal for pig producers is to maximize profit, there is also increasing interest in expressing FE in financial terms (feed cost/pig sold, feed cost/kg weight gain, etc.). Koch *et al*. (1963) realized about the importance of correcting for BW and BW gain and suggested to divide feed intake into two components: i) the expected feed intake for a given level of production (maintenance + growth); and ii) a residual portion independent from production needs. The residual portion, called residual feed intake (**RFI**), indicates how animals deviate from their expected feed intake, with animals having lower RFI being more efficient. As RFI is independent of production, it is a better-suited measure to compare individuals differing in BW and growth rate and it may reflect inherent variation in biological processes (Herd and Arthur, 2009). The latter authors suggested that variation in FE, as measured by RFI, might be explained by variation in five major processes, namely: i) feed intake; ii) digestion; iii) body composition and metabolism; iv) physical activity; and v) thermoregulation. The contribution of these physiological mechanisms to variation in RFI was determined on divergently selected cattle by Richardson and Herd (2004) (Figure 5).

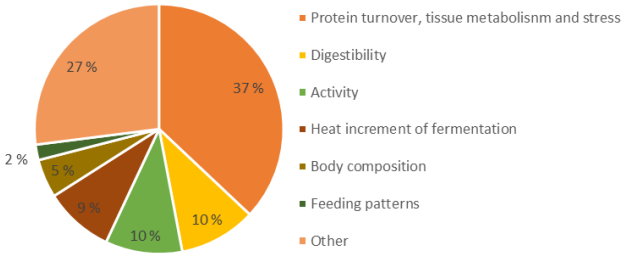

Figure 5. Contributions of biological mechanisms to variation in residual feed intake determined on divergently selected cattle. Adapted from Herd and Arthur (2009).

Residual feed intake seems to be a useful selection criterion for improving FE in pigs, as improved FE has been demonstrated in two independent sets of experimental lines divergently selected for low and high RFI (one in INRA: Gilbert *et al*., 2007, and one in Iowa State University, ISU: Cai *et al.*, 2008). Gilbert *et al*. (2017) recently reviewed the results from the RFI divergent selection carried out at INRA over 10 generations. Similar to Herd and Arthur (2009), the authors suggest that the better FE of the low RFI line could result from the improvement of various biological functions that require energy and nutrients, namely i) improved digestion; ii) better intermediary metabolism and iii) reduced maintenance and activity requirements. A summary of the effects of INRA's selection for low RFI on different

biological functions is provided in Figure 6. The work of this thesis focuses on energy and nutrient digestion and metabolism and, therefore, only these two factors will be further discussed.

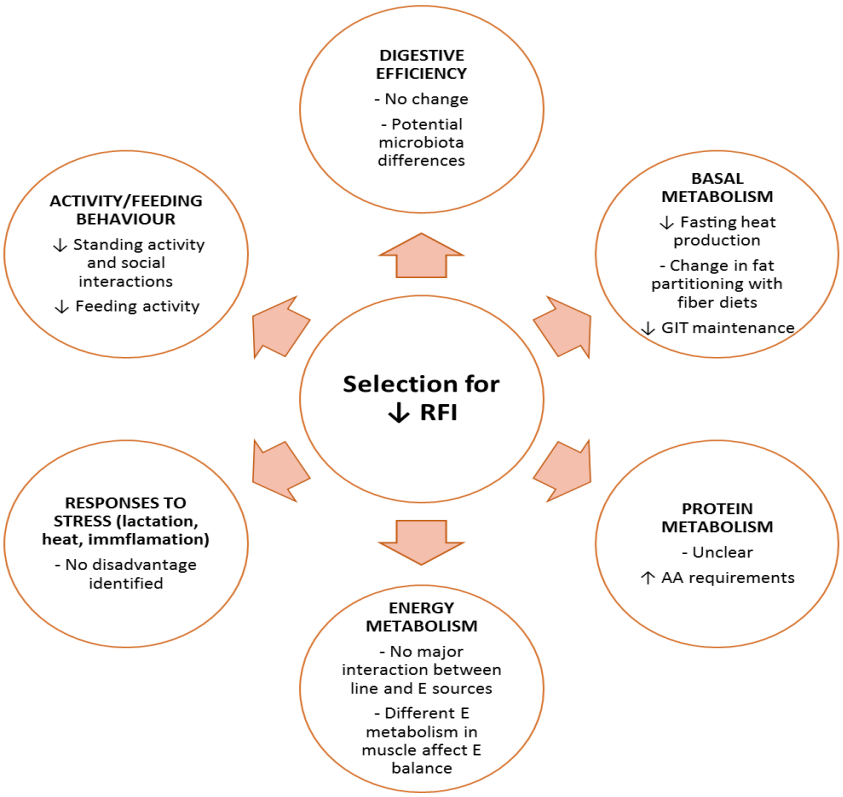

Figure 6. Effects of reducing residual feed intake (RFI) on major biological functions in growing pigs. GIT = gastrointestinal tract, AA = amino acid, E = energy. Adapted from Gilbert *et al*., 2017.

5.4.1. Factors contributing to feed efficiency

5.4.1.1. Digestibility

Improved FE of pigs selected for a low RFI has been mainly established on standard commercial diets (Gilbert *et al.*, 2007; Cai *et al.*, 2008). However, Montagne *et al*. (2014) reported negative effects on FE when high RFI pigs (less efficient) were changed to a high-fiber, low-energy diet, while such effects were not observed for the low RFI pigs (more efficient). Gondret *et al*. (2014) suggested that high FE pigs might have a greater ability to

cope with fibrous diets, which would be important to consider if SBM was to be partially or totally replaced by RSM. According to Herd and Arthur (2009), nutrient digestibility is one of the main factors contributing to differences in RFI. These authors indicated that nutrient digestibility may account for 10% of the variation associated with RFI in cattle. On the other hand, digestibility has been reported to play a minor role in explaining differences in RFI in monogastrics (chickens: Luiting *et al*., 1994; mice: Bunger *et al*., 1998; pigs: Gilbert *et al*., 2017). However, contradictory results are found in the literature both in pigs fed standard diets and in pigs fed high-fiber diets. When feeding standard diets, Barea *et al*. (2010), Montagne *et al*. (2014), and Labussière *et al*. (2015) found that digestive efficiency did not differ between lines divergently selected for RFI at INRA, while other studies (Harris *et al*., 2012; Vigors *et al*., 2016) found greater digestibility values for dry matter (**DM**), N, and gross energy (**GE**) in low RFI pigs. Similarly, a low RFI line had higher capacity to digest a high-fiber diet compared to a high RFI line (Mauch *et al*., 2015), while no effect of selection for RFI on nutrient and energy digestibility was observed in pigs fed a fibrous diet by Montagne *et al*. (2014). In the last study, digestibility decreased similarly in both lines fed the high-fiber diet. High DF can reduce energy and nutrient digestibility in pigs. Therefore, selection for FE (or RFI) under high-fiber dietary conditions could result in pigs with improved digestive efficiency. Indeed, research has shown that there is genetic variability for digestive capacity within a line or breed of growing pigs fed a high-fiber diet (Noblet *et al*., 2013), indicating that there is potential to develop pigs that are more robust and have better ability to cope with local fibrous diets. Improved digestibility of fibrous alternative protein sources, such as RSM, could contribute to a more efficient and sustainable pig production.

5.4.1.2. Body composition and metabolism

Following digestion and absorption, efficiency of nutrient metabolism in different tissues may contribute to variation in overall FE. Animals require nutrients to support both *maintenance* functions and *tissue deposition*. *Maintenance* accounts for about one-third of the energy intake in pigs (Patience, 2012) and minimizing maintenance energy requirements will result in increased proportion of energy available for growth. Basal metabolic rate represents an important fraction of the energy required for maintenance and is often estimated by partitioning total heat production (**HE**) into its different components and determining fasting HE. Fasting HE is lower in low RFI pigs than in high RFI intake pigs (Barea *et al*., 2010; Boddicker *et al*., 2011), indicating that the low RFI pigs have lower maintenance requirements

and therefore can allocate more energy into other functions, such as growth. Gilbert *et al*. (2017) suggested that differences in mitochondria abundance and activity in tissues and differences in size of visceral organs could contribute to the difference in basal metabolic rate between low and high RFI pigs. Stress and immune responses are another component of maintenance requirement and may increase it, as they pose an energy demand on the animals. These functions can influence metabolic priorities through re-allocation of nutrients from growth towards mounting a proper defense response (Patience *et al.*, 2015). There is concern about a potential impact from selecting for low RFI on the ability to re-allocate resources for defense responses. However, in their review, Gilbert *et al*. (2017) concluded that selection for low RFI pigs did not compromise their ability to face environmental challenges (including inflammatory, heat stress, and lactation challenges), which disagrees with the resource allocation theory. Regarding *tissue deposition*, lean deposition has a lower energy cost than fat deposition, but efficiency of lean deposition is more variable than that of fat because of the greater variation in protein turnover than in fat (Herd and Arthur, 2009). In addition, protein turnover (anabolism vs. catabolism) is more variable between organs than fat turnover. Therefore, variation in nutrient partitioning towards lean vs. fat deposition, and differences in organ growth, and in protein and energy metabolism may influence FE. In this respect, Gilbert *et al*. (2007) reported moderately positive (r = 0.44) and negative (r = -0.55) genetic correlations between RFI and carcass back fat thickness and lean mean content, respectively. From the literature, the contribution of changes in protein metabolism to differences in FE is not clear. Similar N utilization and protein deposition was observed between low and high RFI lines from the 6th (Barea *et al*., 2010) and 7th (during post-weaning, Labussière *et al*., 2015) generations, while these parameters were lower in low RFI pigs during the growing period (Renaudeau *et al*., 2013). On the other hand, Harris *et al.* (2012) reported improved N balance in low RFI pigs. Similarly, no line difference in rate of protein synthesis and expression of protein synthesis markers were found in loin muscle of either the INRA (Le Naou *et al.*, 2012) or the ISU (Cruzen *et al.*, 2013) RFI divergent lines, while Vincent *et al*. (2015) observed over-expression of genes related to protein synthesis. Contradictory results regarding differences in protein catabolism between RFI lines are also found in the literature. Concerning energy metabolism, lower glycolytic and oxidative enzyme activities have been observed in skeletal muscles of low vs. high RFI pigs (Le Naou *et al.*, 2012; Faure *et al*., 2013), indicating reduced nutrient catabolism for energy production in the muscle of these pigs. Down-regulation of genes related to mitochondrial metabolism, including some antioxidant proteins, was observed in the loin muscle of low RFI pigs by Vincent *et al*. (2015).

In the same line, Grubbs *et al*. (2013) observed a reduction in electron leakage and production of reactive oxygen species in mitochondria from muscle and liver of low RFI pigs. The observations from the latter studies indicate that improved FE in low RFI pigs could be partially explained by reduced oxidative stress.

Further investigations into the biological mechanisms underlying differences in the various factors contributing to FE and their link to genetic information are definitely needed. In addition, impacts of potential dietary changes in future pig production, such as use of alternative protein sources, on these biological functions also need to be investigated.

6. Methodology

6.1. Methods for determination of nutrient and energy digestibility

6.1.1. Total collection vs. marker technique

Digestibility represents an important part of the present thesis. Different *in vivo* methods were used to measure dietary nutrient and energy digestibility in Experiments 1 and 2. In Experiment 1, digestibility was measured indirectly by the marker technique, while in Experiment 2 digestibility was measured directly by the total collection technique. Therefore, a short explanation and comparison of the two methods will follow.

The total collection technique is the most conventional and reliable way to measure nutrient digestibility. In short, it consists on keeping accurate records of feed intake and feed refusals of the animals. Animals are kept in individual metabolic crates (Figure 7) so that total collection of feces can be performed. This way, accurate records of fecal output can also be made. Nitrogen balance was also estimated in Experiment 2, therefore, urine output was also measured and pooled urine sub-samples were homogenized and analyzed for N.

Figure 7. Metabolic crates used for total collection of urine and feces in Experiment 2.

Then, digestibility of a given nutrient can be calculated as:

$$\text{Nutrient digestibility (\%)} = \frac{\text{Nutrient intake} - \text{Nutrient in feces}}{\text{Nutrient intake}} \times 100$$

Although reliable, the total collection technique is time consuming, laborious, and costly. Other concerns with this method are low feed intake, losses during collection and consequent overestimation of digestibility values, and contamination of feces with urine, hair, and skin.

The marker technique does not need total collection of ileal digesta or feces, or measure of feed intake. Therefore, this method reduces time, labor, and expense invested in digestibility studies. In this so called indicator method (Kotb and Luckey, 1972), an indigestible marker is included in the diet to be tested and representative samples of diets and ileal digesta or feces are taken for chemical analysis of the nutrients of interest and the indigestible reference marker. In Experiment 1, cumulative samples of diets and feces were taken over seven days, while only one sample of ileal digesta was taken at slaughter. It is acknowledged that the ileal digesta samples are not as representative. The digestibility of a given nutrient is calculated by using the change in the ratio of each nutrient in relation to the marker in the diet and in the ileal digesta or feces:

$$\text{Nutrient digestibility (\%)} = 100 - 100 \times \frac{\text{\% Marker in diet} \times \text{\% Nutrient in ileal digesta or feces}}{\text{\% Marker in ileal digesta or feces} \times \text{\% Nutrient in diet}}$$

The markers used for this technique can be internal (a natural component of the feed), or external (added to the feed). Common markers used in animal nutrition include chromic oxide, titanium oxide, ferric oxide, yttrium oxide, lignin, acid-insoluble ash, etc. (Marais, 2000). In Experiment 1, yttrium oxide was used as a marker because of extensive experience and well established detection methods at our laboratory (Austreng *et al.*, 2000; Vhile *et al*., 2007).

6.1.2. Ileal vs. Total tract digestibility

Information presented in this and the following section is limited and largely based on data from Darragh and Hodgkinson (2000), Sauer *et al*. (2000), and Stein *et al.* (2007). For a deeper understanding on these topics, the reader is referred to the cited literature. In Experiment 1, both apparent ileal and total tract nutrient digestibility were estimated. Ileal rather than total tract digestibility is preferred for estimating digestibility of certain nutrients, especially protein and AA. Undigested dietary or endogenous protein can undergo different events when entering the hindgut: i) a proportion of the protein will pass through the hindgut and be excreted in the feces; ii) the rest of the protein will be fermented by the gut microbiota and N will be absorbed (mainly as ammonia) or incorporated as microbial protein, which will be mainly excreted in feces. As a result of the microbial protein fermentation, total tract protein and AA digestibility will most likely be overestimated. Net synthesis of AA in the hindgut can also occur, which could result in underestimation of protein digestibility. Therefore, ileal

digestibility is thought to give a more accurate indication of protein availability for the pig, as it avoids the problems associated with microbial protein fermentation or synthesis in the hindgut.

6.1.3. Apparent vs. True vs. Standardized ileal digestibility

Ileal digestibility of nutrients, protein and AA will be used as an example, can be expressed as AID, TID, or SID, depending on how accurate one wants or needs to be. Accuracy will depend on which proportion of the ileal protein or AA outflow is included in the digestibility calculation. Ileal digesta contains undigested and unabsorbed dietary protein and AA as well as protein and AA of endogenous origin (i.e., endogenously synthesized proteins, and mucin and digestive enzymes secreted into the GIT). Stein *et al*. (2007) refer to the latter as ileal endogenous AA losses (**IAA_{end}**) and divide them into basal IAA_{end} and specific IAA_{end}. Basal IAA_{end} represent the losses intrinsic to the animal, related to DM intake but independent of the diet composition. Specific IAA_{end} represent the extra losses induced by inherent diet/feed ingredient characteristics, such as protein, fiber, and ANF content. Having this in mind, the AID of protein or a given AA is calculated as follows:

$$\text{AID (\%)} = \frac{\text{AA intake} - \text{ileal AA outflow}}{\text{AA intake}} \times 100$$

Measuring ileal digestibility without accounting for IAA_{end} leads to underestimation of the true digestibility and lack of additivity of AID values for individual feed ingredients in diets containing various protein sources, particularly ingredients with low protein content (Stein *et al*., 2005). The lack of additivity has been attributed to the effect of dietary AA level on i) the relative contribution of endogenous AA to total ileal AA flow, which decreases with increasing dietary AA; and ii) the AID values, which will increase in a non-linear fashion with increasing dietary AA level due to the lower relative contribution of endogenous AA to total ileal AA flow. Therefore, accurate determination of IAA_{end} is needed in order to measure ileal digestibility of AA accurately for diet formulation.

Ileal digestibility adjusted for total IAA_{end} is referred to as TID, and can be calculated as:

$$\text{TID (\%)} = \frac{\text{AA intake} - (\text{ileal AA outflow} - \text{total IAAend})}{\text{AA intake}} \times 100$$

However, it is difficult to measure total IAA_{end} accurately and they do not consider the different specific AA losses induced by different feed ingredients. Therefore, the TID AA requirements of the pigs will vary when using feed ingredients that cause different specific $IAA_{end.}$ The scarce information about TID and total IAA_{end} values for the variety of ingredients used in pig diets is the major limitation for using TID in diet formulation.

When only the basal IAA_{end} are corrected for in the digestibility calculation, the values are referred to as SID values:

$$\text{SID (\%)} = \frac{\text{AA intake} - (\text{ileal AA outflow} - \text{basal IAAend})}{\text{AA intake}} \times 100$$

Because only basal IAA_{end} are included in the calculation, SID values are independent of AA dietary level. In addition, SID values differentiate between feeding ingredients that produce different levels of specific IAA_{end}, which is particularly important when using ingredients that contain fiber and ANF, as is the case for RS co-products. Because determining basal IAA_{end} is not as difficult and the AA SID values for common pig feed ingredients are additive in mixed diets (Stein *et al.*, 2005), SID is considered the preferred measure of digestible AA requirement and content in pig diets. Therefore, the diets used in both Experiment 1 and 2 were formulated using table values for the SID of the included feed ingredients.

Apparent ileal and total tract digestibility values were used both in Experiment 1 and 2. Therefore, it is appropriate to acknowledge the issues associated with this way of expressing digestibility, especially because RS co-products were used, which contain fiber and ANF.

6.2. Quantitative determination of nutrient and energy metabolism

The energy available for an animal to use for maintenance and production comes from the chemical energy contained in the nutrients in the diet (carbohydrates, protein, and fat). However, the total amount of GE supplied by the diet is not available for the animal. Available energy in diets for pigs has been characterized as DE, metabolizable energy (**ME**), and NE by considering sequential energy losses during digestion and metabolism from GE in the diet. Digestible energy only accounts for the energy lost in feces, ME also accounts for the energy lost in urine and methane, and in addition to these losses, NE accounts for energy lost as heat during ingestion and metabolism of nutrients. Oxidation of dietary nutrients yields free energy, which can be used to do work (e.g., retention of nutrients and energy), and heat

energy, which cannot do work and is therefore a measure of energetic inefficiency. Oxidation of either carbohydrates, protein, or fat yields different amounts of free energy and heat energy. Namely, the heat loss depends on the type of substrate that is oxidized. Thus, the metabolic processes being responsible for the energy supply to the animal can be determined by measuring HE. Heat production can be measured using different calorimetric methods (Figure 7), either directly in an animal calorimeter, or indirectly by measuring gas exchange associated with the oxidation of energy substrates (carbohydrates, fat, and protein) and calculating HE from the stoichiometry of substrate oxidation.

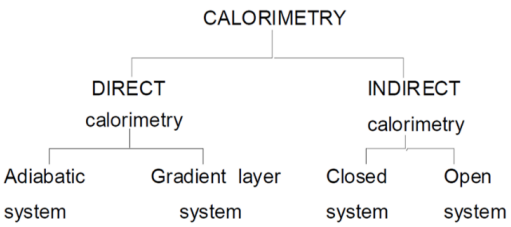

Figure 7. Different calorimetry methods

In Experiment 2, HE was determined by means of indirect calorimetry in an open-air circulation unit and therefore, the method used for this work will be further discussed.

6.2.1. Indirect calorimetry

Total HE can be determined from measurements of gas exchange (O_2 consumed, CO_2 and CH_4 produced) combined with either measurements of N excreted in urine (respiratory quotient, RQ-method, used in Experiment 2) or with measurements of carbon and N balances (CN-method). Gas exchange measurements can be performed in respiration chambers, which can be either a closed or an open-air circuit system (used in Experiment 2). A simple diagram and a picture of the open-air circuit respiration chambers used in Experiment 2 are illustrated in Figure 8 A and B, respectively. Following the diagram, fresh atmospheric air for the test animal is drawn from outside through an air conditioning system to control temperature, humidity, and mixing of the air in the chamber. The inlet air is thoroughly mixed with the air expired by the animal and pumped out of the chamber through a flow meter and different gas sensors. Thereby, total volume of the mixed air is measured continuously and its O_2, CO_2, and CH_4 concentrations are determined in aliquote samples by gas analyzers. Oxygen

consumed, and CO_2 and CH_4 produced by the animal can then be calculated based on volume and composition of the inlet air compared with the outlet air.

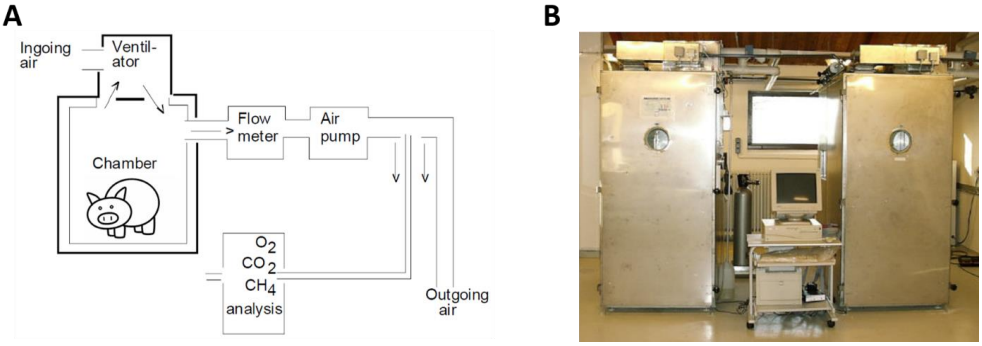

Figure 8. Diagram (A) and picture (B) of the open-air circuit respiration chambers used in Experiment 2.

In Experiment 2, the RQ-method was used to estimate HE. The RQ is the ratio between CO_2 produced and O_2 consumed. Different amounts of O_2 and CO_2 are inhaled and exhaled, respectively, when a certain amount of a specific nutrient, i.e., carbohydrate, fat, or protein, is metabolized. Therefore, it is possible to estimate the relative amounts of the different nutrients being oxidized by measuring O_2 consumption and CO_2 production. If urinary N is also measured, oxidation of protein can be estimated. Once RQ and oxidation of protein are known, oxidation of lipids and carbohydrates can also be estimated. In Experiment 2, total HE and oxidized nutrients were estimated based on gas exchange data and the mean urinary N according to the equations from Brouwer (1965) and Chwalibog *et al*. (1992) (see materials and methods in Paper III), which are based on stoichiometric calculations and constants.

7. Aims of the thesis

The overall aim of the thesis was to investigate the impacts of replacing SBM with RS co-products in diets for pigs on factors that could contribute to their FE. In particular, we aimed at:

1. Evaluating the effects of partially replacing SBM with high-fiber RS co-products on nutrient and energy digestibility of young pigs (Experiment 1, Paper I).

2. Identifying underlying biological mechanisms explaining differences in digestibility under these dietary conditions (Experiment 1, Paper I).

3. Assessing the metabolic effects of this dietary change through metabolomics analysis (Experiment 1, Paper II).

4. Evaluating the effects of partially replacing SBM with increasing levels of RSM on nutrient and energy digestibility of young pigs (Experiment 2, Paper III).

5. Evaluating the effects of partially replacing SBM with increasing levels of RSM on N balance, energy metabolism, and substrate oxidation of young pigs (Experiment 2, Paper III).

It was hypothesized that RS co-products could partially replace SBM in diets for young pigs, but that ANFs in RS could affect the efficiency of the pigs to digest and further utilize dietary nutrients and energy due to changes in digestive enzyme activity, intestinal absorptive surface, energy expenditure, and metabolic pathways responses.

8. Main results and general discussion

8.1. Diets

The coarse RS fraction included in the RSF diet in Experiment 1 was obtained by air classification of a commercial *hexane-extracted* RSM. This type of RSM was chosen as a parent meal because of its lower fat content compared to cold or expeller-pressed RSM, as high-fat material may affect the air-classification process due to agglomeration and stickiness of particles (Hansen *et al*., 2017). On the other hand, the RSM used in Experiment 2 was *expeller-pressed* because this is the most commonly used in animal feed in Scandinavia and we wanted to resemble commercial conditions. Experiment 1 was a pilot study conducted to identify biological markers associated with differences in digestibility and metabolism when feeding RS vs. SBM diets to pigs. The idea is to use these markers in future growth performance experiments with larger number of pigs, where FE will be accurately measured and the pigs genotyped, so that the performance and phenotypic data can be linked to genetic information. As described in section 5.3.1 of the general introduction, fiber is one of the main challenges when using RSM as an alternative to SBM in pig diets. Therefore, high-fiber RS co-products (coarse RS fraction + RS hulls) were used in this experiment, instead of using the parent RSM or the fine RS fraction, to increase the contrast in RS fiber content between the control and the test diet in order to induce clear and detectable effects on the digestibility and biological parameters. The ingredient composition and chemical contents of the diets used in Experiment 1 and 2 are presented in Tables 1 and 2 of Papers I and III, respectively. As expected, partial replacement of SBM and wheat with high-fiber RS co-products resulted in increased contents of ADF (4.2 vs. 8.2%) and NDF (13.3 vs. 15.5%). Similarly, partially replacing SBM and wheat with increasing levels of RSM in Experiment 2 increased the contents of NDF, DF, total NSP, cellulose, and lignin. Regarding content of monomeric sugars, inclusion of RS co-products resulted in higher contents of rhamnose and arabinose, and lower contents of fucose, galactose, xylose, mannose, and uronic acids, which is consistent with the higher and lower presence of these sugars in RSM and SBM, respectively (Bach Knudsen, 2014; see Table 4). Inclusion of RS co-products decreased the starch content and increased the concentration of fat in the test diets in both experiments. Starch content is very low both in RSM and in SBM, however, wheat was replaced along with SBM in both experiments to achieve balanced diets, which explains the reduced starch content in the RS diets. The higher fat content in the RS diets is likely due to the higher fat content in the RS

co-products than in SBM (2.5 and 8.0% in the coarse RS fraction and in the RS hulls, respectively vs. 1.4% in SBM in Experiment 1; and 11.1% in RSM vs. 1.0% in SBM in Experiment 2). Total glucosinolate content was analyzed in the diets in Experiment 1, while it was only analyzed in the RSM in Experiment 2. Inclusion of RS co-products resulted in the presence of 1 mmol glucosinolate/kg in the RSF diet, while glucosinolates where not detected in the control diet. The RSM used in Experiment 2 contained 11.3 mmol glucosinolate/kg, therefore the glucosinolate content in the test diets was estimated from the RSM composition and inclusion level, and assumed to increase proportionally as the level of RSM inclusion increased (1.13, 2.26, and 3.39 mmol/kg in diets RSM10, RSM20, and RSM30, respectively). There were no major differences in GE, CP, and AA concentrations between the control and the RS diets in any of the experiments.

8.2. Health status and growth

Overall, the pigs appeared healthy throughout both of the experiments, except for a mild to moderate diarrhea incidence in some of the pigs both in Experiment 1 and 2, and a pig showing unthriftiness in Experiment 2. Following veterinary recommendations, the pigs affected with diarrhea received a probiotic treatment (Zoolac Propaste; VESO AS) and the pig with unthriftiness got antibiotic treatment (Tribissen Vet. 48%). In general, and despite the higher fiber and glucosinolate contents in the RS-diets, the pigs consumed the diets readily and grew normally. Daily feed intake and weight gain were similar across dietary treatments in both experiments, which was somehow expected because of the restrictive/semi-*ad libitum* feeding regime and the short duration of the experiments, none of them being a performance experiment. Therefore, results about the effects of dietary treatment on performance and growth are not conclusive but rather indicative of the health status of the pigs. Apparent ileal digestibility data from two control pigs in Experiment 1 was omitted because of little presence and liquid ileal digesta. Similarly, two pigs belonging to the RSM10 group in Experiment 2 were excluded from the study because of low feed intake or technical problems during the balance period. Feeding RSF resulted in increased thyroid to BW ratio (0.09 vs. 0.07, P = 0.004) but did not affect the liver to BW ratio of the pigs in Experiment 1 significantly (P = 0.17), although livers of the RSF pigs were numerically heavier. Differences in organ size in Experiment 1 were not supported by histopathological changes related to tissue damage of the thyroid glands nor the livers. Increasing levels of RSM increased (linear P = 0.033) the liver index in Experiment 2 (Paper III, Figure 1). Because of time restriction at sampling in

Experiment 2, thyroid glands could not be dissected out and weighed and, therefore, this data is not available. The dietary effect on organ weight in both experiments was attributed to the presence of glucosinolates in the RS diets (see section 5.3.2. of the general introduction). Because of their ANF effects, a maximum limit of 2.1 mmol total glucosinolate content/kg feed was recommended (EFSA, 2008). Although the total glucosinolate content in the RSF (1.0 mmol/kg) and the RSM10 (1.1 mmol/kg) diets were below the recommended levels, the RSF increased the thyroid weight after only 3 weeks of exposure, which supports the high sensitivity reported for young pigs towards these components (Mejicanos *et al*., 2016). The glucosinolate content in diets RSM20 and RSM30 (2.3 and 3.4 mmol/kg, respectively) was estimated to be above the recommendations and, therefore, the increased liver index observed was not surprising. In agreement with Choi *et al*. (2015), our results indicate that the effect of RSM feeding on liver weight is dose-dependent. However, long-term feeding experiments should be conducted to evaluate the relationship between the effects of RS feeding on thyroid and liver weight and function and the growth performance of the pigs. Anyhow, effects on the size and function of these highly active metabolic organs may have important consequences on the overall metabolism of the pigs and should be considered when using RS in pig diets. Indeed, metabolomics analyses of digesta, liver, and serum samples from the pigs in Experiment 1 demonstrated that the RSF diet elicited several metabolic effects on the pigs, which will be discussed in the next sub-sections. Although blood samples from all pigs in Experiment 1 were analyzed, these results are not shown. However, no differences in standard hematology and most of the clinical chemistry variables were observed between the dietary treatments, except for plasma urea and creatinine levels, and their ratio, which were decreased ($P < 0.01$) by the RSF diet. This could indicate a potential effect of the RSF diet on protein metabolism, although the level of these metabolites were still within the normal range for this age pigs. A clarification is needed for the latter results, as a mistake was made when reporting plasma urea and creatinine levels, which were reduced by the RSF diet, and not increased as reported in Paper I.

8.3. Nutrient and energy digestibility

The digestibility results are presented in Tables 3 and 4 (Paper I) for Experiment 1 and in Table 3 (Paper III) for Experiment 2. Partially replacing wheat and SBM with high-fiber RS co-products reduced ($P < 0.01$) the AID and ATTD of DM, organic matter (**OM**), CP, NDF, ADF, and GE in Experiment 1. Reduced AID of all individual AA (except for methionine)

and ATTD of most monomeric sugars (except for arabinose and rhamnose, which were increased; $P < 0.001$) were also observed in pigs fed the RSF diet. The AID of starch and the ATTD of fat were similar among pigs fed both diets. Similar to Experiment 1, partial replacement of wheat and SBM with increasing levels of RSM in Experiment 2 reduced ($P < 0.01$, linear $P < 0.001$) the ATTD of OM, CP, total carbohydrates, and DF (linear $P < 0.034$), as well as the ATTD of fucose, mannose, glucose, and uronic acids. In contrast, ATTD of fat and arabinose were higher (linear $P < 0.05$) in pigs fed the RSM diets. The similar starch digestibility observed in Experiment 1 could be explained by the main starch source being cereal ingredients and very little coming from SBM or RS co-products. The greater ATTD of arabinose and rhamnose (Experiments 1 and 2), and of fat (Experiment 2) in pigs fed the RS diets is probably due to the higher concentrations of these components in the RS diets. The general reduction in digestibility by the RS diets observed in both experiments is most likely due to the higher fiber content in these diets. High DF has been extensively reported to impair nutrient and energy digestibility in pigs, both at the ileum and total tract level (Jørgensen *et al*., 2007; Wilfart *et al*., 2007a; Len *et al*., 2009a,b). Furthermore, previous research dealing with replacement of SBM with increasing levels of up to 20% solvent-extracted (Landero *et al*., 2011) or expeller-pressed canola meal (Landero *et al*., 2012) also reported a reduction in ATTD of DM, CP, and GE. In agreement with the latter studies, increasing inclusion of RSM reduced the ATTD of energy and nutrients in a linear fashion. In addition to the higher fiber content, the fiber type may affect digestive and metabolic processes differently (Wenk, 2001). In this regard, the inclusion of high-fiber RS co-products nearly doubled the ADF content in the RSF diet (Paper I, Table 2). Although lignin in the diets was not analyzed in Paper I, it was assumed that the lignin content was higher in the RSF diet than in the control diet because of the inclusion of hull-rich RS co-products, which are highly lignified (Bach Knudsen, 2014; Table 4 in the general introduction). This was confirmed in Paper III, where a thorough dietary fiber analysis was performed, and a linear increase in lignin content with increasing inclusion of RSM was shown. Together, higher ADF and lignin contents indicate that the fiber fraction in the RS diets is more insoluble and indigestible, which is supported by the low ADF and NDF digestibility observed in pigs fed the RSF diet. The similar hindgut disappearance rate (measured as ATTD – AID) of ADF and NDF, reported in Paper I for pigs fed both diets is another indication of the low degradability of the fiber fraction in the RSF diet. Higher content and insolubility/indigestibility of the fiber in the RS diets may have reduced digestibility by increasing digesta bulk (Lindberg, 2014) and passage rate, and reducing the retention time of digesta in the small and large intestine (Wilfart *et al*., 2007b). The increased ($P < 0.011$, linear

$P < 0.001$) bulking of feces (g DM/day) observed with increasing inclusion of RSM in Experiment 2 supports this argument (Supplementary Table S1, Paper III). The combination of these three factors may have decreased the exposure time of digesta to enzymatic digestion and microbial fermentation in the small and large intestine, thus explaining the reduction of both AID and ATTD of nutrients, fiber, and energy in pigs fed the RS diets. The rigid lignin-cellulose matrix of the RS co-products (Pustjens *et al*., 2013) may also hinder substrate accessibility and activity of digestive enzymes and microbes, further contributing to the reduced digestibility. In fact, part of the reduction in digestibility by the RS diets may be explained by the low digestibility of the protein and AA in the RS hulls. Rapeseed hulls represent a large proportion of the meal (Carré *et al*., 2016), and they contain a considerable amount of protein (13.2 % CP in the RS hulls used in Experiment 1). However, the highly lignified and complex fiber matrix of the RS hulls may entrap these nutrients and prevent the action of digestive enzymes (Bach Knudsen, 2014), thus reducing their digestibility. Potential additional factors contributing to the reduced CP and AA digestibility by the RS diets are i) increased endogenous losses through physical abrasion of the intestinal mucosa by the insoluble fibers (Montagne *et al*., 2003), and ii) interference of proteolytic enzymes with other ANF in RS, such as tannins and phytic acid (Khajali and Slominski, 2012). The latter argument is supported by the reduced ($P < 0.030$) jejunal trypsin activity reported in pigs fed the RSF diet in Experiment 1 (Paper I, Table 5).

It could be expected that higher DF in the RS diets would lead to higher fermentation. In addition, the overall reduction in AID of nutrients by the RSF diet in Experiment 1 resulted in more nutrients reaching the hindgut and therefore more substrate available for microbial fermentation in these pigs. However, no significant differences were observed in the hindgut fermentation of nutrients between the two treatments (Paper I, Table 3), which indicates that there was no difference in the rate of microbial fermentation in the large intestine of the pigs. There are several possible explanations for the lack of effect of the RSF diet on hindgut fermentation rate: i) the already mentioned increased digesta bulk and passage rate in the large intestine reduced the time of exposure of digesta to microbes and therefore the extra substrate entering the large intestine could not be fermented; ii) the lower degradability of the fiber fraction in the RSF diet; iii) the complex and lignified matrix of the RSF diet may hinder overall nutrient fermentation by microbes; iv) the pigs used in the present experiment were young and therefore their GIT and fermentation capacity are not fully developed (Lindberg, 2014). Nevertheless, approximately 30% of the dietary NDF was fermented in the hindgut of

the pigs fed both diets, indicating a substantial microbial fermentation in the large intestine. It is worth noticing that the rate of decrease of nutrient and energy digestibility in the ileum was maintained in the total tract, while the fermentation capacity in the large intestine was not affected by the diet. This is supported by the metabolomics results from this experiment, which showed similar concentrations of short chain fatty acids (**SCFA**) in cecal and colonic digesta of pigs fed both diets (Paper II, Figure 4 A, B, and C). Similarly, the concentrations of secondary bile acids, which are also microbial metabolites, in the digesta did not differ between the dietary groups (Paper II, Figure 4 E and F). Moreover, increasing levels of RSM in Experiment 2 did not affect the ATTD of T-NSP nor the CH_4 production of the pigs (Paper III, Table 5); further supporting similar fermentation rates among pigs fed all diets.

On the other hand, the concentrations of SCFA in digesta from both dietary groups increased gradually from the duodenum towards the colon. Similarly, the concentrations of primary bile acids were higher in digesta from the small intestine (Paper II, Figure 4 D), while secondary bile acids were more abundant in digesta from the large intestine. The latter observations are consistent with the localization and metabolic function of the gut microbiota, which is more abundant and active in the large intestine compared to the small intestine. In addition, metabolomics analyses of digesta identified sinapine, sinapic acid, and gluconapin as robust exposure markers to the RSF diet because of their presence in the digesta of the pigs fed the RSF diet and their near-absence in the digesta of the control pigs (Paper II, Figure 2 B, C, D, and E). Vice versa, daidzein was identified as an exposure marker to the control diet because of its presence and absence in the digesta from the control and RSF pigs, respectively (Paper II, Figure 2 B and Supplementary Figure S1). These observations are rational considering that these specific phytochemicals are present and particularly abundant in RS (Mailer *et al*., 2008) and in soybean (Jung *et al*., 2000), respectively. Because of the continuous consistency of the data, the authors feel confident about the results from all the metabolomics analyses presented in Paper II. Metabolomics analyses of digesta samples were also consistent with the digestibility results published in Paper I. Overall, lower concentrations of most individual free AA were found in the duodenum (numerically lower) and jejunum ($P < 0.05$) of the RSF pigs, while these concentrations were generally higher ($P < 0.05$) in their ileum (Paper II, Figure 3). The concentration of total free AA was also lower ($P < 0.05$) in the jejunum of the pigs fed the RSF diet (Paper II, Figure 3 B). These observations are consistent with the lower jejunal trypsin activity and with the reduced AID of CP and AA reported for these pigs in Paper I. Despite the delay and reduction in protein

digestion by the RSF diet, no difference in the concentration of total free AA in the liver was observed between the dietary groups. Curiously, greater concentrations of γ-aminobutyric acid (**GABA**) were found in the digesta from all the GIT sections ($P < 0.05$) as well as in the liver ($P < 0.01$) of the pigs fed the RSF diet compared to the control pigs (Paper II, Figure 3 J and Supplementary table S4). GABA, a non-proteinogenic AA, is a main inhibitory neurotransmitter in the body and it is involved in many physiological processes, including gastrointestinal functions. There are GABA receptors expressed along the GIT and they are associated with the modulation of gut motility and secretory activities, among other functions (Auteri *et al*., 2015). Interestingly, sinapic acid, the most abundant phenolic component in RS, has been reported to potentiate GABA signals (Yoon *et al*., 2007), and it was also found in greater concentrations in the digesta of the RSF pigs. Whether the increase in GABA concentrations and its co-occurrence with sinapic acid in the intestinal lumen could contribute to the delayed and reduced digestibility by the RS diets warrants further investigation.

One of the objectives in Experiment 1 was to identify biological mechanisms underpinning differences in digestibility when feeding RS vs. SBM diets to pigs. Feeding the RSF diet did not affect any of the histomorphological parameters measured (Paper I, Figure 2). However, jejunal trypsin activity was lower in pigs fed the RSF compared to control, which coincides with the reduced AID of CP and AA observed in these pigs. Likewise, feeding the RSF diet did not affect amylase or maltase activities (Paper I, Table 5), which also coincides with the similar AID of starch between both dietary groups. Thus, our results indicate that the reduced digestibility by the RSF was not associated with changes in digestive and absorptive intestinal surfaces, but it correlated with digestive enzyme activities.

Apart from the differences in digestibility between the pigs fed the two diets, there was a considerable variation in AID of CP and ATTD of NDF among individual pigs within both dietary treatments in Experiment 1 (Paper I, Figure 1). Unfortunately, the experimental design did not allow us to test the heritability of this variation. However, within-population genetic variability of nutrient and energy digestibility has been reported by others in Large White pigs from four different sires fed a high-fiber diet (Noblet *et al*., 2013). Therefore, these authors suggested that the variation in digestive capacity may be heritable.

From the observations on AID and ATTD of energy and nutrients in both experiments (Papers I and III), and the observations on digesta metabolome, enzyme activity, and intestinal morphology in Experiment 1 (Papers I and II):

i) the digestive efficiency was reduced when using RS co-products as an alternative to SBM in pig diets. This could potentially impair the FE of the pigs when using local ingredients, which will not help improving the sustainability and self-sufficiency of European pig production.

ii) the reduced digestibility in pigs fed RS co-products may not be because of lack of adaptation of the GIT, but potentially caused by inhibition of enzyme activity and/or lack of enzyme/microbiota-substrate interaction resulting from entrapped nutrients in the complex fiber matrix, and/or interference with other ANF.

8.4. Nutrient and energy metabolism

Although feeding the RSF did not lead to changes in the concentration of total free AA in the liver of the pigs, metabolomics analysis of serum detected greater concentrations ($P < 0.05$) of total and specific individual AA in these pigs (Paper II, Supplementary Table S5). The increased total free AA pool in serum may be explained by a reduction in protein synthesis or an increase in protein degradation. The latter argument is supported by an up-regulation of the urea cycle in the liver of the RSF pigs, as indicated by i) the reduced concentrations of arginine ($P < 0.05$), citrulline ($P < 0.05$), and ornithine ($P = 0.06$), which are intermediate metabolites of the urea cycle, in the liver (Paper II, Figure 5 E and Supplementary Table S4); and ii) the increased concentrations ($P < 0.05$) of N carriers, namely glutamine and glutamic acid, in the serum of these pigs (Paper II, Supplementary Table S5). However, as mentioned in section 8.2, the reduced ($P < 0.01$) plasma urea and creatinine levels found in these pigs (Paper I, data not shown) oppose this argument. In addition, the concentrations of total plasma protein and aspartate amino transferase (another marker for protein degradation), were not affected by the RSF diet (Paper I, data not shown). The observations from Experiment 1 indicate contradictory effects of the RSF diet on protein metabolism. Protein turnover is an energy-costly process for all animal species, and variation in protein metabolism has been associated with differences in efficiency of protein utilization in chickens (Tomas *et al*., 1991). These authors found that reduced protein degradation rates in muscle led to improved efficiency of protein retention. Therefore, effects of increasing RSM inclusion on N balance were investigated in Experiment 2 and presented in Paper III, Table 4. Despite less N being available for absorption due to the reduced ATTD of CP (mentioned in section 8.3.) and the lower ($P < 0.003$, linear $P < 0.001$) digested N (**DN**) with increasing RSM inclusion, retained N (**RN**) did not differ among dietary groups. This was also the case when expressing RN in

proportion of ingested N (**IN**) and DN, which indicates that the RSM diets did not affect the efficiency of utilization of DN for retention (RN:DN). This is in agreement with Len *et al*. (2007), who reported that inclusion of fiber in pig diets did not affect RN:DN. Total N excretion was also similar across dietary treatments. However, increasing inclusion of RSM induced a shift in N excretion from urine to feces, as indicated by a decrease (linear $P < 0.019$) in urinary N (UN) and an increase ($P < 0.003$, linear $P < 0.001$) in fecal N (FN), with a subsequent reduction ($P < 0.006$, linear $P < 0.001$) in UN:FN. High DF, especially soluble fiber, has been previously shown to induce a repartition of N from urine to feces in pigs due to higher N excretion in the form of bacterial protein (Bindelle *et al.,* 2009). However, the fiber fraction in the RSM diets is more insoluble than in the control diet, so it is suggested that the shift in N excretion may be associated with a considerable proportion of the dietary N being part of the complex RSM fiber matrix and thus having a low digestibility (Jensen *et al*., 1990). Anyhow, the shift in N excretion could represent an environmental advantage of replacing SBM with RSM, as most of the ammonia in pig manure originates from the breakdown of urea in urine rather than from the breakdown of protein in feces (Aarnink *et al*., 1993). Together, the observations from Experiments 1 and 2 indicate that replacing SBM with RS co-products did not have major effects on protein metabolism.

Several observations from the metabolomics analyses of liver and serum samples showed that feeding the RSF diet affected various metabolites associated with oxidative stress and redox balance: i) decreased ($P < 0.05$) concentration of ascorbic acid, an antioxidant, and increased concentrations of oxidized metabolites, including oxidized glutathione (GSSG, $P < 0.01$), cysteine-glutathione (Cys-GS, $P < 0.01$), and cysteine ($P < 0.01$) in the liver of these pigs (Paper II, Figure 5 C); ii) increased ($P < 0.01$) concentration of pyroglutamate, a metabolite marker of oxidative stress, in the liver of these pigs (Paper II, Figure 5 C); iii) increased concentrations of butanal, a degradation product of *in vivo* lipid peroxidation, in the liver ($P = 0.06$) and serum ($P < 0.05$) of these pigs (Paper II, Figure 5 C and 6 C, respectively). Lipid peroxidation constitutes a response to oxidative stress. Increased ($P < 0.01$) concentrations of oleic acid-containing phosphatidylcholines were found in the liver and serum of the pigs fed the RSF diet, which is consistent with the higher content of oleic acid in RS than in soybeans (USDA-FCD, 2017). On the other hand, lower concentrations of DHA-containing phosphatidylcholines were found in the liver ($P < 0.01$) and serum ($P < 0.05$) of the RSF pigs, despite the higher content of α-linolenic acid, a precursor for DHA, in RS than in soybeans (USDA-FCD, 2017). The latter observations may be explained by a higher susceptibility of

α-linolenic acid and DHA, which are ω-3 fatty acids, to lipid peroxidation and further supports increased lipid peroxidation by the RSF diet. The observed disruption of the oxidative stress status of the pigs fed the RSF diet is consistent with the enlarged thyroid glands of these pigs but is in contrast to the antioxidant properties associated with several RS components such as vitamin C (Krumbein *et al.*, 2005), sinapic acid and other phenolic acids (Nićiforović and Abramovič, 2014), and isothiocyanates (de Figueiredo *et al*., 2013). Stress situations may require resource reallocation and increase maintenance energy requirements to amount an appropriate stress response, consequently reducing energy available for growth (Rauw, 2012). Similarly, increased organ weights may increase energy use for maintenance and decrease the energy available for retention. In this regard, enlarged thyroid glands were observed in pigs fed the RSF in Experiment 1. Thyroid glands could not be dissected out and weighed in Experiment 2, but increased liver index was observed with increasing inclusion of RSM. Although not measured in any of our experiments because of time limitation at sampling, it is known that pigs respond to high DF by increasing viscera weight and volume (Jørgensen *et al*., 1996), which can also increase energy and AA requirements, and potentially impair FE (Nyachoti *et al*., 2000). Together, variation in metabolism, stress status, and organ weight may affect the energy expenditure of the pigs. Despite the increased weight of the liver and the potential effects of RSM on the weight of other organs and on the oxidative stress status of the pigs (as observed in Experiment 1), increasing RSM inclusion did not affect HE and energy retention (**RE**) of the pigs, also when expressed in proportion to ME intake (Paper III, Table 5). This is in agreement with Hansen *et al*. (2006), who found that inclusion of different fiber sources did not affect HE:ME nor RE:ME in growing pigs. However, the difference in DF content in the latter (5-11%) and present (17-20%) studies might not have been large enough to induce an effect, as greater difference in DF content (6-27%) increased HE:ME and decreased RE:ME in the study of Jørgensen *et al*. (1996). In agreement with our results, previous research on the effects of RSM glucosinolates on HE in pigs reported no major changes in HE:ME despite observing increased liver, kidneys, and thyroid weights (Fandrejewski *et al*., 1994) or reduced thyroid hormones in plasma (Buchmann and Wenk, 1989). In addition to similar RE:ME, the ME requirement for maintenance (**ME_m**) of 825 $kJ/kg^{0.60}$ stated by NRC (2012) is intermediate to the two estimates of ME_m (700 vs. 900 $kJ/kg^{0.60}$) obtained in Experiment 2 (Paper III, Figure 3). Similarly, our estimates for the efficiency of utilization of ME for retention in body tissues (**k_g** = 0.70 and 0.80, Paper III, Figure 3) are within the range reported in the literature (k_g = 0.69 to 0.82, Thorbek *et al*.,

1984). The latter observations indicate that increasing RSM inclusion did not have major effects on the ME_m and k_g of the pigs.

From the observations on liver and serum metabolome in Experiment 1 (Paper II) and the observations on N and energy metabolism in Experiment 2 (Paper III):

i) changes in protein metabolism were observed after replacing SBM with RS co-products, as indicated by the reduced concentrations of urea cycle intermediate metabolites in the liver, increased concentrations of N carriers in serum, and reduced plasma urea and creatinine levels found in pigs fed the RSF diet. However, these results seem contradictory and further long-term investigations are needed to get more knowledge about the effects of feeding RS co-products on protein metabolism at a molecular level.

ii) changes in protein metabolism in Experiment 1 did not translate into changes in RN or efficiency of utilization of DN for retention with increasing inclusion of RSM in Experiment 2.

iii) inclusion of RS co-products had an effect on the redox and oxidative stress status of the pigs, which warrants further research.

iv) increasing inclusion of RSM did not affect HE or the efficiency of utilization of ME for retention.

v) estimated ME_m and k_g for all pigs in Experiment 2 were within normal ranges.

9. Concluding remarks and future perspectives

Taking together the results from the present thesis, it can be concluded that:

- The digestive efficiency of young pigs was reduced when using RS co-products as an alternative protein source to SBM.
- The reduced digestibility was not associated with changes in intestinal morphology, but it correlated with digestive enzyme activities.
- Feeding RS co-products increased GABA concentrations in the digesta along the entire GIT and in the liver. The relationship between this marker and nutrient digestibility and FE should be assessed in future studies.
- There was considerable individual variation in nutrient digestibility among individual pigs within both dietary treatments. Whether this variation in digestibility is heritable should be investigated in future experiments to evaluate the possibility of selecting pigs with improved digestive capacity when feeding RS diets.
- Feeding RS co-products enlarged the thyroid (Experiment 1) and liver (Experiment 2) to BW ratios after three weeks. Potential effects of longer exposure on, FE, metabolism, and growth performance should be assessed.
- Replacing SBM with up to 30% of RSM did not compromise overall protein and energy metabolism, or the efficiency of utilization of DN or ME for retention.
- However, feeding RS co-products affected protein metabolism at the molecular level, although this effect is not clear and necessitates further research.
- Feeding RS co-products induced a change on the redox and oxidative stress status of the pigs. Future research should be directed to identify the specific source of oxidative stress, investigate the relationship between changes in oxidative stress and changes in digestibility, metabolism, and ultimately FE, and assess potential dietary interventions to minimize oxidative stress when using RS co-products.

Overall, if RS co-products are to be used as an alternative to SBM in pig diets without compromising FE, efforts should focus in developing robust pig genotypes with greater capacity to digest recalcitrant fibers and in increasing RS fiber degradability through processing.

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11. Papers

Paper I

Livestock Science 203 (2017) 1–9

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

High-fiber rapeseed co-product diet for Norwegian Landrace pigs: Effect on digestibility

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ARTICLE INFO

Keywords:
Digestibility
Feed efficiency
Fiber
Landrace pigs
Rapeseed meal
Soybean meal

ABSTRACT

The effect of partially replacing soybean meal (SBM) and wheat with high-fiber rapeseed (RS) co-products on the nutrient and energy digestibility of 40 Norwegian Landrace pigs (17.8 ± 2.7 kg initial BW) was investigated. Pigs were fed a pelleted diet containing 200 g/kg of a coarse fraction of air-classified rapeseed meal (RSM) and 40 g/kg of RS hulls or a SBM control diet (20 pigs/dietary treatment) for 3 wk to estimate apparent ileal (AID) or total tract (ATTD) digestibility of energy and nutrients, organ weight, intestinal histomorphology, and digestive enzyme activities of individual pigs. Feeding high-fiber RS co-products increased ($P = 0.004$) the thyroid to body weight ratio and reduced ($P < 0.05$) the AID and ATTD of energy, dry matter, organic matter, crude protein (CP), neutral detergent fiber, acid detergent fiber, P, and most of the amino acids (AA) and monosaccharides. The reduction in digestibility was not associated with morphological changes in ileum or colon. The reduced AID of CP and AA coincided with a decrease ($P = 0.030$) in trypsin activity in the jejunum. The AID of starch was not affected by the dietary treatment, which also coincided with similar amylase and maltase activities in the jejunum. Variation in nutrient digestibility was observed among individual pigs within each dietary treatment. In conclusion, feeding high-fiber RS co-products to pigs enlarged the thyroid gland and reduced the AID and ATTD of most nutrients and energy. The reduction in digestibility was not associated with changes in intestinal morphology, but correlated with digestive enzyme activities.

1. Introduction

The European pig industry is heavily dependent on imported feed ingredients, especially soybean meal (SBM) as a protein source in commercial diets (FEFAC, 2015). Increased and more efficient use of local protein sources, such as rapeseed (RS), could improve the sustainability and self-sufficiency of pig production in Europe. European RS production is rapidly increasing, mainly because of higher demands from the biofuel industry (Messerschmidt et al., 2014). Rapeseed meal (RSM), a co-product from RS processing for oil and biofuel production, has potential to replace a significant proportion of SBM in pig diets (Weightman et al., 2014). Life cycle assessment studies of the environmental impacts of feed production have shown that global warming potential decreased up to 10% when SBM is replaced with RSM in diets for pigs (Van Zanten et al., 2015). However, the use of RSM in pig diets is associated with reduced feed intake, growth rate, and nutrient utilization (Landero et al., 2011; Seneviratne et al., 2011; Torres-Pitarch et al., 2014). These effects have been attributed to the high dietary fiber (DF) content and the presence of several antinutritional factors (ANF) in RSM, including glucosinolates (Mejicanos et al., 2016).

High content of DF in pig diets is associated with impaired nutrient utilization and reduced net energy values (Noblet and Le Goff, 2001), although the magnitude of such negative impacts will be determined by the fiber source, relative solubility and fermentability, and the age and genotype of the animal (Lindberg, 2014). Indigenous pig breeds have been shown to digest fiber better than exotic breeds genetically selected for improved growth performance (Len et al., 2009a, 2009b; Urriola and Stein, 2012). Improvements in feed efficiency (FE) are crucial for a more economically and environmentally sustainable pork production. According to Herd and Arthur (2009), nutrient digestibility is one of the factors contributing to variation in FE. Fiber digestibility is more variable and lower than that of other main nutrients (Jha and Berrocoso, 2015), and thus the use of high-fiber diets may affect the progress of

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http://dx.doi.org/10.1016/j.livsci.2017.06.008
Received 27 January 2017; Received in revised form 2 June 2017; Accepted 16 June 2017

selection for improved FE. The Norwegian Landrace breed has a history of selection for improved FE beginning in the 1960s (Kolstad and Vangen, 1996). However, genetic selection has emphasized traits such as average daily gain and carcass lean of pigs fed high-quality diets (Kolstad and Vangen, 1996), which indirectly affect FE. Thus, the Norwegian Landrace pigs may have great potential for a high FE when fed high-quality diets, but this may not be applicable when fed diets with higher fiber content and ANF. Genetic variability of organic matter (OM), N and energy digestibility in Large White pigs fed a high-fiber diet has been reported (Noblet et al., 2013). We hypothesized that replacing SBM with high-fiber RS co-products affects the capacity of Norwegian Landrace pigs to digest nutrients and energy and that there is individual variation in digestibility among pigs fed such diets. Therefore, the objectives of this study were to evaluate nutrient and energy digestibility of Norwegian Landrace weanling pigs fed standard or a high-fiber RS diet, and to identify underlying biological mechanisms associated with variation in digestibility.

2. Materials and methods

The research protocol was reviewed and approved by the Norwegian Food Safety Authority. The trial was conducted at the experimental farm of the Norwegian University of Life Sciences (NMBU), Ås, Norway.

2.1. Animals, housing and allotment

The experiment was conducted as a randomized complete block design with 2 periods, 2 dietary treatments and 10 replicates per treatment per period (total of 20 replicates per treatment). Each experimental period lasted 3 wk, and consisted of a 2-wk adaptation period followed by a 1-wk period of feces and data collection. For each period, 20 castrated male pigs (Norwegian Landrace; Norsvin, Hamar, Norway) with an average initial BW of 16.1 ± 2.2 and 19.6 ± 1.8 kg, respectively, were obtained from 2 different multiplier herds. In total, 40 pigs from 14 different litters with 2 or 4 pigs per litter were used. Upon arrival, pigs were allocated into 10 metabolic crates (1.2 × 1.4 m) in pairs, based on similar BW and litter. Pigs within the same crate were assigned randomly to 1 of the 2 dietary treatments. Each crate was equipped with a self-feeder and a low-pressure nipple drinker and had a partially covered rubber slatted flooring. Pigs were provided ad libitum access to water except at the time of feeding. Pigs were offered toys according to the Norwegian animal welfare legislation (Lovdata, 2003). The room temperature was kept at 21 ± 4 °C and a 12-h light/12-h dark cycle was provided for the duration of the experiment. The clinical health status of the pigs was monitored daily.

2.2. Dietary treatments and feeding

The dietary treatments were: 1) a Norwegian commercial diet based on wheat, barley and SBM (Control) and 2) a diet in which wheat and SBM were partially replaced by 20% coarse RSM and 4% pure RS hulls (RSF). The coarse RSM was produced by air-classification of a commercial hexane-extracted RSM (Bunge, Warsaw, Poland), which separated a coarse high-fiber fraction and a fine low-fiber fraction with a higher protein content (Hansen et al., 2017). The parent RSM was jet-milled (JMX-200; Comex AS, Rud, Norway) to an average particle size of 35 μm. The RSM fractions were obtained after a multiple separation process using air-classification at 3 different rotor speeds (2200, 1900, and 1700 rpm), where the lower bulk density fractions were separated, and the coarse fraction was used for further separation (ACX-200; Comex AS). After 3 fractionation steps, the remaining coarse fraction had a particle size of 74 μm and a yield of 56.4% of the parent RSM. The RS hulls were obtained by grinding whole seeds (Askim Frukt- og Bærpresseri AS; Askim, Norway) with a roller mill (DT900-12; CPM-Roskamp, Waterloo, IA, United States), and separating the low bulk density hulls with a laboratory air-classifier. The chemical composition of the coarse RSM fraction and the RS hulls used in this experiment is shown in the footnote of Table 1.

Table 1
Dietary composition of experimental diets.

	Dietary treatments[a]	
Ingredient, g/kg as-fed	Control	RSF
Wheat[b]	629.1	506.5
Barley[c]	100.0	100.0
Soybean meal[d]	140.0	30.0
Coarse rapeseed meal[e]	–	200.0
Rapeseed hulls[f]	–	40.0
Fish meal	40.0	40.0
Soybean oil	50.0	50.0
Monocalcium phosphate	16.4	9.1
Limestone	11.3	11.2
L-Lys·HCl	3.4	3.4
DL-Met	0.5	0.5
L-Thr	1.3	1.3
L-Trp	0.2	0.2
Sodium chloride	4.0	4.0
Vitamin-trace mineral premix[g]	3.2	3.2
Attractant[h]	0.5	0.5
Y_2O_3	0.1	0.1

[a] Control diet based on wheat and soybean meal; RSF = high-fiber rapeseed diet.
[b] Whole wheat: 86.4% dry matter (DM), 11.1% crude protein (CP), 1.6% ether extract (EE), 58.1% starch, 9.0% neutral detergent fiber (NDF), 2.2% acid detergent fiber (ADF), 1.4% ash.
[c] Barley: 86.2% DM, 7.4% CP, 1.3% EE, 53.5% starch, 16.0% NDF, 5.1% ADF, 1.6% ash.
[d] Soybean meal: 89.0% DM, 43.3% CP, 1.4% EE, 1.4% starch, 8.9% NDF, 5.7% ADF, 5.4% ash.
[e] Coarse fraction from an air-classified hexane-extracted rapeseed meal: 90.0% DM, 31.2% CP, 2.5% EE, 26.2% NDF, 18.6% ADF, 6.7% ash.
[f] Rapeseed hulls: 88.8% DM, 13.2% CP, 8.0% EE, 55.1% NDF, 48.6% ADF, 4.4% ash.
[g] Provided per kilogram of diet: 90 mg Zn (ZnO); 90 mg Fe ($FeSO_4$); 45 mg Mn (MnO); 19.5 mg Cu ($CuSO_4$); 0.45 mg I ($Ca(IO_3)_2$); 5700 IU vitamin A; 4500 IU cholecalciferol; 100.7 mg dl-α-tocopheryl acetate; 2.40 mg menadione; 9.0 mg riboflavin; 36.0 mg D-pantothenic acid; 12.0 μg cyanocobalamine; 12.0 mg niacin; 0.24 mg biotin; and 1.8 mg folic acid.
[h] Maxarome; Felleskjøpet, Kambo, Norway.

The diets were formulated to meet or exceed the requirements for indispensable amino acids and all other nutrients and energy for pigs of this age (NRC, 2012), and were subsequently mixed and pelleted at the NMBU Center for Feed Technology (Ås, Norway). Yttrium (III) oxide was included (0.01%) as an inert marker for digestibility calculations. The composition and chemical contents of the diets are presented in Table 1 and Table 2, respectively.

Upon arrival at the research facility, pigs were offered a commercial weaner diet, which was gradually replaced (over 3 d) by 1 of the 2 experimental diets. The animals were fed equal meals twice daily at 0800 and 1500 h, with the total amount of feed corresponding to 3.5% of their BW per day. Pigs within the same crate were separated for 15 min by a physical barrier at every meal to allow individual feeding. Water was added to the feed immediately before feeding at a ratio of 2:1 (w/w). All pigs were weighed weekly, and daily feed allowance was adjusted accordingly.

2.3. Sample collection and processing

During the collection period, feces were obtained daily by grab sampling directly from pen floors or by rectal stimulation. Feces from each pig over the 7-d period were pooled and frozen at −20 °C. Upon completion of the trial, feces were freeze-dried, ground through a 1-mm screen in an ultra centrifugal mill (ZM 100; Retsch, Haan, Germany) and mixed before chemical analyses to determine apparent total tract digestibility (ATTD) of energy and nutrients. Feces and ileal digesta were also ground through a 0.5-mm screen for determination of starch

Table 2
Analyzed chemical concentrations of experimental diets.

Item, g/kg of DM	Dietary treatments[a]	
	Control	RSF
Gross energy, MJ/kg	17.6	17.8
Dry matter, g/kg	908.4	906.1
Crude protein	201.8	201.8
Ether extract	79.2	87.7
Starch	402.1	370.8
Neutral detergent fiber	113.0	154.8
Acid detergent fiber	41.8	82.3
Ash	57.0	60.8
P	9.7	8.7
Y	0.1	0.1
Amino acid		
Ala	8.9	9.1
Arg	11.5	11.1
Asp	17.1	15.3
Cys	3.7	4.5
Glu	43.6	41.3
Gly	9.5	10.1
His	5.0	5.1
Ile	8.4	8.3
Leu	14.7	14.4
Lys	12.8	13.2
Met	4.0	4.3
Phe	9.0	8.2
Pro	14.7	15.0
Ser	10.4	10.0
Thr	9.4	10.4
Trp	2.8	2.7
Tyr	5.1	5.2
Val	9.2	9.8
Total amino acids	199.8	198.0
Monosaccharides		
Arabinose	13.3	20.2
Fucose	4.2	4.2
Galactose	12.2	11.1
Glucosamine	0.4	0.7
Total glucose	588.9	457.7
Rhamnose	1.1	2.3
Xylose and Mannose	19.7	18.8
Total glucosinolates, mmol/kg	–	1.0

[a] Control diet based on wheat and soybean meal; RSF = high-fiber rapeseed diet.

and amino acid content. Cumulative samples of the diets were also obtained during the collection period and ground through a 1 or 0.5-mm screen before chemical analyses, as explained for the feces.

Blood samples were collected from all pigs at the start of the experiment and 1 d before slaughter. Approximately 10 mL pre-prandial blood was collected into EDTA and lithium heparin-coated tubes (Vacuette; Greiner Bio-One, Kremsmünster, Austria) via venipuncture in the jugular vein/bijugular trunk. After collection, plasma was harvested by centrifugation at 2,000 × *g* for 10 min at 4 °C and kept on ice until transport, along with EDTA whole blood, to the laboratory for analysis of standard clinical chemistry and hematology variables.

At the end of each experimental period, pigs were euthanized using a captive bolt pistol followed by exsanguination. All pigs received a normal morning meal 2.5–3 h before slaughter to ensure the presence of digesta along the gastrointestinal tract (GIT). An incision was made to expose the abdominal cavity, and the entire GIT was immediately removed. Approximately 25 cm sections of the duodenum (25 cm from the pyloric sphincter), mid-jejunum, ileum (20 cm anterior to the ileocecal valve), cecal apex, and the central flexure of the spiral colon were tied off with cotton string, excised and placed in aluminum trays on ice. Tissues from distal jejunal lymph node, liver, thyroid gland, kidney, spleen, heart and lungs were sampled from standardized locations. The livers and thyroid glands were weighed before samples of these organs were taken. Each animal and its organs were evaluated macroscopically by a trained pathologist. Tissues and digesta from the different intestinal segments were further sampled for microbial investigation, histology, enzyme activity, transcriptomics, and metabolomics analyses. Only the results from histology and enzyme activity are included in the present paper. The digesta samples were snap-frozen in liquid nitrogen and the tissue samples were rinsed with PBS and put on dry ice. All samples were stored at −80 °C until analysis. Digesta from the last 2 m of the small intestine were collected, stored at −20 °C, and processed as previously discussed for the feces before chemical analyses to determine apparent ileal digestibility (AID). Samples for histology were fixed in 10% buffered formalin solution for 48 h, embedded in paraffin and sectioned in an automatic microtome. Two micron thick sections were mounted on glass slides and stained with hematoxylin-eosin for histological evaluation.

2.4. Histopathology

The morphological evaluation was performed blindly and all assessments were conducted by the same trained pathologist. Histological sections of all the harvested organs were examined in a microscope (Axio Imager Z2; ZEISS, Jena, Germany), and digital images were obtained using a color camera (Axiocam 506 color; ZEISS). The evaluation of intestinal morphology was performed following a modified version of the protocol described by Day et al. (2008), made to fit normal intestinal morphology in the pig. The morphological assessment included evaluation of epithelial damage, formation of crypt dilation and crypt abscesses, mucosa fibrosis, lacteal dilation, follicle atrophy, number of intraepithelial lymphocytes (IELs), presence of infectious agents, and infiltration of leucocytes (i.e., lymphocytes, plasma cells, eosinophils, neutrophils, and macrophages). The results were recorded semi-quantitatively as normal (0), mild (1), moderate (2), and severe (3) changes.

All micrographs were captured with the same ×10 objective magnification in 6 different locations of each intestinal section (i.e., base, middle, and apex areas of intestinal plicas, both from plicas with and without Peyer's Patches). Villi height (VH) and crypt depth (CD) were measured in pixels/inch using the software program Image J-Fiji (Schindelin et al., 2012), and the VH to CD ratio was calculated by dividing VH with CD. One rarely encounters many villi in full length and all perpendicular to the lumen in a row in one section. Therefore, only the longest villi were selected and measured from the base of the crypt to the tip of the villus. Between 3 and 6 villi were measured in each of the 6 different areas of every section. Crypts were selected when the crypt epithelium was visible from the *Lamina muscularis mucosae* and measured from this point to the crypt-villus junction.

2.5. Digestive enzyme activity

Tissue samples from the jejunum were thawed and the mucosa was scraped carefully with a glass slide. Approximately 40 mg of the mucosal scrapings and 70 mg of digesta were homogenized in 1.5 mL of ice-cold water (Milli-Q) using a bead mill (TissueLyser; Qiagen Retsch, Haan, Germany) and sonicated in an ice-cold bath (T 460/H; Elma Schmidbauer GmbH, Ransbach-Baumbach, Germany). The homogenate was centrifuged at 21,100 × *g* for 10 min at 4 °C. The supernatant was analyzed for protein concentration and enzyme activities. The total protein concentration was determined (Bradford, 1976). The activities of trypsin and amylase in jejunal digesta were measured using commercial kits (Abcam, Cambridge, UK). The activity of maltase in jejunal mucosa was assayed following an adaptation of the method described by Dahlqvist (1968) and using a commercial glucose assay kit (Sigma-Aldrich, Saint Louis, MO, United States).

2.6. Chemical analyses

All chemical analyses on ileal digesta and feces were performed in duplicate on freeze-dried samples. Triplicate analyses were conducted with feed samples. Samples of dietary ingredients, diets, ileal digesta,

and feces were analyzed for dry matter (DM) by drying to constant weight at 104 °C (EC, 1971b), ash by incineration at 550 °C (EC, 1971a), crude protein (CP) by Kjeldahl N × 6.25 (EC, 1993), starch according to the method described by McCleary et al. (1994), acid detergent fiber (ADF) and neutral detergent fiber (NDF) using a fiber analyzer system (Ankom200; ANKOM Technologies, Fairport, NY, United States) with filter bags (Ankom F58; ANKOM Technologies). Gross energy content was determined with an adiabatic bomb calorimeter (Parr 1281; Parr Instruments, Moline, IL, United States) according to ISO (1998). Ether extract (EE) was determined in diets and feces after extraction with petroleum ether and acetone (70/30) using an accelerated solvent extractor (Dionex ASE 200; Dionex Corp., Sunnyvale, CA, United States). Amino acid (AA) analysis (except tryptophan) of diets and ileal digesta was performed according to EC (2009) on an amino acid analyzer (Biochrom 30; Biochrom Ltd., Cambridge, United Kingdom). Tryptophan was analyzed according to EC (2009) on a high performance liquid chromatography system (Dionex UltiMate 3000; Dionex Softron GmbH, Germering, Germany) with a fluorescence detector (Shimadzu RF-535; Shimadzu Corp., Kyoto, Japan). Monosaccharides in feces and diets were analyzed on a high performance anion exchange chromatography system (Dionex ICS3000; Dionex Corp.) with pulsed amperometric detection and equipped with a CarboPac PA1 column (2 × 250 mm) connected to a guard of the same type (2 × 50 mm), after hydrolysis of the samples in trifluoroacetic acid (TFA), essentially as described by Manns et al. (2014) but with the following modifications. A flow rate of 0.25 mL/min was used with a "reversed" gradient developing from 26 to 0 mM KOH in 9.5 min, kept at 0 mM for 2.5 min and elevated to 100 mM for the next 2 min using a chromatography management system (Dionex Chromeleon; Dionex Corp.). The samples were analyzed for oligosaccharides after TFA hydrolysis and no oligosaccharides were detected, indicating that the degradation of polymeric constituents by TFA was complete. Total glucosinolate analysis of the diets was performed according to EC (1990). For the determination of Y and P concentrations in feed, ileal digesta, and feces, samples were first digested with concentrated ultrapure HNO_3 at 250 °C using a microwave (Milestone UltraClave III; Milestone, Sorisole, Italy). Samples were then diluted (to 10% HNO_3 concentration), and Y and P were analyzed with an inductively coupled plasma mass spectroscopy system (Agilent 8800 Triple Quadrupole; Agilent Technologies, Santa Clara, CA, United States). Standard clinical chemistry and hematology panels for pigs were analyzed in plasma and whole blood, respectively, according to certified procedures at the Central Laboratory of NMBU School of Veterinary Medicine.

2.7. Calculations and statistical analyses

The AID and ATTD of nutrients and energy was calculated by the indirect method, as described by Maynard and Loosli (1969), using Y_2O_3 as the inert marker (Austreng et al., 2000). Hindgut disappearance of nutrients and energy was calculated as the difference between the ATTD and AID of nutrients and energy (Urriola and Stein, 2012). Liver and thyroid to body weight (BW) ratios were calculated by dividing the weight of the organ with the BW.

The AID, ATTD, hindgut disappearance, blood and plasma measurements and organ index data were subjected to two-way analysis of variance using the general linear model procedure (SAS, 1990). According to a randomized complete block design, the fixed effects of diet (n = 2) and litter (n = 14) were included in the main model. The ileal data from 2 pigs fed the control diet were excluded because of little presence and very liquid ileal digesta. Treatment means were separated using the least-squares means test. For the histology data, mean results for each of the locations were plotted in a statistical software (GraphPad Prism 7.0; GraphPad Software, Inc., La Jolla, CA, United States) and a one-way analysis of variance test or *t*-test was performed. Differences between dietary treatments were considered significant if $P < 0.05$, and were considered a trend if the *P*-value was between 0.05 and 0.10. Results are presented as the LS-means for each treatment, and variance is expressed as the standard deviation or the pooled standard error of the mean. Pigs were considered the experimental unit for all analyses.

3. Results

3.1. Diets

The analyzed composition of the 2 experimental diets was consistent with calculated values from ingredient composition and inclusion rates used in the formulation of the experimental diets. Overall, the CP, GE and AA concentrations were similar for the 2 diets. The largest differences in the AA content were observed for methionine, cysteine, and threonine, which were slightly higher in the RSF diet compared with the control diet. The RSF diet contained slightly more ash and EE than the control diet, and starch content decreased when replacing SBM and wheat with RS co-products (40.2 vs. 37.1% in control and RSF diets, respectively). Inversely, NDF and ADF increased with increasing inclusion of RS co-products (11.3 vs. 15.5% for NDF and 4.2 vs. 8.2% for ADF, in the control and RSF diets, respectively). For monosaccharide content, the RSF diet had more arabinose, glucosamine, and rhamnose, and less total glucose than the control diet.

3.2. Health status and growth

All animals appeared healthy and active throughout the experiment, except for one outbreak of a mild to moderate diarrhea in 8 pigs that lasted 1–5 d during the collection week of the second period. The diarrhea incidence was independent of dietary treatment, and the affected pigs immediately received a probiotic treatment (ZooLac Propaste; VESO AS, Oslo, Norway) following veterinary recommendations. Feces from affected animals were only collected when the fecal consistency (data not shown) was normalized to avoid interference with the digestibility values. Pigs fed both diets consumed their feed readily and grew normally. The initial and final BW averaged 17.8 ± 2.8 and 28.3 ± 4.3 kg for pigs fed the control diet, and 17.9 ± 2.6 and 28.0 ± 4.0 kg for pigs fed RSF diet. The average daily feed intake was 728.7 ± 106.8 and 731.9 ± 102.8 g/d for pigs fed the control and RSF diets, respectively. The liver to BW ratio (data not shown) did not differ between the control and RSF groups, while an increased thyroid to BW ratio was observed in pigs fed the RSF diet (0.09 vs. 0.07; $P = 0.004$). There were no differences in the hematology and most of the clinical chemistry variables (data not shown) between the dietary treatments, except for plasma urea and creatinine levels, which were increased ($P < 0.01$) in pigs fed RSF compared with pigs fed the control diet. The levels of these metabolites were within the normal range for this age pigs.

3.3. Digestibility of dietary components

The AID of DM, OM, CP, NDF, ADF, energy, and P was affected by diet ($P < 0.01$), and all values were lower for pigs fed RSF compared with those fed the control diet (Fig. 1 and Table 3). There was no dietary effect on the AID of starch. Feeding the RSF diet also resulted in a reduction ($P < 0.01$) in ATTD of DM, OM, CP, NDF, ADF, energy and P. The ATTD of EE and starch was similar among pigs fed both diets. There was no effect of diet on the hindgut disappearance of DM, OM, CP, starch, NDF, ADF, energy and P. The AID of total AA and all individual AA (except for methionine) was reduced ($P < 0.05$) in pigs fed the RSF diet (Table 4). Similarly to the ATTD of NDF and ADF, the ATTD of fucose, galactose, and total glucose was reduced ($P < 0.001$) in the pigs fed RSF compared with pigs fed the control diet (Table 4). In contrast, pigs fed the RSF diet had greater ($P < 0.001$) ATTD of arabinose, rhamnose, and glucosamine than pigs fed the control diet, while ATTD of xylose and mannose was not affected by diet.

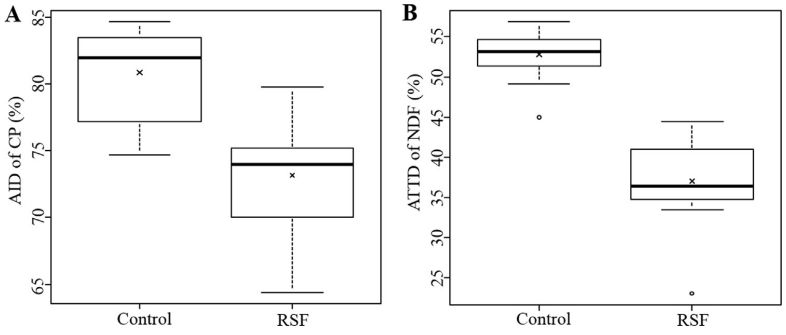

Fig. 1. Effects of feeding a high-fiber rapeseed diet (RSF) as compared to a soybean meal-based diet (Control) for 3 wk on: (A) apparent ileal digestibility of crude protein (CP, n = 18 for Control and 20 for RSF) and (B) apparent total tract digestibility of neutral detergent fiber (NDF, n = 20 for both groups) in Norwegian Landrace weanling pigs.

Table 3
Effects of feeding a high-fiber rapeseed diet for 3 wk on apparent ileal digestibility, total tract digestibility, and hindgut disappearance of nutrients, energy and phosphorous in Norwegian Landrace weanling pigs.[a]

	Dietary treatments[b]		
Item	Control	RSF	*P*-value
Apparent ileal digestibility, %			
Dry matter	72.8 ± 3.8	65.7 ± 3.7	< 0.001
Organic matter	75.1 ± 3.5	68.2 ± 3.5	< 0.001
Gross energy	76.1 ± 3.2	69.6 ± 3.5	< 0.001
Starch	95.8 ± 1.9	95.7 ± 2.3	0.941
Neutral detergent fiber	20.0 ± 12.8	7.4 ± 14.0	0.009
Acid detergent fiber	29.9 ± 13.1	−4.0 ± 12.2	< 0.001
P	55.8 ± 6.6	47.1 ± 5.0	< 0.001
Apparent total tract digestibility, %			
Dry matter	85.9 ± 0.8	79.2 ± 0.9	< 0.001
Organic matter	87.9 ± 0.9	81.3 ± 1.0	< 0.001
Gross energy	85.9 ± 1.0	79.5 ± 1.1	< 0.001
Crude protein (N × 6.25)	84.4 ± 1.8	77.6 ± 1.8	< 0.001
Ether extract	84.5 ± 2.1	84.2 ± 2.1	0.609
Starch	99.6 ± 0.1	99.5 ± 0.1	0.010
Acid detergent fiber	39.2 ± 6.4	10.3 ± 5.3	< 0.001
P	57.2 ± 2.0	48.3 ± 2.3	< 0.001
Hindgut disappearance,[c]%			
Dry matter	12.9 ± 3.9	13.6 ± 3.7	0.630
Organic matter	12.6 ± 3.7	13.1 ± 3.5	0.691
Gross energy	9.6 ± 3.5	9.8 ± 3.5	0.835
Crude protein (N × 6.25)	4.2 ± 2.7	4.7 ± 3.8	0.644
Starch	3.8 ± 2.0	3.8 ± 2.3	0.955
Neutral detergent fiber	31.6 ± 13.8	29.4 ± 15.1	0.643
Acid detergent fiber	9.0 ± 14.6	14.2 ± 13.7	0.249
P	1.5 ± 6.3	1.2 ± 5.7	0.871

[a] Values are least-squares means ± standard deviation of the mean, n = 20 except for apparent ileal digestibility and hindgut disappearance values for the control group where n = 18.
[b] Control diet based on wheat and soybean meal; RSF = high-fiber rapeseed diet.
[c] Hindgut disappearance = apparent total tract digestibility – apparent ileal digestibility of nutrients or energy.

Table 4
Effects of feeding a high-fiber rapeseed diet for 3 wk on apparent ileal digestibility of amino acids and apparent total tract digestibility of monosaccharides in Norwegian Landrace weanling pigs.[a]

	Dietary treatments[b]		
Chemical constituent	Control	RSF	*P*-value
Apparent ileal digestibility, %			
Ala	78.7 ± 4.5	75.2 ± 4.9	0.028
Arg	87.2 ± 2.1	83.1 ± 3.2	< 0.001
Asp	77.1 ± 4.2	70.2 ± 4.6	< 0.001
Cys	76.5 ± 4.3	69.3 ± 5.0	< 0.001
Glu	88.7 ± 2.2	85.8 ± 2.9	0.002
Gly	69.1 ± 6.6	63.4 ± 7.7	0.018
His	84.7 ± 2.5	80.7 ± 3.1	< 0.001
Ile	84.3 ± 3.1	78.2 ± 3.6	< 0.001
Leu	84.9 ± 2.7	80.2 ± 3.6	< 0.001
Lys	87.1 ± 2.8	81.2 ± 3.6	< 0.001
Met	86.8 ± 3.8	84.8 ± 3.4	0.15
Phe	84.4 ± 2.8	79.7 ± 3.4	< 0.001
Pro	82.0 ± 5.5	73.2 ± 7.1	< 0.001
Ser	83.9 ± 2.9	77.8 ± 2.8	< 0.001
Thr	81.4 ± 3.5	75.8 ± 2.6	< 0.001
Trp	79.8 ± 3.5	72.2 ± 4.9	< 0.001
Tyr	84.9 ± 3.6	78.3 ± 3.5	< 0.001
Val	81.7 ± 3.1	75.3 ± 3.6	< 0.001
Total amino acid	83.5 ± 3.0	78.3 ± 3.6	< 0.001
Apparent total tract digestibility, %			
Arabinose	57.6 ± 4.4	71.8 ± 3.3	< 0.001
Fucose	94.0 ± 0.7	91.4 ± 1.1	< 0.001
Galactose	92.2 ± 0.9	83.2 ± 1.8	< 0.001
Glucosamine	−128.6 ± 33.6	−69.7 ± 25.4	< 0.001
Glucose	99.9 ± 0.1	99.7 ± 0.1	< 0.001
Rhamnose	55.5 ± 8.9	63.3 ± 5.4	< 0.001
Xylose and mannose	81.4 ± 2.9	80.7 ± 3.3	0.472

[a] Values are least-squares means ± standard deviation of the mean, n = 20 except for apparent ileal digestibility and hindgut disappearance values for the control group where n = 18.
[b] Control diet based on wheat and soybean meal; RSF = high-fiber rapeseed diet.

Individual variation in digestibility among pigs within the same dietary treatment was observed in the terminal ileum and in the total tract (Fig. 1). The AID of CP in pigs fed the control diet ranged from 74.7% to 84.7%, with an average of 80.9%. The AID of CP in the pigs fed RSF ranged from 64.4% to 79.8%, with an average of 73.2%. Similarly, the ATTD of NDF ranged from 45.0% to 56.9% (average 52.8%) and from 23.0% to 44.5% (average 37.0%) in pigs fed control and RSF diets, respectively. Individual pig variation in fermentation capacity was also observed among pigs within the same dietary treatment (Table 3). Hindgut disappearance of CP in pigs fed the control diet ranged from 0.4% to 10.3% (average 3.9%) and from −3.1% to 13.6% (average 4.6%) in pigs fed the RSF diet. Hindgut disappearance of NDF ranged from −2.8% to 60.9% (average 33.5%) and from 1.5% to 57.4% (average 30.2%) in pigs fed control and RSF diets, respectively.

3.4. Macroscopic and histopathological evaluation

Data from the macroscopic evaluation and the histological assessment, except for VH, CD, and VH:CP, are not presented. Macroscopic evaluation of the intestines of all pigs showed no pathological conditions. Similarly, histological assessment of the various intestinal segments did not show epithelial damage in any of the pigs, and there was no sign of crypt dilation, crypt abscesses, mucosa fibrosis or follicle atrophy. No differences in VH, CD and VH:CD in ileum and CD in colon segments were observed between pigs fed the different diets (Fig. 2), nor were there differences in these measurements between pigs from the 2 different experimental periods (data not shown). A very mild to moderate multifocal infiltration of neutrophils was observed in the lamina propria and in the epithelium of the colon of some pigs. This very

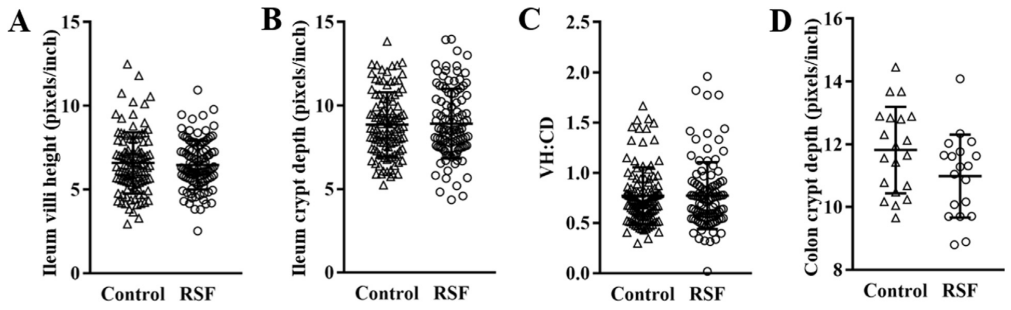

Fig. 2. Effects of feeding a high-fiber rapeseed diet (RSF) as compared to a soybean meal-based diet (Control) for 3 wk on: (A) ileum villi height (VH, n = 18 for Control and 20 for RSF); (B) ileum crypt depth (CD, n = 18 for Control and 20 for RSF); ileum VH:CD (n = 18 for Control and 20 for RSF); and (D) colon crypt depth (n = 18 for Control and 20 for RSF) in Norwegian Landrace pigs. Measurements are presented in pixels/inch, 11.5 pixels/in. = 500 μm.

mild degree of inflammation was observed in 13 pigs fed the control diet and 8 pigs fed RSF. Generally there were none to a minimal number of neutrophils in the lamina propria in all other intestinal segments. *Cryptosporidium* sp. was observed in the enterocytic brush border in the jejunum and ileum of 2 pigs (no other pathology was identified in the intestines of these pigs). There was a consistent increase of IELs in all intestinal segments of the pigs from the second period, regardless of dietary treatment and diarrhea incidence. Histological evaluation of the lungs showed less severe ($P = 0.060$) pneumonic lesions in pigs fed RSF diet compared to the pigs fed the control diet (data not shown). No histopathological changes were observed when evaluating myocardium, liver, kidney and thyroid gland from pigs in either dietary treatment.

3.5. Digestive enzyme activity

Amylase activity in jejunal digesta and maltase activity in jejunal mucosa did not differ between dietary treatments while feeding the RSF diet reduced ($P = 0.030$) trypsin activity in the jejunal digesta of the pigs (Table 5).

4. Discussion

In Europe, the heavy reliance on imported SBM as a protein source in pig diets is questionable from a food security point of view, making it necessary to search for alternative protein sources and develop robust genotype pigs that perform well when fed such diets. Therefore, our objectives were to evaluate the energy and nutrient digestibility of Norwegian Landrace weanling pigs when switched from a conventional SBM-based to a RS-based diet and to identify biological mechanisms associated with differences in digestibility.

Fiber content in RSM is considerably higher than in SBM because of the greater proportion of hulls relative to seed mass (Mejicanos et al., 2016). Over 70% of the RS fiber is concentrated in the hulls, which serves as the main reservoir for non-starch polysaccharides and lignin (Carré et al., 2016). A coarse fraction of air-classified RSM and pure RS hulls were therefore used in the present experiment to increase the contrast in fiber level and fiber composition between treatments and accentuate the specific effects of the RS fiber on digestibility. Consequently, the ADF and NDF concentrations in the diet increased when replacing wheat and SBM with the high-fiber RS co-products, which was the major difference between the dietary treatments. Similarly, methionine and cysteine contents were greater in the RSF diet, as RSM contains more sulphur AA when compared to SBM (Newkirk et al., 2003).

Table 5
Effects of feeding a high-fiber rapeseed diet for 3 wk on jejunal digestive enzyme activities in Norwegian Landrace weanling pigs.[a]

	Dietary treatments[b]			
Enzyme activities, U/mg protein	Control	RSF	SEM	*P*-value
Trypsin	4468	3231	392	0.030
Amylase	31,241	25,942	4469	0.396
Maltase	1.5	1.4	0.1	0.881

[a] Values are least-squares means and pooled standard error of the mean (SEM), n = 20.
[b] Control diet based on wheat and soybean meal; RSF = high-fiber rapeseed diet.

Norwegian Landrace pigs have a high FE when fed standard diets (Kolstad and Vangen, 1996). In this paper, we refer to standard diets as high-energy density and -protein diets based on conventionally used ingredients. Energy and nutrient digestion of feed has been reported as one of the physiological processes contributing to variation in the FE of an animal (Herd and Arthur, 2009). Therefore, it was important to assess differences in the capacity of Norwegian Landrace pigs to digest energy and nutrients in the diet. The observed reduction in AID and ATTD of most nutrients and energy, including most AA and monosaccharides, by the RSF diet may be attributed to the higher fiber content. It is well known that high fiber content reduces the digestibility of energy and dietary components both at the ileum and total tract level (Len et al., 2009a, 2009b; Wilfart et al., 2007a; Yin et al., 2000). Furthermore, the decreased nutrient and energy digestibility observed in the present study is consistent with previous research where increasing dietary levels of up to 20% solvent-extracted (Landero et al., 2011) or expeller-pressed canola meal (Landero et al., 2012), to replace SBM, linearly reduced ATTD of energy, DM and CP in weanling pigs. Similarly, Sanjayan et al. (2014) reported that increasing dietary inclusion of 20–25% of 2 types of canola meal (*Brassica juncea* yellow and *Brassica napus* black) led to a reduction in ATTD of DM, CP and energy in weanling pigs. These authors attributed this effect to an increased NDF content, which was lower than in the present experiment.

In addition to the level of fiber, the fiber type or source has specific effects on the digestion and absorption processes (Wenk, 2001). Fiber in different feedstuffs vary in solubility, degree of lignification and fermentability, that will in turn affect the physico-chemical properties of the diet, which are important for its utilization in pigs (Bach Knudsen and Jørgensen, 2001). The ADF content was almost twice as high in the RSF diet compared to the control diet, indicating that the fiber fraction in this diet was more insoluble than in the control diet. Lignin is highly resistant to degradation and is known to cause a considerable reduction in digestive processes (Wenk, 2001). A larger amount of lignin was expected in the RSF diet because lignin concentration in RSM is considerably greater than in SBM, especially because of the high degree of lignification of the RS hulls compared with soybean hulls (26.2 vs. 2.1% of DM, Bach Knudsen, 2014), and our RSF diet contained a coarse RSM fraction and pure RS hulls. Consequently, the inclusion of RS co-products may have resulted in a more insoluble and indigestible DF

fraction, which may explain the reduced digestibility of ADF and NDF of the pigs fed the RSF diet, as previously shown in several studies (Urriola and Stein, 2010; Wilfart et al., 2007a). In fact, the low ADF and NDF digestibility in pigs fed the RSF diet indicates that the fiber fraction in this diet is highly resistant to digestion. The negative AID of ADF obtained in pigs fed the RSF diet may be an artifact because of endogenous losses, as significant amounts of non-dietary material may be co-analyzed with fiber in ileal digesta and feces (Montoya et al., 2016), but it indicates that this fraction is predominantly indigestible in the small intestine. Insoluble fiber increases passage rate and decreases mean retention time in the small and large intestine of pigs (Wilfart et al., 2007b). Thus, feeding the RSF diet may have reduced the time that the digesta was exposed to enzymatic degradation and microbial fermentation, which may have contributed to the reduced AID and ATTD of nutrients and energy in pigs fed this diet. Rapeseed meal also has a rigid lignin-cellulose matrix (Pustjens et al., 2013) that can further hinder the accessibility and action of digestive enzymes (Hansen, 1986), and may have contributed to the reduced nutrient digestibility observed in the ileum. The reduction in digestibility by the RSF may also be partially explained by the low digestibility of the protein and AA present in the RS hulls. The hull fraction represents about 30% of the RSM (Mejicanos et al., 2016) and the RS hulls used in the present experiment contained 13.2% of CP. The high lignification of RS hulls may create a complex fiber matrix that binds and encapsulates protein and AA, preventing the action of digestive enzymes (Bach Knudsen, 2014), and thus reducing their digestibility. Lindberg (2014) reported an increased digesta bulk caused by insoluble fiber and this may be an additional factor reducing the digestibility of the RSF diet in the present experiment. Insoluble fiber appears to increase endogenous losses through physical abrasion, scraping the mucin from the intestinal mucosa (Montagne et al., 2003), and may have contributed to the reduction in the apparent digestibility of CP and AA by the RSF diet.

Majority of the starch in the diets originated from cereal ingredients, with minimal contribution from SBM or RS co-products. As a consequence, starch digestibility of both diets was similar. The greater ATTD of arabinose and rhamnose in pigs fed the RSF diet may be attributed to the higher content of these monosaccharides in the RSF diet. The negative ATTD of glucosamine is most likely because of the low concentration of glucosamine in the diets and its presence in endogenous losses, as glucosamine is one of the main carbohydrates in mucin (Montagne et al., 2003) and bacterial cell walls (Ward, 1973). Noblet et al. (2013) observed within-population genetic variability of nutrient and energy digestibility in Large White growing pigs from 4 different sires fed a high-fiber diet. These authors suggested that this genetic variability in digestive efficiency may be heritable. The design of the present experiment did not allow for the evaluation of a heritable effect on digestibility. However, the individual variation in AID of CP, ATTD of NDF and in hindgut fermentation of CP and NDF indicates that there are differences in the digestion and fermentation processes among pigs. Whether this variation is heritable should be estimated to investigate the possibility to select pigs for an increased ileal and total tract digestibility when fed high-fiber RS diets. Noblet et al. (2013) hypothesized that differences in N absorption between different half-sib families may have occurred at the hindgut level. In the present experiment, the difference in CP digestibility among pigs was also observed at the ileum level. We acknowledge the limitations of the slaughter procedure for collecting representative ileal digesta samples, which may contribute to the variation observed in the AID data.

Rapeseed meal contains several ANF, such as tannins, sinapine, glucosinolates, and phytic acid, which could interfere with proteolytic enzymes and reduce P bioavailability (Khajali and Slominski, 2012). This may partly explain the decrease in trypsin activity and further contribute to the reduced CP, AA, and P digestibility in the pigs fed the RSF diet. Breakdown products from glucosinolates in RSM have been shown to affect palatability and impair feed intake, alter thyroid function by inhibiting production of thyroid hormones, and impair liver and kidney function (Mejicanos et al., 2016). The total glucosinolate content in the RSF diet was below the recommended limit of 2.1 mmol/kg feed for pigs (EFSA, 2008). Interestingly, an increased thyroid to BW ratio was observed in pigs fed the RSF diet, indicating a possible negative impact on thyroid function even at low inclusion levels and short time of exposure. However, histological assessment of thyroid gland did not confirm any changes related to tissue damage in pigs fed RSF diet. As reviewed by Mejicanos et al. (2016), enlarged thyroid after short-term exposure to low dietary concentrations of glucosinolates indicates that young pigs are highly sensitive to these components. In contrast, liver to BW ratio was not affected by the RSF diet, which is supported by the absence of histopathological changes in the liver. However, long-term experiments are needed to further evaluate potential negative effects because enlarged livers after 13 wk of exposure to RSM has been reported (Choi et al., 2015).

The VH and VH:CD have been commonly used as general indicators of the digestibility in the small intestine, with shorter villi and reduced VH:CD being considered as detrimental for digestion and absorption processes (Montagne et al., 2003). Feeding the RSF diet did not affect VH and VH:CD in the ileum or colonic CD compared with the control diet, indicating that the reduced AID and ATTD of nutrients and energy observed in this study cannot be explained by a decrease in digestive and absorptive intestinal surfaces. Pin et al. (2012) developed a model using rodent data and determined that at least 14 d are required to observe changes in the intestinal crypt structure after a dietary modification. The lack of changes in the intestinal architecture observed in this study after 21 d indicates that changes in intestinal architecture may occur after a longer exposure to the experimental diet, as reported by Chen et al. (2015) after feeding weaned piglets with different fiber sources for 30 d. Variations in brush border enzymatic activities are often linked to morphological changes in the intestine (Kelly et al., 1991). Montagne et al. (2003) suggested that an increase in VH:CD may enhance the hydrolytic capacity of the intestinal epithelium. The lack of effect of feeding the RSF diet on maltase activity, a brush border disaccharidase, coincides with the absence of intestinal morphological changes. Pure lignin and cellulose have been shown to strongly inhibit pancreatic amylase and trypsin activities in vitro (Hansen, 1986). In our study, feeding the RSF diet did not affect amylase activity but reduced trypsin activity in the jejunal digesta. Overall, the reduction in AID and ATTD of most nutrients and energy by the RSF diet was not associated with changes in ileal or colonic morphology. However, the reduced AID of CP and AA coincided with a decrease in trypsin activity, and the lack of a dietary effect on AID of starch coincided with similar amylase and maltase activities in the jejunum. Chen et al. (2015) found that weaned piglets fed a diet containing 10% of wheat bran for 30 d had the lowest ATTD of GE, DM, OM and CP compared with pigs fed diets containing 10% of soybean or pea fiber, although they had greater jejunal villi length and greater digestive enzyme activities. Our results indicate that the AID and ATTD of nutrients in pigs fed the RSF diet did not correlate with the intestinal morphology features measured, but correlated with the digestive enzyme activities.

Taken together, from the observations on AID and ATTD of nutrients, enzyme activity, and intestinal morphology, the authors suggest that the decrease in nutrient digestibility in pigs fed RS co-products as compared with SBM may not be because of lack of adaptation in the GIT, but potentially caused by inhibition of enzyme activity and/or lack of enzyme-substrate interaction resulting from entrapped nutrients in the fiber matrix (Grundy et al., 2016), and/or interference with other ANF. Entrapment of nutrients seems to be commonly observed with insoluble fibers, of which RS hulls have a high content. Therefore, we speculate that selection of pig genotypes for improved digestibility when feeding high-fiber RS diets may give rise to pigs with greater ability to digest recalcitrant and insoluble fiber (Hedemann et al., 2006; Pustjens et al., 2014).

5. Conclusion

In conclusion, partial replacement of wheat and SBM with high-fiber RS co-products in pig diets increased the thyroid to BW ratio and reduced the AID and ATTD of most nutrients and energy. Individual variation in nutrient digestibility was observed among pigs within each dietary treatment. The reduction in nutrient digestibility was not associated with changes in ileal and/or colonic morphology, but correlated with a decrease in trypsin activity in the jejunum.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

We thank D-K. Forberg, S. Herlofsen Nes, M. Henne, and L. Andreassen from the pig research facilities and the laboratory staff at NMBU, especially R. Ånestad, for their practical and technical help. This research was financially supported by FeedMileage-Efficient use of Feed Resources for a Sustainable Norwegian Food Production (Research Council of Norway, Oslo, Norway; grant no. 233685/E50), and Foods of Norway, Centre for Research-based Innovation (Research Council of Norway; grant no. 237841/030).

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Paper II

Running head: Metabolic effects of feeding rapeseed to pigs

Identification of redox imbalance as a prominent metabolic response elicited by rapeseed feeding in swine metabolome[1]

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[1]This study was financially supported by FeedMileage-Efficient use of Feed Resources for a Sustainable Norwegian Food Production (Research Council of Norway, Oslo, Norway; grant no. 233685/E50). Dr. Chi Chen's research is partially supported by a USDA Agricultural Experiment Station project MIN-18-092.

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Abstract

Rapeseed (**RS**) is an abundant and inexpensive source of energy and amino acids (**AA**) in diets for monogastrics and a sustainable alternative to soybean meal. It also contains diverse bioactive phytochemicals that could have antinutritional effects at high dose. When the RS-derived feed ingredients (**RSF**) are used in swine diets, the uptake of these nutrients and phytochemicals is expected to affect the metabolic system. In this study, two groups of young pigs (17.8 ± 2.7 kg initial BW) were equally fed a soybean meal-based control diet and a RSF-based diet, respectively, for 3 wk. Digesta, liver, and serum samples from these pigs were examined by liquid chromatography-mass spectrometry (**LC-MS**)-based metabolomic analysis to determine the metabolic effects of the two diets. Analyses of digesta samples revealed that sinapine, sinapic acid, and gluconapin were robust exposure markers of RS. The distribution of free AA along the intestine of RSF pigs was consistent with the reduced apparent ileal digestibility of AA observed in these pigs. Despite its higher fiber content, the RSF diet did not affect microbial metabolites in the digesta, including short-chain fatty acids and secondary bile acids. Analyses of the liver and serum samples revealed that RSF altered the levels of AA metabolites involved in the urea cycle and one-carbon metabolism. More importantly, the detection of increased concentrations of multiple oxidized metabolites and aldehydes as well as decreased concentrations of ascorbic acid and docosahexanoic acid in liver and serum clearly indicated that RSF disrupted redox balance in young pigs. These metabolic changes warrant further investigation on their correlations with performance and health in long-term feeding trials as well as nutritional and processing interventions that allow greater utilization of RSF in diets for swine.

Key words: amino acid metabolism, metabolomics, rapeseed, redox balance, swine

Introduction

Rapeseed (**RS;** *Brassica* sp) has an amino acid (**AA**) profile and energy content comparable to soybean (Rutkowski, 1971). RS-derived feed ingredients (**RSF**), such as RS meal (**RSM**), are considered an economical source of AA and energy for monogastrics. However, widespread adoption of RSF is limited because RS components, mainly fiber and phytochemicals, have negative impacts on animal growth performance and health. For example, high fiber content in RSF may interfere with digestibility of other nutrients (Noblet

et al., 2013). More importantly, bioactive phytochemicals in RSF, including glucosinolates, phenolic acids, and erucic acid, function as anti-nutrients at high doses (Mailer *et al*., 2008). Glucosinolates are thioglucose compounds that have goitrogenic and hepatotoxic effects at high doses (Mawson *et al*., 1994a; Tripathi and Mishra, 2007), while phenolic acids may decrease palatability of the diet by contributing to a bitter taste and astringency of RSF. Overall, the influence of RSF on animal growth performance is largely determined by the balance between the nutritional and anti-nutritional events elicited by RS components.

Feed efficiency is of great interest for pig and poultry production because feed represents over 70% of the total production cost. Feed efficiency is the result of digestion, absorption, distribution, metabolism, and excretion of nutrients in feed. These processes are responsible for linking the diet and growth performance responses, and have been reported as major contributors to variation in feed efficiency (Herd and Arthur, 2009). Because RS contains abundant nutrients and diverse bioactive phytochemicals, the disposition of RSF is expected to result in unique changes and features in the body through both direct contribution and indirect regulation, especially in the metabolome. However, knowledge on the metabolic effects of RSF is primarily limited to growth performance, energy and nutrient digestibility, and detection of RS phytochemicals. A comprehensive investigation on the metabolic effects of RS in pigs has not been reported. In this study, the metabolic effects of RSF were assessed by comparing metabolic differences between young pigs fed a SBM-based control diet and pigs fed a RS-based diet using liquid chromatography-mass spectrometry (**LC-MS**)-based metabolomic analysis.

Materials and methods

Chemicals and reagents.

Amino acid standards, n-butanol, and sodium pyruvate, were purchased from Sigma-Aldrich (St. Louis, MO); LC-MS-grade water, acetonitrile (**ACN**), and formic acid were obtained from Fisher Scientific (Houston, TX); 2,2′-dipyridyl disulfide (**DPDS**) were from MP Biomedicals (Santa Ana, CA); dansyl chloride (**DC**) was purchased from Acros Organics (Morris Plains, NJ); 2-hydrazinoquinoline (**HQ**) and triphenylphosphine (**TPP**) were obtained from Alfa Aesar (Haverhill, MA), and *p*-chlorol-L-phenylalanine was from Alexis Biochemicals (San Diego, CA).

Animals, dietary treatments, and sample collection.

The design, procedures, and methods of animal feeding have been reported (Pérez de Nanclares *et al.*, 2017). Briefly, 40 Norwegian Landrace castrated pigs (17.8 ± 2.7 kg initial BW) were assigned to one of two dietary treatments (20 pigs/diet) at the experimental farm of the Norwegian University of Life Sciences. Dietary treatments consisted of feeding a SBM-based control diet or a RSF where SBM was partially replaced with high-fiber RS co-products (**Table S1** and **S2**). High-fiber RS co-products were to increase the difference in fiber content and fiber composition between the diets (Pérez de Nanclares *et al.*, 2017). These co-products consisted of 20% of a coarse fraction from air-classified RSM and 4% of pure RS hulls. Pigs were fed twice daily an amount of their respective experimental diets equivalent to 3.5% of BW. After three wk of feeding, the pigs were time fed and sacrificed, and digesta samples from five different sites along the intestinal tract (duodenum, jejunum, ileum, cecum, and colon), along with serum and liver samples were collected, snap frozen,and stored at -80 °C for metabolomic analysis. Ileal digesta samples were also processed and chemically analyzed for determination of apparent ileal digestibility (**AID**) of crude protein (**CP**) and AA as described by (Pérez de Nanclares et al., 2017).

Metabolomics

The LC-MS-based metabolomic analysis comprised sample preparation, chemical derivatization, LC-MS analysis, data deconvolution and processing, multivariate data analysis (**MDA**), and marker characterization and quantification as previously described (Chen *et al*., 2007).

Sample preparation.

Digesta samples (duodenum, jejunum, ileum, cecum, and colon) were prepared by mixing with 50% aqueous ACN in 1:9 (w/v) ratio and then centrifuged at 18,000 × g for 10 min to obtain digesta extract supernatants. For serum samples, deproteinization was conducted by mixing one volume of serum with 19 volumes of 66% aqueous ACN and then centrifuging at 18,000 × g for 10 min to obtain the supernatants. Liver tissue samples were fractionated using a modified Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 100 mg of liver sample were homogenized in 0.5 mL of methanol and then mixed with 0.5 mL of chloroform and 0.4 mL of deionized water. After 10 min centrifugation at 18,000 × g, the upper aqueous fraction

was harvested and the chloroform fraction was dried with nitrogen gas and then reconstituted in n-butanol.

Chemical derivatization.

For detecting metabolites containing amino functional groups in their structure, the samples were derivatized with DC prior to the LC-MS analysis. Briefly, 5 μL of sample or standard was mixed with 5 μL of 100 μmol/L *p*-chlorophenylalanine (internal standard), 50 μL of 10 mmol/L sodium carbonate, and 100 μL of DC solution (3 mg/mL in acetone). The mixture was incubated at 25 °C for 15 min and centrifuged at 18,000 × g for 10 min, and the supernatant was transferred into a sample vial for LC-MS analysis. Samples were derivatized with HQ prior to the LC-MS analysis to detect carboxylic acids, aldehydes and ketones (Lu *et al.*, 2013). Briefly, 2 μL of sample was added into a 100 μL of freshly prepared ACN solution containing 1 mmol/L DPDS, 1 mmol/L TPP, and 1 mmol/L HQ. The reaction mixture was incubated at 60 °C for 30 min, chilled on ice, and then mixed with 100 μL of ice-cold deionized water. After centrifugation at 18,000 × g for 10 min, the supernatant was transferred into a HPLC vial for LC-MS analysis.

Conditions of LC-MS analysis.

Comprehensive coverage of the metabolome was achieved using four different LC-MS conditions to analyze lipids and hydrophilic metabolites, as well as HQ- and DC-derivatized metabolites in digesta, serum, and liver extracts. A 5 μL aliquot was injected into an ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (**UPLC-QTOFMS**) system (Waters, Milford, MA) and separated by a BEH C18 column (Waters) with a gradient of mobile phase ranging from water to 95% aqueous ACN containing 0.1% formic acid over a 10-min run. Capillary voltage and cone voltage for electrospray ionization were maintained at 3 kV and 30 V for positive mode detection, respectively. Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M + H]^+$ = 556.2771 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx^{TM} software

(Waters) in centroided format. Additional structural information was obtained by tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 40 eV.

Data analysis and visualization.

After data acquisition in the UPLC-QTOFMS system, chromatographic and spectral data of samples were deconvoluted by MarkerLynx™ software (Waters) to provide a multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*) and ion abundance. The abundance of each ion was calculated by normalizing the single ion counts (**SIC**) *versus* the total ion counts (**TIC**) in the entire chromatogram. The data matrix was then exported into SIMCA-P+™ software (Umetrics, Kinnelon, NJ) and transformed by Pareto scaling. Multivariate data analysis, including principal components analysis (**PCA**) and partial least squares-discriminant analysis (**PLS-DA**), were used to model the digesta, serum and liver samples for the control and RS treatment groups. Metabolite markers were identified by analyzing ions contributing to sample separation in MDA models. After Z score transformation, the concentrations or relative abundances of identified metabolite markers in the samples were presented in heat maps generated by the R program (http://www.R-project.org), and correlations among these metabolite markers were defined by hierarchical clustering analysis.

Characterization, quantification, and pathway analysis of metabolite markers.

The chemical identities of metabolite markers were determined by accurate mass measurement, using elemental composition analysis, by searching the Human Metabolome Database (**HMDB**), the Kyoto Encyclopedia of Genes and Genomes (**KEGG**), and Lipid Maps databases using MassTRIX search engine (http://masstrix3.helmholtz-muenchen.de/masstrix3/) (Suhre and Schmitt-Kopplin, 2008), as well as MS/MS fragmentation and comparisons with authentic standards if available. Individual metabolite concentrations were determined by calculating the ratio between the peak area of each metabolite and the peak area of the internal standard, and fitting with a standard curve using QuanLynx™ software (Waters). The relevant pathway analysis of metabolite markers was performed by MetaboAnalyst 3.0 (www.metaboanalyst.ca) using KEGG pathway database.

Statistical analysis.

The AID data were subjected to two-way analysis of variance (ANOVA) using the general linear model (**GLM**) procedure of the SAS software (SAS Institute Inc.,Cary, NC). Treatment means were separated using the least-squares (**LS**) means test (Pérez de Nanclares *et al*., 2017). Statistical analysis of metabolomics parameters was performed as two-tailed Student's *t*-tests for unpaired data. Results are presented as mean ± standard deviation (**SD**). Differences between dietary treatments were considered significant if $P < 0.05$, and were considered a trend if the *P*-value was between 0.05 and 0.10.

Results

Effects of RSF on the digestibility of protein and AA.

The performance of pigs fed the control diet and RSF, including their physiological status and digestion efficiency, was compared, and the results have been reported (Pérez de Nanclares *et al*., 2017). Feeding RSF did not affect the histomorphology of ileum and colon, but enlarged the thyroid gland. In addition, RSF reduced the AID and total tract digestibility (**ATTD**) of energy and nutrients, which coincided with a decreased trypsin activity in the jejunum (Pérez de Nanclares *et al*., 2017). The AID of CP, total AA, and all individual AA (except for Met) were decreased by the RSF ($P < 0.05$; **Figure 1**).

Effects of RSF on digesta metabolite composition in small and large intestines.

A comprehensive coverage of small-molecule nutrients and metabolites in digesta extracts, including phytochemicals, AA, organic acids, bile acids, and microbial metabolites, was achieved through a combination of chemical derivatization, and optimized chromatographic and spectroscopic conditions. In the PCA model of pooled LC-MS data, small intestine samples (duodenum, jejunum, and ileum) from both dietary treatments were separated clearly from their corresponding large intestine samples (cecum and colon) along the principal component (**PC**) 1 of the model (**Figure 2A**). Furthermore, the control samples from all the intestinal sites were also separated from their corresponding RSF samples along the PC 3 of the model (**Figure 2A**). The markers contributing to the separation between the control and RSF samples, as well as the markers that changed along the intestinal tract, were revealed in the loadings plot, and further characterized by structural and quantitative analyses (**Figure**

2B). Phytochemicals from RS and soybean were the major contributors to the differences between digesta samples from the two dietary treatments. Sinapine, sinapic acid, and gluconapin were identified as exposure markers of RSF because of their presence in the digesta of the pigs fed RSF and their near-absence in the digesta of the pigs fed the control diet (**Figure 2C-E**). Sinapic acid and sinapine, the choline conjugate of sinapic acid, were present along the entire intestinal tract after RSF feeding (**Figure 2C-D**). In contrast, gluconapin, a glucosinolate, was mainly present in the small intestine, but nearly absent in the large intestine (**Figure 2E**). Daidzein, a soy isoflavone, was positively correlated to control feeding because of its high abundance in the ileum and cecum of control pigs as compared to the RSF pigs (**Figure S1**).

Distribution of free AA (FAA) along the intestinal tract was assessed by quantitative analysis and clustering analysis, resulting in the observation of two similar clusters of proteinogenic AA for both dietary treatments in the heat map (**Figure 3A**). One AA cluster, including Gly, Thr, Met, Lys, Arg, and aromatic AA (Phe, Tyr, and Trp), was more abundant in both duodenum and jejunum, while the other cluster, including Ala, His, Ser, Pro, branched-chain AA (Val, Leu, Iso), and acidic AA (ASP, Asn, Glu, and Gln), had higher concentrations in jejunum (**Figure 3A**). Subtle differences in the distribution of proteinogenic AA were observed between the two dietary treatments (**Table S3**). Compared to the control, the RSF pigs had lesser concentrations ($P < 0.05$) of total AA, Phe, Met, Val, His, and Ala in jejunum, but greater concentrations ($P < 0.05$) of Phe, Trp, Val, His, Thr, and Ala in the ileum (**Figure 3B-I**). Interestingly, from duodenum to cecum, gamma-aminobutyric acid (**GABA**), a non-proteinogenic AA and also a neurotransmitter, was present in greater ($P < 0.05$) concentration in all section of the GI of pigs fed RSF compared to the control pigs (**Figure 3J**).

The influences of RS feeding on the metabolism of the intestinal microbiota was evaluated by measuring the concentrations of major SCFA and bile acids in digesta, which are two groups of metabolites either formed or metabolized by the microbiota. The results from quantitative analysis of SCFA and bile acids showed no differences between the two dietary treatments (**Figure 4A-F**). As expected, the concentrations of acetic, propionic, and butyric acids were greater in the large intestine than in the small intestine of both dietary treatments (**Figure 4A-C**). The concentration of taurochenodeoxycholic acid, a primary bile acid, was greater in the small intestine than in the large intestine of pigs fed both dietary treatments (**Figure 4D**). In contrast, the concentration of lithocholic acid, a secondary bile acid, was greater ($P < 0.05$) in the large intestine than in the small intestine of pigs fed both dietary

treatments (**Figure 4E**). Considerable amounts of hyodeoxycholic acid were detected along the entire intestinal tract of pigs fed both diets (**Figure 4F**).

Effects of RSF on the hepatic metabolome.

Metabolomic analysis of liver extracts revealed RS-induced metabolic changes in the liver since control and RSF samples from individual pigs were separated in a PLS-DA model (**Figure 5A**). The hepatic metabolites contributing to the separation of the samples from the two dietary treatments were identified in the loadings plot and structurally elucidated by authentic standards or MSMS analysis (**Figure 5B**). Feeding RSF affected multiple metabolites associated with oxidative stress and redox balance. Decreased concentration of ascorbic acid ($P < 0.05$), an antioxidant, was observed in the livers of RSF pigs, while increased concentrations of oxidized disulfide metabolites, including oxidized glutathione (**GSSG**, $P < 0.01$), cysteine-glutathione (**Cys-GS**, $P < 0.01$), and cysteine ($P = 0.06$), were observed in the same samples (**Figure 5C**). Moreover, pyroglutamate ($P < 0.05$), a metabolite marker of oxidative stress, and butanal ($P = 0.06$), a degradation product of *in vivo* lipid peroxidation, were also increased by RSF (**Figure 5C**). Total FAA and most individual FAA in the liver were comparable between control and RSF treatments (**Table S4**). Feeding the RSF diet affected two major donors of one-carbon units to the folate cycle differently by increasing ($P < 0.05$) glycine while decreasing ($P < 0.05$) serine concentrations in the liver (**Figure 5D**). The concentrations of arginine ($P < 0.05$), ornithine ($P = 0.06$), and citrulline ($P < 0.05$), which are involved in the urea cycle, were reduced in the liver of RSF pigs compared to that of control pigs (**Figure 5E**). The composition of phospholipids in the liver was also affected by RSF, because RSF increased ($P < 0.01$) the levels of two C18 fatty acid-containing phosphatidylcholines (**PC**), PC (18:0, 18:1) and PC (18:1: 18:2), and decreased ($P < 0.01$) the concentrations of two docosahexaenoic acid (**DHA**)-containing PC, PC(16:0, 22:6) and PC(18:0, 22:6) (**Figure 5F**).

Effects of RSF on the serum metabolome.

Quantitative analysis of serum FAA showed that RSF resulted in a greater concentration of total FAA in serum, which were mainly due to an increase in Glu, Gln, Val, and Thr concentrations (**Table S5**). Metabolomic analysis revealed a clear separation between the control and RSF treatments in serum samples in the scores plot of a PLS-DA model (**Figure 6A**). Serum metabolites contributing to the separation between the two dietary treatments

were identified in the loadings plot, and structurally elucidated by authentic standards or MSMS analysis (**Figure 6B**). Butanal and 2-oxoglutaric acid were identified as markers of RSF because their concentrations were greater ($P < 0.05$) in the serum of these pigs compared to the control (**Figure 6C**). In addition, three of the four phospholipid markers identified in the liver after RS feeding changed consistently in serum, with increased ($P < 0.01$) PC (18:1: 18:2) and reduced ($P < 0.05$) PC (16:0, 22:6) and PC (18:0, 22:6; **Figure 6D**).

Discussion

Sustainability of pork production demands new approaches to reduce carbon, water, and land consumption. Replacing soybean meal with RSF decreases the environmental impact of pork production (van Zanten *et al*., 2015). However, antinutritional factors in RS and their negative effects on growth performance limit the inclusion of RSF in swine diets. Since metabolism is the foundation of nutrient digestibility and growth performance, examining the metabolic effects of RSF could guide the interventions aiming to help pigs cope with antinutritional factors in RSF.

Due to differences between RS and soybean on nutrient and phytochemical compositions, feeding pigs with the products of these two oilseeds are expected to yield different metabolic profiles. Metabolomic analysis in this study confirmed this hypothesis through modeling the digesta, liver, and serum samples from young pigs fed SBM and RS-based diets. The broad impacts of RS on the metabolome of young pigs are reflected by the structural and functional diversity of the metabolites responsive to RSF. Discussions on the significance of these metabolites in monitoring RS feeding and their potential associations with the performance and the health of the pigs are based on the sources and functions of these metabolites, including exposure markers, microbial metabolites, protein digestion and amino acid metabolism, and oxidative stress.

Phytochemicals as exposure markers. Detecting the presence of phenolics and glucosinolates in digesta of pigs fed the RS diet was expected because they are the most abundant and bioactive phytochemicals in RS (Mailer *et al*., 2008). However, the concentrations of these components in pig digesta and their distribution along the different segments of small and large intestines have not been reported in previous studies. In the present study, digesta samples from five sites of the small and large intestines were separated in a multivariate model, largely based on their anatomical locations in the intestinal tract (**Figure 2A**). This

pattern of distribution reflected gradual transformation of feed components, including phytochemicals, along the intestinal tract through digestion, absorption, microbial metabolism, and excretion. A prominent observation from the quantitative analysis of RS phytochemicals was the detection of high concentrations of sinapine (up to 1 mg per g wet weight) in the digesta along the entire intestinal tract of RSF pigs. This is consistent with the fact that sinapine is highly abundant in RS and canola seeds, accounting for about 80% of their total phenolic content, and present at a concentration of about 10 g/kg in defatted canola seeds and press cake (Khattab *et al*., 2010; Nićiforović and Abramovič, 2014). Sinapic acid was also detected in the digesta along the entire intestinal tract in the RSF pigs. This was in contrast to the observation in broiler chickens, in which up to 0.1% of dietary sinapic acid was almost completely absorbed in the small intestine, with little presence in the large intestine (Qiao *et al*., 2008). However, because sinapine is the choline ester of sinapic acid, continuous hydrolysis of sinapine in RSF pigs was likely a major source of the sinapic acid detected along the intestinal tract, including the large intestine. Gluconapin is the most abundant glucosinolate in RS (Mawson *et al*., 1993a). The consistent presence of this chemical in the small intestine and its disappearance in the large intestine agrees with previous observations, indicating that glucosinolates are resistant to digestive enzymes, but susceptible to the myrosinase-like activities of cecal microbiota, such as lactic acid bacteria, *Enterobacteriaceae*, *Bifidobaicterium*, and *Bacteroides spp* (Lai *et al*., 2010; Mullaney *et al.*, 2013). It should be noted that the degradation of gluconapin and other glucosinolates in the cecum can produce corresponding isothiocyanates, which are bioactive compounds that mediate antioxidant responses in the body (Mawson *et al*., 1993b). The identification of daidzein as a robust exposure marker in the digesta of control pigs was also expected because this isoflavone is abundant and naturally occurring in legumes (Jung *et al*., 2000). The observation of greater abundance of daidzein in the ileum and cecum and less abundance in the duodenum, jejunum, and colon may reflect the fact that daidzein, as an aglycone of soybean isoflavones, is formed through β-glucosidases-mediated hydrolysis in jejunum and then degraded by microbial metabolism in cecum and colon (Day *et al*., 1998; Zubik and Meydani, 2003).

Microbial metabolites. The prominence of microbial metabolites, especially SCFA and bile acids, in the chemical composition of digesta was illustrated by their clear contribution to the separation of the small intestine samples from the large intestine sample in the metabolomic model (**Figure 2A-B**). The distribution of SCFA, primary bile acids, and secondary bile acids

was consistent with the localization and metabolic function of the microbiota in the large intestine. The RSF diet in this study is a high-fiber diet based on its levels of NDF and ADF (**Table S2**). Considering the extensive interactions between fiber and gut microbiota (Simpson and Campbell, 2015), including the metabolic interactions (Koh *et al*., 2016), changes of microbial metabolites in the digesta of RSF pigs were initially expected. However, no differences in the concentrations of SCFA and bile acids in the digesta were observed between the two dietary treatments. This observation, together with the fact that the RSF diet did not affect the histomorphology of the ileum and colon of the pigs (Pérez de Nanclares *et al*., 2017), suggests that the fiber content and/or type in the RSF diet might not significantly affect the metabolic functions of microbiota and the intestinal tissues housing the microbiota during the three-wk feeding period. Whether long-term feeding of RS could affect the microbiota and microbial metabolism requires further investigation.

Protein digestion and AA metabolism. An observation associated with protein digestion in the intestinal tract was the presence of two major clusters of FAA in the heat map of FAA concentrations of the digesta samples from both dietary treatments (**Figure 3A**). One FAA cluster was present in greater concentrations in jejunum, while the other FAA cluster was present in greater concentrations in both duodenum and jejunum. This observation indicates that individual AA do not share the same temporal-spacial profile along the intestinal tract when released from the digestion of the proteins in these two diets. This pattern of FAA clustering may be the result of different site-specific proteolytic enzyme activities and different efficiency in site-specific AA absorption due to the distribution of proteolytic enzymes and transporters for individual AA in the intestinal tract (Erickson and Kim, 1990). Furthermore, the FAA pool only explains a small fraction of the total AA absorption because a large proportion of AA is directly absorbed as di- and tripeptides (Gilbert *et al*., 2008).

The control and RSF diets had relatively comparable levels and composition of AA (**Table S2**). However, differences in the FAA profiles of digesta, liver, and serum samples were observed between two dietary groups (**Table S3**, **S4**, and **S5**). The lower concentration of total FAA in the jejunal digesta of the RSF pigs, together with the reduced AID of CP and AA observed in these pigs, indicates a delay in the protein digestion process in these pigs. This is supported by the reduced trypsin activity in the jejunal digesta of the RSF pigs reported before (Pérez de Nanclares *et al*., 2017). Interestingly, the delay and reduction in protein digestion induced by RS feeding did not lead to significant changes in the concentration of

total FAA in the liver. In fact, greater concentrations of total FAA and specific individual AA were observed in the serum of the RSF pigs. The increase in the serum FAA pool may be due to reduced protein synthesis, a metabolic process directly associated with growth performance, or to an increase in protein degradation. The latter possibility is supported by the up-regulation of the hepatic urea cycle observed in the RSF pigs, as indicated by the reduced concentrations of intermediates of the urea cycle (Arg, Cit, and Orn) in the liver and the increase of nitrogen carriers in the serum (Glu and Gln) of the RSF pigs. In addition to the changes in total FAA, specific individual AA were also affected by RS feeding. Hepatic free Ser and Gly, two important intermediate metabolites in one-carbon metabolism, were decreased and increased by the RS diet, respectively. Serine hydroxymethyltransferase (**SHMT**) is the primary enzyme in the conversion of serine into glycine (Narkewicz *et al*., 1996). It is unknown if RS feeding could affect the expression level and the activity of this enzyme. Moreover, the concentrations of GABA, a minor non-proteinogenic AA and an inhibitory neurotransmitter, dramatically increased from duodenum to cecum, as well as in the liver after RSF feeding. GABA is found in many plants, including *Brassica* species such as Chinese cabbage (Bouche and Fromm, 2004; Kim *et al*., 2013). Three types of GABA receptors are expressed along the intestinal tract and modulate both motor and secretory activities (Auteri *et al*., 2015; Krantis, 2000). Interestingly, sinapic acid, the most abundant phenolic compound in RS, was reported to potentiate GABA signals (Yoon *et al.*, 2007). Because it is unknown whether the increase of GABA and its co-presence with sinapic acid in the intestinal lumen could contribute to the RS-elicited changes in AA digestibility, further investigation is warranted.

Oxidative stress. The decrease of ascorbic acid and the increase of multiple oxidative stress markers, including oxidized thiol metabolites and pyroglutamate were the most prominent effects of RSF on hepatic and serum metabolome. These observations indicate a disruption in the redox balance status of pigs fed RSF. This observation is consistent with the organ toxicity observed in previous RS feeding experiments (Mawson *et al*., 1994b). However, our results are in contrast to the antioxidant properties assigned to certain RS components based on the following facts: 1) RS is a rich source of vitamin C (Krumbein *et al*., 2005); 2) sinapic acid and other phenolic acids in RS can function as antioxidants (Gaspar *et al*., 2010; Nićiforović and Abramovič, 2014); and 3) isothiocyanates, the degradation products of glucosinolates, are robust inducers of antioxidant enzymes (de Figueiredo *et al.*, 2013). It is likely that the amount of RS co-products in the diet used in the present experiment may have played a major

role in the observed disruption of redox homeostasis. In fact, enlarged thyroid glands were observed in the RSF pigs (Pérez de Nanclares *et al*., 2017). It is likely that this goitrogenic dose caused a strong pro-oxidant effect that overrode the antioxidant activity of RS components. Future studies using a dose titration of RS and antioxidants are needed to validate this hypothesis. Despite the apparent development of oxidative stress, short-term RS feeding did not affect the growth of the pigs in the current study. However, negative influences of RS feeding on growth performance have been observed in longer-term experiments (Choi *et al*., 2015; Kracht *et al*., 2004), as well as in our following long-term feeding study (data not shown). The relationship between the changes in oxidative stress induced by feeding RS and the changes in FE and growth performance remains to be elucidated.

Lipid peroxidation is a notable response among the diverse and extensive consequences of oxidative stress. Butanal is an aldehydic product of lipid peroxidation (Esterbauer *et al.*, 1982), and its concentrations in serum and liver was increased by feeding the RS diet. The higher concentrations of oleic acid-containing PC observed in the liver and serum of pigs fed the RS diet is consistent with the fact that RS (canola) oil contains more oleic acid than soybean oil (USDA Food Composition Databases, https://ndb.nal.usda.gov/ndb/). However, although RS oil has a greater concentration of α-linolenic acid, a precursor for DHA, than soybean oil (USDA Food Composition Databases, https://ndb.nal.usda.gov/ndb/), DHA-containing PC were decreased in the liver and serum of RSF pigs. A plausible explanation is that both α-linolenic acid and DHA, which are omega-3 fatty acids, are more susceptible to lipid peroxidation compared with oleic acid. Therefore, the observed change in PC may be attributed to the oxidative stress. In fact, when RS products were fed together with selenium, an important co-factor of the glutathione peroxidase complex, greater concentrations of omega-3 fatty acids, including DHA, were detected in meat and back fat of growing-finishing pigs (Gjerlaug-Enger *et al.*, 2015). Therefore, alleviation of oxidative stress may be an effective approach for improving animal growth performance when feeding RS diets to pigs.

In summary, the disposition of RS phytochemicals and nutrients, as well as their influences on microbial, lipid, and antioxidant metabolism, were examined through both untargeted metabolomic analysis and targeted quantitative analysis of digesta, liver, and serum samples from young pigs fed a RS diet compared to a SBM-control diet. RS feeding had limited effects on microbial and AA metabolism, but it had a prominent negative influence on the antioxidant homeostasis of young pigs. Identifying the specific source or sources of oxidative stress, investigating its impact on feed efficiency and growth performance, and exploring potential

444 dietary interventions will be meaningful topics in further research on using RS co-products as
445 a source of energy, protein, and other nutrients in animal feeds.

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Figure captions

Figure 1. Apparent ileal digestibility (AID) of CP and AA in young pigs fed with soybean-based diet (control) or rapeseed feeds for three wk. Values (%) are least-squares means, n = 18 for control group and 20 for the RSF group.

Figure 2. Identification of exposure markers through metabolomic modeling of digesta samples after feeding young pigs with soybean-based control or rapeseed-based diets for 3 wk. Data from Liquid Chromatography Mass Spectroscopy (LC-MS) analysis of digesta samples (duodenum, jejunum, ileum, cecum, and colon) were processed by principal component analysis (PCA) for identifying rapeseed-induced metabolic changes and temporal-spatial distribution of metabolites along the intestinal tract. Concentrations and distribution of rapeseed phytochemicals across the intestine were determined. **A.** Scores plot of PCA model. **B.** Loadings plot of the PCA model. Major metabolites contributing to the separation between the control and rapeseed samples as well as the separation between the small and large intestine samples are labeled. ↑ indicates positive correlation. **C.** Sinapine. **D**. Sinapic acid. **E.** Gluconapin.

Figure 3. Influences of rapeseed on the distribution of free AA (FAA) along the intestinal tract: (D = duodenum, J = jejunum, I = ileum, Ce = cecum, Co = colon). Concentrations of FAA along the intestinal tract were quantified and then processed by clustering analysis. **A**. Heat map of the FAA distribution along the intestinal tract. ↑ indicates positive correlation. **B.** Total free AA (FAA). **C.** Phenylalanine. **D.** Tryptophan. **E.** Methionine. **F.** Valine. **G.** Histidine. **H.** Threonine. **I.** Alanine. **J.** Gamma Amino Butyric Acid (GABA). Significant differences between the control and Rapeseed Feed (RSF) samples are marked by * ($P < 0.05$) and ** ($P < 0.01$).

Figure 4. Distribution of metabolites related to microbial metabolism in the intestine after feeding young pigs with soybean meal-based control or rapeseed feed (RSF) for three wk. Concentrations of short-chain fatty acids and bile acids were determined. **A.** Acetic acid. **B.**

584 Propionic acid. **C.** Butyric acid. **D.** Hyodeoxycholic acid. **G.** Lithocholic acid. **H.**
585 Taurochenodeoxycholic acid.

586 **Figure 5.** Influences of rapeseed feeding on pig hepatic metabolome. Data from liquid
587 chromatogramphy mass spectroscopy (LC-MS) analysis of aqueous and lipid fractions of liver
588 samples were processed by partial least squares-discriminant analysis (PLS-DA) to identify
589 rapeseed-induced metabolic changes in the liver. **A**. Scores plot of PLS-DA model. **B**.
590 Loadings plot of PLS-DA model. ↑ indicates positive correlation. Major metabolites
591 contributing to the separation between the soybean meal-control and the rapeseed samples are
592 labeled. **C.** Metabolites associated with redox balance. **D.** Serine and glycine. **E.** Urea cycle
593 metabolites. **F.** Phosphatidylcholine metabolites. Significant differences between the control
594 and RSF samples are marked by * ($P < 0.05$) and ** ($P < 0.01$).

595

596 **Figure 6.** Influences of rapeseed feeding on pig serum metabolome. Data from liquid
597 chromatography-mass spectroscopy (LC-MS) analysis of serum samples were processed by
598 partial least squares-discriminatory analysis (PLS-DA) for identifying rapeseed-induced
599 metabolic changes in serum. **A**. Scores plot of PLS-DA model. **B**. Loadings plot of PLS-DA
600 model. Major metabolites contributing to the separation between the soybean meal-control and
601 the rapeseed samples are labeled. ↑ indicates positive correlation. **C.** Metabolites associated
602 with redox balance. **D.** Phosphatidylcholine metabolites. Significant differences between the
603 control and RSF samples are marked by * ($P < 0.05$) and ** ($P < 0.01$).

Figures

Figure 1

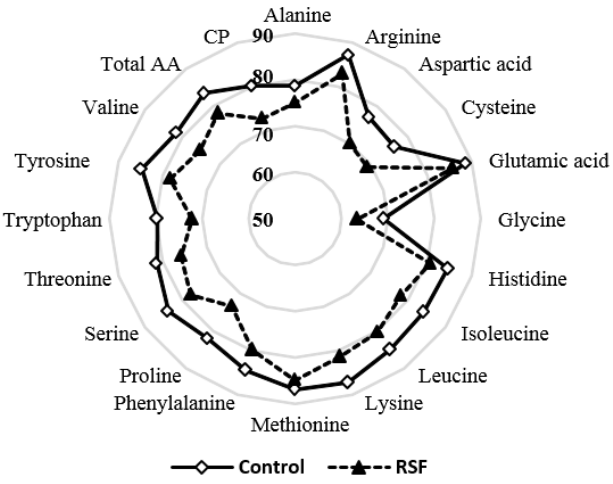


Figure 2

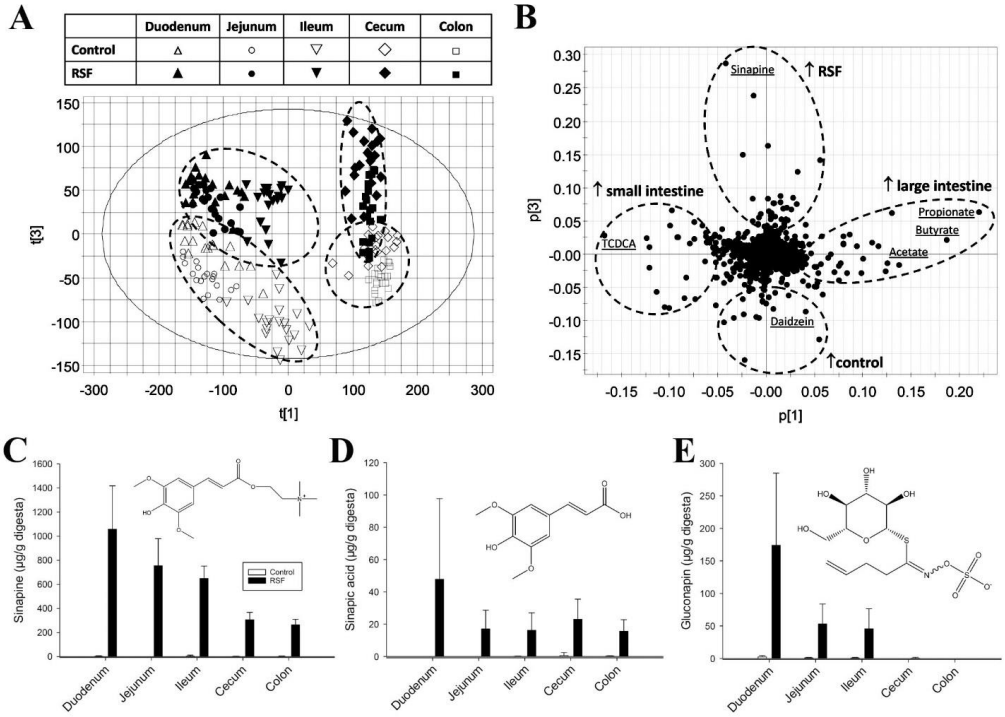

A
Duodenum
Jejunum
Ileum
Cecum
Colon
Control
RSF
t[1]
t[3]
B
Sinapine
↑ RSF
↑ small intestine
↑ large intestine
Propionate
Butyrate
TCDCA
Acetate
Daidzein
↑control
p[1]
p[3]
C
Sinapine (μg/g digesta)
Control
RSF
D
Sinapic acid (μg/g digesta)
E
Gluconapin (μg/g digesta)

Figure 3

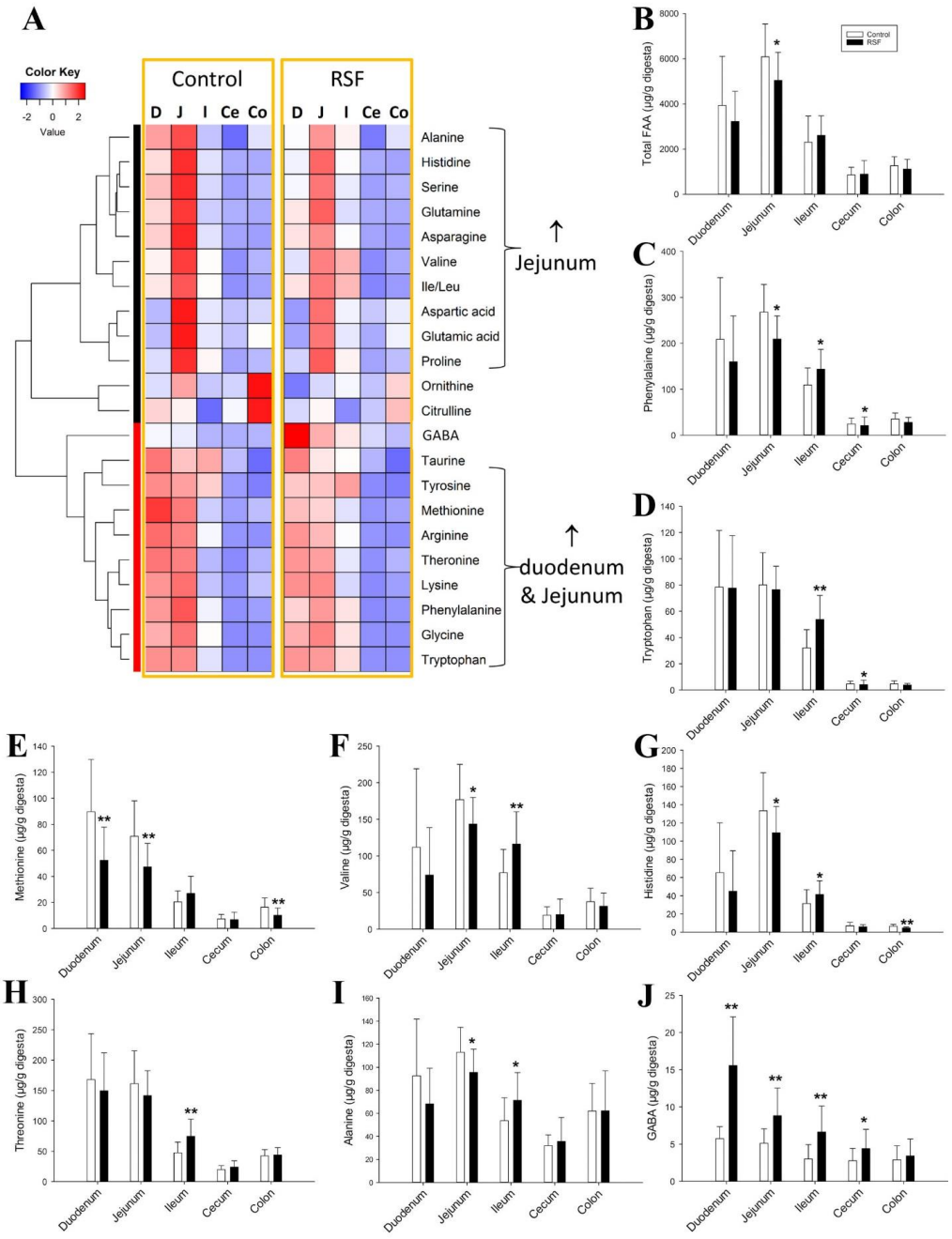

A
Color Key
-2 0 2
Value
Control
RSF
D J I Ce Co
Alanine
Histidine
Serine
Glutamine
Asparagine
Valine
Ile/Leu
Aspartic acid
Glutamic acid
Proline
Ornithine
Citrulline
GABA
Taurine
Tyrosine
Methionine
Arginine
Theronine
Lysine
Phenylalanine
Glycine
Tryptophan
Jejunum
duodenum & Jejunum
B
Total FAA (μg/g digesta)
Control
RSF
C
Phenylalaine (μg/g digesta)
D
Tryptophan (μg/g digesta)
E
Methionine (μg/g digesta)
F
Valine (μg/g digesta)
G
Histidine (μg/g digesta)
H
Threonine (μg/g digesta)
I
Alanine (μg/g digesta)
J
GABA (μg/g digesta)
Duodenum
Jejunum
Ileum
Cecum
Colon

Figure 4

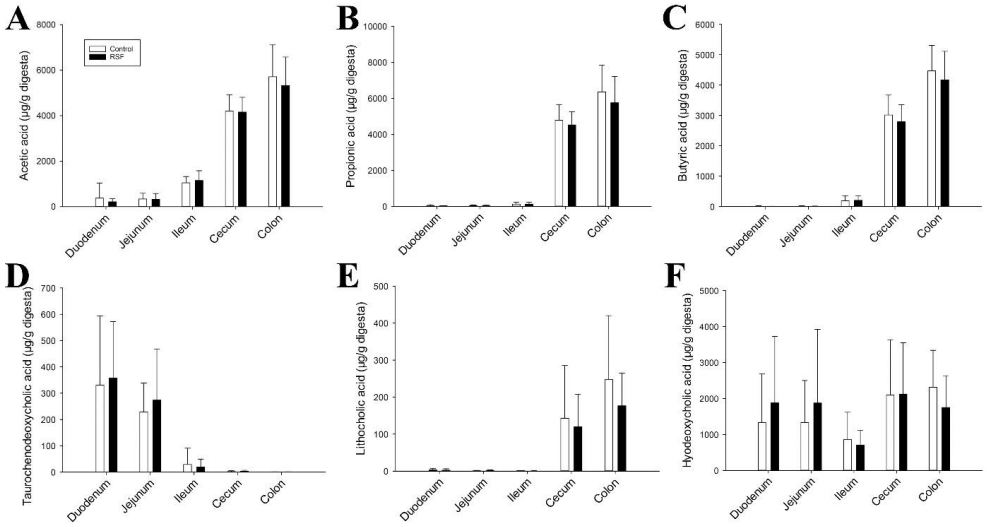

A
Acetic acid (μg/g digesta)
Control
RSF
Duodenum
Jejunum
Ileum
Cecum
Colon
B
Propionic acid (μg/g digesta)
C
Butyric acid (μg/g digesta)
D
Taurochenodeoxycholic acid (μg/g digesta)
E
Lithocholic acid (μg/g digesta)
F
Hyodeoxycholic acid (μg/g digesta)

Figure 5

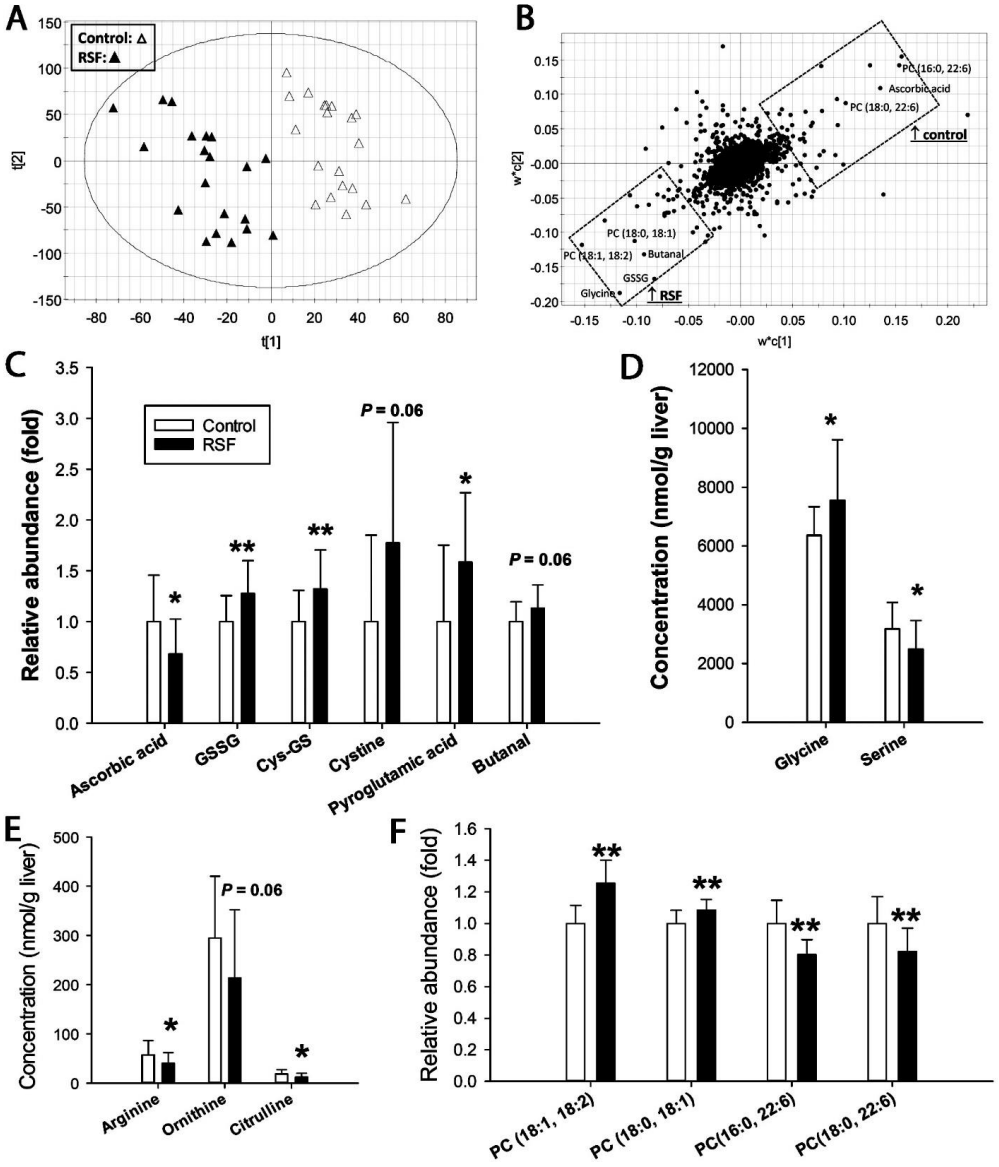
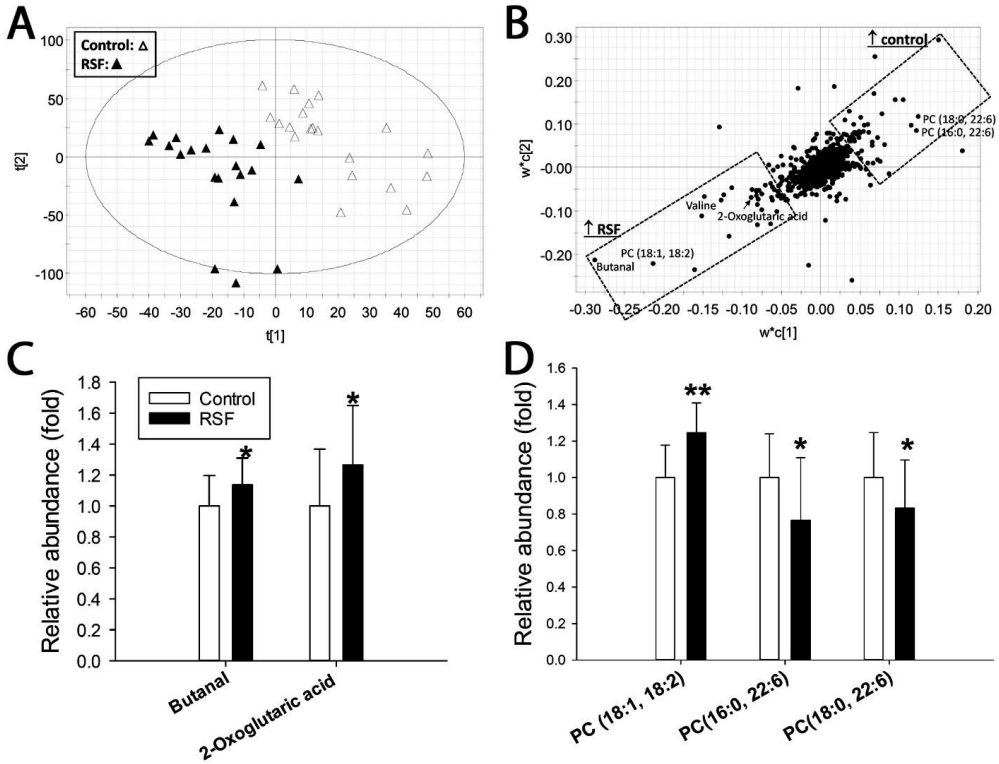

A
Control: △
RSF: ▲
t[2]
t[1]
B
PC (16:0, 22:6)
Ascorbic acid
PC (18:0, 22:6)
↑ control
PC (18:0, 18:1)
PC (18:1, 18:2)
Butanal
GSSG
Glycine
↑ RSF
w*c[2]
w*c[1]
C
Control
RSF
Relative abundance (fold)
P = 0.06
P = 0.06
Ascorbic acid
GSSG
Cys-GS
Cystine
Pyroglutamic acid
Butanal
D
Concentration (nmol/g liver)
Glycine
Serine
E
Concentration (nmol/g liver)
P = 0.06
Arginine
Ornithine
Citrulline
F
Relative abundance (fold)
PC (18:1, 18:2)
PC (18:0, 18:1)
PC(16:0, 22:6)
PC(18:0, 22:6)

Figure 6


A
Control: △
RSF: ▲
t[2]
t[1]
B
↑ control
↑ RSF
PC (18:0, 22:6)
PC (16:0, 22:6)
Valine
2-Oxoglutaric acid
PC (18:1, 18:2)
Butanal
w*c[2]
w*c[1]
C
Control
RSF
Relative abundance (fold)
Butanal
2-Oxoglutaric acid
D
Relative abundance (fold)
PC (18:1, 18:2)
PC(16:0, 22:6)
PC(18:0, 22:6)

Supplemental information for "Identification of redox imbalance as a prominent metabolic response elicited by rapeseed feeding in swine metabolome" C. Chen, M. Pérez de Nanclares, J. F. Kurtz, M. P. Trudeau, L. Wang, D. Yao, M. Saqui-Salces, P. E. Urriola, L. T. Mydland, G. C. Shurson, M. Overland

Table S1. Dietary composition of experimental diets

Ingredient, g/kg as-fed	**Control**[a]	**Rapeseed based feed**[a]
Wheat[b]	629.1	506.5
Barley[c]	100.0	100.0
Soybean meal[d]	140.0	30.0
Coarse rapeseed meal[e]	–	200.0
Rapeseed hulls[f]	–	40.0
Fish meal	40.0	40.0
Soybean oil	50.0	50.0
Monocalcium phosphate	16.4	9.1
Limestone	11.3	11.2
L-Lys·HCl	3.4	3.4
DL-Met	0.5	0.5
L-Thr	1.3	1.3
L-Trp	0.2	0.2
Sodium chloride	4.0	4.0
Vitamin and trace mineral premix[g]	3.2	3.2
Attractant[h]	0.5	0.5
Marker (Y_2O_3)	0.1	0.1

[a] Control diet based on wheat and soybean meal; RSF = rapeseed-based feed.
[b] Whole wheat: 86.4% DM, 11.1% CP, 1.6% EE, 58.1% starch, 9.0% NDF, 2.2% ADF, 1.4% ash.
[c] Barley: 86.2% DM, 7.4% CP, 1.3% EE, 53.5% starch, 16.0% NDF, 5.1% ADF, 1.6% ash.
[d] Soybean meal: 89.0% DM, 43.3% CP, 1.4% EE, 1.4% starch, 8.9% NDF, 5.7% ADF, 5.4% ash.
[e] Coarse fraction from an air-classified hexane-extracted rapeseed meal: 90.0% DM, 31.2% CP, 2.5% EE, 26.2% NDF, 18.6% ADF, 6.7% ash.
[f] Rapeseed hulls: 88.8% DM, 13.2% CP, 8.0% EE, 55.1% NDF, 48.6% ADF, 4.4% ash.
[g] Provided per kilogram of diet: 90 mg Zn (ZnO); 90 mg Fe ($FeSO_4$); 45 mg Mn (MnO); 19.5 mg Cu ($CuSO_4$); 0.45 mg I ($Ca(IO_3)_2$); 5700 IU vitamin A; 4500 IU cholecalciferol; 100.7 mg dl-α-tocopheryl acetate; 2.40 mg menadione; 9.0 mg riboflavin; 36.0 mg D-pantothenic acid; 12.0 μg cyanocobalamine; 12.0 mg niacin; 0.24 mg biotin; and 1.8 mg folic acid.
[h] Maxarome; Felleskjøpet, Kambo, Norway.

Table S2. Measured concentrations of chemical components in experimental diets.

Item, g/kg of DM	Control	Rapeseed based Feed
Gross energy, MJ/kg	17.6	17.8
DM, g/kg	908.4	906.1
CP, %	201.8	201.8
Ether extract, %	79.2	87.7
Starch, %	402.1	370.8
NDF, %	113.0	154.8
ADF, %	41.8	82.3
Ash, %	57.0	60.8
P, %	9.7	8.7
Y, %	0.1	0.1
Amino acid, %		
Ala	8.9	9.1
Arg	11.5	11.1
Asp	17.1	15.3
Cys	3.7	4.5
Glu	43.6	41.3
Gly	9.5	10.1
His	5.0	5.1
Ile	8.4	8.3
Leu	14.7	14.4
Lys	12.8	13.2
Met	4.0	4.3
Phe	9.0	8.2
Pro	14.7	15.0
Ser	10.4	10.0
Thr	9.4	10.4
Trp	2.8	2.7
Tyr	5.1	5.2
Val	9.2	9.8
Total amino acids, %	199.8	198.0
Monosaccharides, %		
Arabinose	13.3	20.2
Fucose	4.2	4.2
Galactose	12.2	11.1
Glucosamine	0.4	0.7
Total glucose	588.9	457.7
Rhamnose	1.1	2.3
Xylose and Mannose	19.7	18.8
Total glucosinolates, mmol/kg	–	1.0

Table S3. Concentration of free AA (µg/g digesta, mean ± SD) in digesta of pigs fed a corn and soybean meal based diet (Control) and a diet based on rapeseed products (RSF)

Amino acid	Duodenum		Jejunum		Ileum		Cecum		Colon	
	Control	RSF	Control	RSF	Control	RSF	Control	RSF	Control	RSF
Alanine	92.57 ± 49.27	68.12 ± 30.95	113.03 ± 21.60	95.55 ± 20.21	53.62 ± 19.84	71.35 ± 24.04	31.99 ± 9.35	35.69 ± 20.74	62.06 ± 23.96	62.15 ± 34.78
Arginine	562.05 ± 381.98	477.74 ± 326.74	507.08 ± 128.07	393.93 ± 125.74	234.18 ± 81.03	236.01 ± 73.77	19.92 ± 18.70	12.74 ± 9.93	9.30 ± 4.82	7.17 ± 5.10
Asparagine	78.38 ± 40.88	67.95 ± 37.86	171.98 ± 66.1	115.16 ± 39.68**	36.79 ± 21.83	49.33 ± 21.99	0.51 ± 0.60	0.69 ± 0.55	0.18 ± 0.15	0.24 ± 0.13
Aspartic acid	139.54 ± 41.47	109.37 ± 32.62	469.87 ± 241.77	372.72 ± 189.56	194.21 ± 129.51	205.18 ± 113.12	151.38 ± 54.54	159.70 ± 66.46	172.86 ± 63.60	198.68 ± 97.72
Citrulline	14.07 ± 11.42	9.17 ± 5.00	12.14 ± 8.47	11.76 ± 8.42	2.64 ± 1.79	4.47 ± 4.23	10.91 ± 7.03	8.11 ± 4.93	25.01 ± 10.27	15.35 ± 6.37**
GABA	5.42 ± 2.05	15.55 ± 6.54**	5.10 ± 1.95	8.82 ± 3.73**	2.99 ± 1.95	6.63 ± 3.50**	2.77 ± 1.67	4.40 ± 2.58*	2.89 ± 1.90	3.41 ± 2.27
Glutamic acid	281.08 ± 128.76	201.65 ± 76.18	1172.31 ± 563.24	884.57 ± 390.12	407.00 ± 285.65	402.32 ± 230.34	291.72 ± 133.34	221.63 ± 106.82	477.58 ± 197.77	435.16 ± 223.14
Glutamine	301.05 ± 228.42	274.50 ± 229.23	689.63 ± 162.64	595.46 ± 207.21	97.67 ± 35.29	125.08 ± 50.20	12.53 ± 7.74	11.64 ± 5.38	7.47 ± 2.93	7.61 ± 3.55
Glycine	485.85 ± 369.98	482.12 ± 362.44	611.65 ± 221.51	569.95 ± 278.27	304.10 ± 195.39	362.65 ± 139.28	49.58 ± 22.27	59.77 ± 35.58	57.58 ± 20.52	55.07 ± 17.52
Histidine	57.64 ± 44.42	36.88 ± 26.74*	133.36 ± 41.95	109.31 ± 28.82	31.47 ± 15.18	41.36 ± 15.14*	7.00 ± 3.67	5.54 ± 1.55	6.54 ± 1.96	4.58 ± 1.05**
Ile/Leu	88.37 ± 51.31	92.08 ± 54.39	171.99 ± 46.54	151.01 ± 38.95	77.00 ± 27.95	116.94 ± 34.36	12.85 ± 8.05	8.38 ± 4.44	25.96 ± 11.37	20.94 ± 9.86
Lysine	538.56 ± 302.48	551.55 ± 250.15	617.59 ± 163.33	560.11 ± 183.42	181.37 ± 76.22	228.13 ± 83.77	78.56 ± 41.00	47.06 ± 15.89	177.44 ± 66.84	101.46 ± 41.20**
Methionine	89.72 ± 40.17	52.36 ± 25.48**	70.80 ± 27.22	47.31 ± 18.02**	20.47 ± 8.39	26.94 ± 13.19	7.38 ± 3.53	5.53 ± 2.25	16.39 ± 7.13	10.12 ± 5.59
Ornithine	6.32 ± 5.84	2.91 ± 2.53	10.79 ± 13.07	6.15 ± 8.46	5.74 ± 7.89	7.25 ± 8.03	5.97 ± 4.21	5.44 ± 4.55	16.32 ± 10.12	9.28 ± 7.45*
Phenylalanine	208.53 ± 134.55	159.77 ± 99.89	267.84 ± 60.12	209.41 ± 50.23**	109.33 ± 36.82	143.51 ± 43.42*	24.41 ± 12.91	17.13 ± 6.45	35.25 ± 12.87	27.86 ± 10.70
Proline	53.52 ± 32.49	47.83 ± 28.26	216.99 ± 48.65	184.50 ± 68.62	83.30 ± 51.24	84.44 ± 45.63	17.31 ± 13.55	16.64 ± 8.32	31.10 ± 16.70	34.48 ± 19.03
Serine	131.76 ± 91.73	79.46 ± 54.69*	232.31 ± 71.96	182.38 ± 53.96*	57.32 ± 19.88	72.36 ± 33.50	19.08 ± 9.03	19.48 ± 7.07	35.94 ± 9.50	31.94 ± 9.36
Taurine	41.13 ± 26.87	39.71 ± 23.99	30.01 ± 24.38	25.48 ± 27.00	34.10 ± 14.49	22.28 ± 15.25	12.88 ± 11.38	11.25 ± 8.67	0.98 ± 0.37	0.79 ± 0.42
Theronine	167.87 ± 75.66	149.44± 62.51	161.24 ± 54.08	141.56 ± 41.25	47.43 ± 17.64	74.58 ± 28.38	19.81 ± 6.63	22.11 ± 5.61	42.45 ± 10.27	43.86 ± 11.98
Tryptophan	78.38 ± 43.16	77.62 ± 40.02	80.11 ± 24.55	76.46 ± 17.84	32.01 ± 13.91	53.72 ± 18.26	4.63 ± 2.17	3.41 ± 1.31	4.61 ± 2.33	3.80 ± 1.13
Tyrosine	169.25 ± 97.78	128.74 ± 92.36	155.42 ± 64.27	131.47 ± 58.59	126.52 ± 53.66	154.49 ± 80.09	31.29 ± 16.31	22.28 ± 7.46	17.45 ± 11.42	15.67 ± 9.77
Valine	82.13 ± 62.00	61.49 ± 33.72	176.86 ± 48.14	143.63 ± 36.36*	77.05 ± 31.95	116.22 ± 44.04**	18.91 ± 11.56	15.80 ± 10.93	37.51 ± 18.33	31.29 ± 17.96
Total AA	3935.84 ± 2174.30	3223.04 ± 1338.69	6089.97 ± 1451.23	5046.26 ± 1239.32*	2308.24 ± 1156.86	2609.93 ± 862.11	859.03 ± 338.29	892.27 ± 602.90	1262.86 ± 397.27	1121.04 ± 426.23

Table S4. Concentrations of free amino acids (nmole/g liver, mean ± SD) in the liver of pigs fed a corn soybean meal based diet and a diet based on rapeseed products (RSF)

Amino acids	**Control**	**RSF**
Alanine	2849.84 ± 1223.14	2216 ± 1068.8
Arginine	57.04 ± 29.28	40.04 ± 21.87*
Asparagine	245.4 ± 62.54	195.68 ± 75.72*
Aspartic acid	918.56 ± 638.41	865.92 ± 667.66
Citruline	18.32 ± 8.94	12.12 ± 8.02*
GABA	16 ± 6.78	27.08 ± 13.87**
Glutamic acid	2637.32 ± 1387.41	2038.8 ± 1492.46
Glutamine	2151.12 ± 760.62	2202.4 ± 927.95
Glycine	6358.92 ± 979.69	7549.08 ± 2060.55*
Histidine	295.32 ± 60	283.04 ± 88.18
Iso/leucine	632.12 ± 170.28	539.4 ± 204.37
Lysine	170.44 ± 74.1	138.12 ± 84.7
Methionine	94.52 ± 45.34	112.68 ± 48.51
Ornithine	294.56 ± 125.59	213.4 ± 138.7
Phenylalanine	329.12 ± 72.25	290.72 ± 93.07
Proline	1921.36 ± 322.34	1968.64 ± 606.39
Serine	3181.16 ± 902.63	2486.72 ± 976.06*
Threonine	1096.4 ± 277.37	1185.4 ± 430.45
Tryptophan	26.88 ± 5.13	24.52 ± 7.17
Tyrosine	64.52 ± 40.36	43.64 ± 51.37
Valine	671.52 ± 192.96	614.16 ± 247.85
Total AA	24030.44 ± 5704.91	23047.56 ± 7491.46

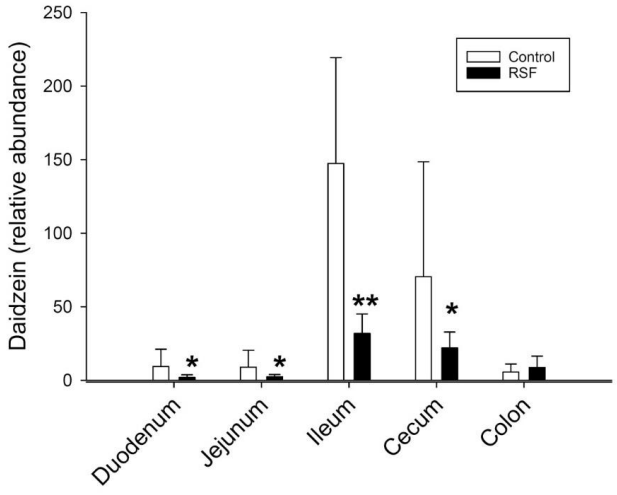
*: $P < 0.05$. **: $P < 0.01$.

Table S5. Concentrations of free amino acids (μM, mean ± SD) in serum of pigs fed a corn-soybean meal based diet and a diet based on rapeseed products (RSF)

Amino acids	**Control**	**RSF**
Alanine	444.18 ± 107.9	472.67 ± 82.08
Arginine	18.19 ± 4.63	18.96 ± 6.06
Asparagine	86.06 ± 30.45	92.49 ± 19.85
Aspartic acid	149.39 ± 73.84	194.52 ± 72.9
Citrulline	42.13 ± 11.72	40.54 ± 10.25
GABA	0.96 ± 1.35	0.44 ± 0.53
Glutamic acid	1177.58 ± 503.22	1556.34 ± 545.89*
Glutamine	398.9 ± 92.28	464.68 ± 73.63*
Glycine	1727.24 ± 352.05	1912.33 ± 316.32
Histidine	122.64 ± 39.37	111.62 ± 29.07
Ile/Leu	84.27 ± 22.06	96.56 ± 19.17
Lysine	137.59 ± 35.07	158.56 ± 52.58
Methionine	231.91 ± 74.04	236.47 ± 65.46
Ornithine	60.15 ± 19.7	67.12 ± 22.99
Phenylalanine	60.97 ± 14.92	61.86 ± 10.23
Proline	415.56 ± 84.55	456.39 ± 68.89
Serine	296.48 ± 90.13	326.31 ± 88.87
Taurine	193.07 ± 63.39	213.04 ± 56.64
Theronine	413.28 ± 150.9	578.37 ± 213.74*
Tryptophan	35.3 ± 13.64	36.3 ± 10.69
Tyrosine	215 ± 80.07	191.57 ± 46.35
Valine	293.14 ± 72.8	386.12 ± 76.18**
Total AA	6603.94 ± 1648.15	7673.27 ± 1182.26*

*: $P < 0.05$. **: $P < 0.01$.

Figure S1. Distribution of daidzein along the intestinal tract of pigs fed a corn-soybean meal based diet (control) and a diet containing rapeseed products (RSF). Relative abundance of daidzein in the digesta samples was determined by calculating the ratio of its ion counts versus the total ion counts of a sample (× 10,000). Significant differences between the control and RSF samples are marked by * ($P < 0.05$) and ** ($P < 0.01$).



Paper III

Increasing levels of rapeseed meal in diets for pigs: Effects on protein and energy metabolism

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Short title: Metabolism in pigs fed rapeseed meal diets

Abstract

The heavy reliance on imported soybean meal (**SBM**) as a protein source makes it necessary for the European pig industry to search for alternatives and to develop robust pigs that perform efficiently when fed such ingredients. Digestion and metabolism are major physiological processes contributing to variation in feed efficiency. Therefore, an experiment was conducted to assess the effects of replacing SBM with increasing levels of rapeseed meal (**RSM**) in diets for young pigs on apparent total tract digestibility (**ATTD**) of energy and nutrients, nitrogen (**N**) balance, energy metabolism, and substrate oxidation. Four diets were fed to 32 pigs (22.7 ± 4.1 kg initial BW) for three weeks. The diets consisted of a control cereal grain-SBM basal diet and three test diets where SBM and wheat were partially replaced with 10, 20, and 30% of RSM. Increasing level of RSM in the diets linearly reduced ATTD of organic matter, crude protein, total carbohydrates, dietary fiber, fat, and energy. Utilization of digested nitrogen (**DN**) for N retention and total N excretion were not affected by RSM inclusion, however, RSM inclusion induced a shift in N excretion from urine to feces. Despite a linear increase in liver index, heat production and utilization of metabolizable energy (**ME**) for retention were not affected by increasing RSM inclusion. In conclusion, replacing SBM with up to 30% of RSM in diets for young pigs reduced the digestive efficiency, but did not compromise protein and energy metabolism or efficiency of utilization of DN or ME for retention.

Keywords: digestibility, energy metabolism, heat production, nitrogen retention, rapeseed meal

Implications

Alternative protein feedstuffs to imported soybean meal (SBM) are needed to improve the sustainability and self-sufficiency of the European pig production. Rapeseed meal (RSM) is abundant and has a high protein content and a balanced amino acid profile and could serve as an alternative to SBM. However, RSM contains higher amounts of fiber and other antinutritional factors that could affect pig performance. The present experiment showed that SBM can be partially replaced with up to 30% RSM without compromising protein and

energy metabolism or efficiency of utilization of nitrogen or energy for retention in young pigs.

Introduction

Rapeseed meal (**RSM**), the co-product from rapeseed (**RS**) processing for oil extraction, is increasingly available in Europe due to the rapid growth of the biofuels industry (Carré and Pouzet, 2014). Increased and efficient use of RSM as an alternative protein source to soybean meal (**SBM**) could decrease the dependency on imports and improve the sustainability and self-sufficiency of European pig production. Because feed represents up to 70% of the total cost for pig producers, small improvements or even similar feed efficiency when using RSM compared with SBM could enhance profitability. In this regard, digestive and metabolic efficiencies are key factors affecting the net efficiency of any pig system. Le Goff and Noblet (2001) reported that the digestive loss of nutrients and energy in most pig rearing systems ranges from 15 to 25% of the total intake. Variation in energy and nutrient metabolism and partitioning following digestion will further determine the net efficiency of pigs (Gilbert *et al.*, 2017). Rapeseed meal has a higher fiber content than SBM and contains other anti-nutritional factors (**ANF**) such as tannins, phytic acid, and glucosinolates, which could interfere with the digestive and metabolic processes of the pigs (Mejicanos *et al.*, 2016). High dietary fiber (**DF**) is associated with impaired nutrient utilization and reduced net energy (**NE**) values in the diet (Noblet and Le Goff, 2001) and increased viscera weight in pigs (Jørgensen *et al.*, 1996). Because visceral organ mass is a major contributor to fasting heat production (van Milgen *et al.*, 1998), influence of RSM-feeding on organ size may affect the total heat production of the pigs. Goitrogenic effects associated with glucosinolates in RSM could also lead to changes in basal metabolic rate and overall energy expenditure (Kim, 2008), and in protein turnover (Hoquette *et al.*, 1998).

Given this scenario, an in depth understanding of the effects of RSM-based diets on the digestive and metabolic efficiency of pigs is of major interest. The objective was to investigate the effects of increasing inclusion of RSM, replacing SBM in isonitrogenous and isoenergetic diets, on nutrient and energy digestibility, nitrogen (**N**) retention, and energy metabolism by means of balance and respiration experiments with young pigs. We hypothesised that replacing SBM with RSM at iso-NE and iso-standardized ileal digestible (**SID**) crude protein (**CP**) levels would reduce nutrient and energy digestibility, N retention, and increase energy expenditure in young pigs.

Materials and methods

The research protocol complied with the guidelines of The Animal Experiments Inspectorate, Ministry of Environment and Food, Copenhagen, Denmark, regarding animal experimentation and care. The experiment was conducted at the Rørrendegård experimental farm of the University of Copenhagen in Tåstrup, Denmark.

Animals and experimental design

The experiment was conducted as a randomized complete block design with four experimental diets, four periods and two replicates per diet per period (a total of eight replicate pigs per diet). Each experimental period lasted three weeks, and consisted of a 2-week adaptation period followed by a 4-day balance period, which included a 22-h respiration experiment by means of indirect calorimetry in an open-air circulation system (Chwalibog *et al.*, 2004). In each period, eight castrated crossbred pigs (Danish Landrace/Yorkshire × Duroc) from two litters were obtained from a commercial herd. One pig from each litter was allocated to one of the four dietary treatments. The pigs from periods 1-4 had an average initial BW of 19.2 ± 3.2, 21.9 ± 4.2, 24.5 ± 4.0 and 25.1 ± 2.4 kg, respectively.

Diets

The RSM was a commercial expeller-pressed RSM (Mestilla UAB, Klaipėda, Lithuania). The four experimental diets were: a control diet (RSM0) based on wheat, barley, oats and SBM, with no RSM, and three treatment diets (RSM10, RSM20, and RSM30), in which SBM and wheat were partially replaced with 10, 20 and 30% of RSM, respectively (Table 1). The diets were optimized based on the chemical analyses and on SID table values for the feed ingredients (Sauvant *et al.*, 2004). The NE values for all the ingredients were calculated based on the Dutch energy evaluation system (CVB). All four diets were optimized to meet or exceed the requirements for essential amino acids and all other nutrients and energy for pigs of this age (NRC, 2012). The diets were subsequently mixed and pelleted at the Centre for Feed Technology (Fôrtek) of the Norwegian University of Life Sciences (Ås, Norway).

Housing and feeding

During the adaptation period, pigs were housed in pairs in pens with concrete floors covered with straw. During the balance periods, pigs were housed individually in stainless steel metabolic cages (1.65 m × 0.75 m) with a slatted floor of round bars and devices for quantitative collection of feces, urine, and feed residues. All pigs were provided with a rubber mat in the front of the metabolism cages.

Upon arrival at the experimental farm, pigs were offered a commercial weaner diet, which was gradually replaced by one of the four experimental diets. Pigs within the same pen were assigned randomly to one of the four experimental diets and they were separated at every meal to allow individual feeding. Pigs were fed equal meals twice daily at 0900 and 1500 h throughout the experimental period, except during the respiration experiment, when they were fed one full meal shortly after transfer into the respiration chambers. Daily feed allowance was adjusted to a level where pigs had a minimum of feed residues, i.e. were fed as close to *ad libitum* as possible. Water was added to the feed immediately before feeding at a ratio of 2:1 (w/w). Pigs were provided *ad libitum* access to water from drinking nipples. The temperature was kept at 20 ± 2 °C and a 12-h light/12-h dark cycle was provided for the duration of the experiment.

Experimental procedures

Pigs were weighed the Friday prior to the start of the balance period and the following Friday, when the balance period ended. The collection was conducted between 0800 and 1200 h every day. Feed residues, feces, and urine were quantitatively collected from each individual pig, weighed, and frozen at -20 °C. Urine was collected in bottles containing 10 ml of 5% sulphuric acid, weighed, and 10% of urine from the daily samples were frozen at -20 °C. The bars inside the metabolic cages, the rubber mat, and the collection plate were rinsed with 1% citric acid solution daily after completion of the collection procedures, and the resulting slurry was collected in separate containers, weighed, and frozen at -20 °C. Diets were sampled daily during the balance periods and pooled before preparation for chemical analyses. Following each balance period, feed residues, feces, urine, and slurry were thawed, homogenized, and representative samples were taken and frozen at -20 °C for later chemical analyses.

The respiration experiments were performed in two open-air-circuit respiration chambers with a volume of 3500 l and constructed for animals with a live weight range of 5-200 kg. Pigs

were brought into the respiration chambers between 0930 and 1030 h at the assigned day for the respiration experiment. Each pig was measured for 22 h, starting at 1100 h and finishing at 0900 h the following morning. For a detailed description of the construction and function of the respiration chambers, as well as for the calibration and measurement procedures, see Chwalibog *et al.* (2004). At the end of each experimental period, pigs were euthanized and the liver was removed and weighed.

Sample processing and chemical analyses

Samples of feces were freeze-dried, and feces and diets were ground through a 1 mm screen and homogenized prior to chemical analyses. Feed residues were analyzed for dry matter (**DM**) and it was assumed that the chemical composition of the DM was equal to that in the DM of the feed. Wet feces were analyzed for DM and N, and freeze-dried feces for ash, fat, gross energy (**GE**), and fiber contents. Urine and slurry were analyzed for N.

Dry matter was measured by drying to constant weight at 105 °C, and ash was determined by incineration at 525 °C. Nitrogen was determined by the Kjeldahl method using the Tecator-Kjeltec system 1030 (Tecator AB, Höganäs, Sweeden) and CP calculated as N × 6.25. Fat content was determined by petroleum ether extraction in a Soxtec system 2043 (Foss, Hillerød, Denmark) after HCl hydrolysis. Total carbohydrate and lignin (**CHO + L**) content in the diet and feces was calculated from the previous analyses as:

$$CHO + L = DM - ash - CP - fat.$$

Gross energy was determined using an IKA Calorimeter system (IKA Gmbh and Co. KG, Staufen, Germany) and starch was analyzed according to Bach Knudsen (1997). Neutral detergent fiber (**NDF**) in the diets was determined using the Ankom200 Fiber Analyzer system with F58 Ankom filter bags (ANKOM Technologies, Fairport, NY, USA). Amino acid (**AA**) analysis and tryptophan in the diets were analyzed according to Commission dir. No 152/2009/EC. Amino acids were determined on a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK) while tryptophan was analyzed on a Dionex UltiMate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Total, soluble, and insoluble non-starch polysaccharides (**T-NSP**, **S-NSP** and **I-NSP**, respectively), and their constituent sugars were determined as alditol acetates by gas-liquid chromatography (**GLC**) for neutral sugars, and by a colorimetric method for uronic acids in three parallel runs as described by

Bach Knudsen (1997). Klason lignin was measured gravimetrically as the residue resistant to sulphuric acid hydrolysis as described by Theander *et al.* (1994). The following parameters were calculated from the previous analyses:

Total non-cellulosic polysaccharides (**T-NCP**) = rhamnose + fucose + arabinose + xylose + mannose + galactose + glucose + uronic acids

$Cellulose_{DF}$ = T-NSP – T-NCP

Insoluble-NCP (I-NCP) = I-NSP – $Cellulose_{DF}$

Soluble-NCP (S-NCP) = T-NCP – I-NCP

DF = T-NSP + Klason lignin

Total glucosinolate analysis of the RSM was performed according to Commission dir. No 1864/90/EEC.

Calculations

The apparent total tract digestibility (**ATTD**) of individual nutrients and energy was calculated according to the equation:

ATTD (% of intake) = [Intake (g) – Fecal excretion (g)/ Intake (g)] × 100

Based on 4 days quantitative collection of urine and feces, digested nitrogen (**DN**, g) was calculated as ingested nitrogen (**IN**) minus fecal nitrogen (**FN**). Retained nitrogen (**RN**, g) was calculated as DN minus urinary nitrogen (**UN**), and N in slurry. Energy in urine (**UE**, kJ) was calculated as 53.5, kJ/g × UN, g (Chwalibog *et al.*, 2004). Energy in methane was calculated as 39.6 kJ/l × CH_4, l (Brouwer, 1965). Metabolizable energy (**ME**, kJ) intake was calculated by subtracting fecal energy (**FE**), UE, and energy in methane from the GE intake. Heat production (**HE**) was calculated based on the 22-h measurements of gas exchange and the mean UN according to Brouwer (1965) as follows:

HE, kJ = 16.18 kJ/l × O_2, l + 5.02 × CO_2, l – 2.17 × CH_4, l – 5.99 kJ/g × UN, g

Retained energy (**RE**, kJ) was calculated as the difference between ME and HE. Energy retained as protein (**RPE**, kJ) was calculated from the N balance as RN, g × 6.25 × 23.86, kJ/g, and energy retained as fat (**RFE**, kJ) as the difference between total RE and RPE. The

respiratory quotient (**RQ**) was determined as the ratio between CO_2 production and O_2 consumption.

The oxidation of protein (**OXP**), carbohydrate (**OXCHO**), and fat (**OXF**) was calculated according to Chwalibog *et al.* (1992) and validated for RQ values below and above one (Chwalibog and Thorbek, 1995):

$$\text{OXP, kJ} = \text{UN, g} \times 6.25 \times 18.42\text{, kJ/g}$$

$$\text{OXCHO, kJ} = (-2.968 \times \text{O}_2\text{, l} + 4.174 \times \text{CO}_2\text{, l} - 1.761 \times \text{CH}_4\text{, l} - 2.446 \times \text{UN, g}) \times 17.58 \text{ kJ/g}$$

$$\text{OXF, kJ} = (1.719 \times \text{O}_2\text{, l} - 1.719 \times \text{CO}_2\text{, l} - 1.719 \times \text{CH}_4\text{, l} - 1.963 \times \text{UN, g}) \times 39.76 \text{ kJ/g}$$

The ME requirement for maintenance (**ME_m**) and the efficiency of utilization of ME for retention in body tissues (**k_g**) were estimated by two linear regression approaches, one where $ME^{0.60}$ was regressed on $RE^{0.60}$, where the intercept (**I**) denoted the ME_m value and k_g was the reciprocal of the slope (**b**). In a second approach $RE/kg^{0.60}$ was regressed on $ME/kg^{0.60}$, and ME_m was calculated as (-I/b) and the b-value was the k_g (Thorbek *et al.*, 1984). The scaling factor 0.60 was chosen because this is presently used by NRC (2012) for growing pigs.

Liver index was calculated as the ratio of liver weight to metabolic BW ($g/kg^{0.75}$).

Statistical analysis

Statistical analysis of general animal performance, ATTD, N balance, energy metabolism, and substrate oxidation data was performed using the general linear model procedure of SAS (1990) for a randomized complete block design, with individual pigs as the experimental unit. The fixed effects of diet (n = 4) and period (n = 4), and the effect of pig live weight as covariate for the general animal performance and ATTD analyses, were included in the statistical model. As the interaction between diet and period was non-significant it was removed from the model. The effect of the covariate pig live weight on ATTD was non-significant and therefore not included in the analysis. Treatment means were separated using the least squares-means test. Polynomial contrasts were used to determine the linear, quadratic, and cubic effects of increasing level of RSM in the diets. Results are presented as LS-means for each dietary treatment, and variance is expressed as the pooled standard error of the mean. Effects were considered significant if $P < 0.05$ and a tendency if $0.05 < P < 0.10$.

Linear regression analyses for determination of ME_m and k_g were performed using the procedure REG in SAS (1990).

Results

Diets

Partial replacement of SBM with increasing levels of RSM was reflected in the CHO + L composition of the diets (Table 2). The contents of T-NSP, cellulose, lignin, DF, and NDF increased with increasing RSM inclusion, while the starch content decreased. Regarding monomeric residues of NSP, RSM inclusion resulted in increased contents of rhamnose, arabinose, and uronic acids. Inversely, the contents of xylose, mannose, and galactose decreased with increasing RSM inclusion. The CP and GE concentrations were similar among diets, with slightly higher CP content in the RSM0. There were no major differences in the concentrations of essential AA between the diets and the concentrations of the five first limiting AA exceeded the requirements of the pigs (NRC, 2012). The RSM used in this experiment contained 11.3 μmol/g total glucosinolates, resulting in 1.13, 2.26, and 3.39 mmol/kg total glucosinolate calculated content in the RSM10, RSM20, and RSM30 diets, respectively.

Health status, intake of nutrients and performance

In general, the pigs had a good health status throughout the experiment, but some pigs experienced transitional loose stools. One pig was excluded from the study due to low feed intake and another pig was excluded due to technical problems during the balance period. Both pigs belonged to the RSM10 group. One pig on the RSM20 diet received antibiotic treatment (Tribrissen® Vet. 48%, Copenhagen, Denmark) following veterinary recommendations due to unthriftiness. The pigs consumed the diets readily and they performed well even on the diet with the highest RSM inclusion level. The BW, daily weight gain, and daily feed intake during the balance periods were similar among dietary groups, while the feces dry weight increased ($P = 0.011$, linear $P = 0.001$) with increasing inclusion of RSM in the diets (Supplementary Table S1). The liver index of the pigs increased (linear $P = 0.033$) with increasing inclusion of RSM (Figure 1).

Digestibility of dietary components

Partially replacing SBM with increasing levels of RSM reduced ($P < 0.01$, linear $P < 0.001$) the ATTD of organic matter (**OM**), CP, CHO, and energy (Table 3). The ATTD of fat ($P = 0.089$, linear $P = 0.038$) increased and ATTD of DF (linear $P = 0.034$) decreased with increasing RSM inclusion, while ATTD of T-NSP and cellulose was not affected by diet ($P > 0.10$). Regarding individual monomeric residues of NSP, the ATTD of fucose, mannose, glucose, and uronic acids was reduced ($P < 0.01$, linear $P < 0.001$) while ATTD of arabinose increased ($P = 0.013$, linear $P = 0.001$) with increasing RSM in the diet. Increasing dietary levels of RSM resulted in a tendency towards a linearly lower ATTD of xylose (linear $P = 0.063$) while the ATTD of rhamnose and galactose was unaffected by diet ($P > 0.10$).

Nitrogen metabolism

Increasing RSM levels in the diet resulted in a slightly lower (linear $P = 0.094$) intake of N (Table 4). Due to the numerically lower IN and the reduced ATTD of CP, the amount of DN decreased (linear $P = 0.003$, cubic $P = 0.054$) in pigs fed increasing levels of RSM. There was no dietary effect on the total N excretion ($P > 0.10$). Excretion of N in the feces increased linearly ($P = 0.003$, linear $P = 0.001$) while excretion of N in urine decreased (linear $P = 0.019$) with increasing RSM inclusion. Consequently, increasing dietary levels of RSM resulted in a decreased UN:FN ($P = 0.006$, linear $P < 0.001$). Retained nitrogen was not affected by diet ($P > 0.10$), nor was the efficiency of utilization of IN or DN for retention (RN:IN and RN:DN, both $P > 0.10$).

Energy metabolism

Due to the reduced ATTD of energy, there was a tendency towards a lower intake of DE (linear $P = 0.096$) in pigs fed increasing levels of RSM, while intake of ME ($P > 0.10$) was not affected by diet (Table 5). The HE was similar for all dietary treatments ($P = 0.082$, cubic $P = 0.057$), with the highest numerical value in the RSM0 group. The calculations of nutrient oxidation in proportion to HE showed that RSM inclusion tended to linearly reduce ($P = 0.062$) OXP, while having a cubic effect on OXCHO ($P = 0.061$) and OXF ($P = 0.067$). The pigs fed the diets with lower RSM inclusion (RSM0 and RSM10) had numerically lower OXF:HE and higher OXCHO:HE (Figure 2). Retained energy ($P > 0.10$) was not affected by diet, nor when it was expressed in proportion to ME ($P > 0.10$). Partitioning of RE between

protein and fat was not affected by diet ($P > 0.10$). Oxygen consumption and CH_4 production were not affected by diet ($P > 0.10$), while CO_2 production decreased in pigs fed increasing levels of RSM ($P = 0.082$, linear $P = 0.023$). The RQ was affected by diet ($P = 0.049$, linear P = 0.077, cubic $P = 0.023$), with higher values in pigs fed lower levels of RSM, namely RSM0 and RSM10, than pigs fed RSM20 and RSM30.

Because there was no difference in RE:ME among groups, linear regression analyses of $ME^{0.60}$ on $RE^{0.60}$ and of $RE/kg^{0.60}$ on $ME/kg^{0.60}$ were performed on all pigs. The analyses gave the following significant ($P < 0.001$) equations (Figure 3):

$$ME^{0.60} = 902 (\pm 105.3) + 1.257 (\pm 0.090) \times RE^{0.60}; R^2 = 0.87, CV = 6.1 \quad (1)$$

$$RE^{0.60} = - 485 (\pm 117.6) + 0.695 (\pm 0.050) \times ME^{0.60}; R^2 = 0.87, CV = 9.3 \quad (2)$$

The resulting ME_m estimate from equation (1) was close to 900 $kJ/kg^{0.60}$ whereas the corresponding estimate from equation (2) (-I/b) was 700 $kJ/kg^{0.60}$. The k_g value of equation (1) (1/b) was 0.80 whereas that found with equation (2) was 0.70.

Discussion

The European pig industry needs alternatives to imported SBM and robust pigs that perform efficiently when fed such ingredients. Therefore, our objective was to replace SBM with increasing levels of RSM, and to investigate the digestive and metabolic efficiencies of young pigs under these conditions.

Increasing inclusion of RSM in the diets, partially replacing SBM and wheat, led to increased dietary fat because of the higher fat content in RSM than in SBM (11.1% vs. 1.0%), and decreased starch levels because wheat was replaced along with SBM to achieve balanced diets. The fiber content in RSM is considerably higher than that in SBM (Bach Knudsen, 2014), therefore, replacing SBM with increasing levels of RSM resulted in gradually higher content of DF, T-NSP, cellulose, NDF, and lignin. Despite the higher fiber and glucosinolate levels, all diets were readily consumed and the pigs performed well even on the RSM30 diet. The increasing DF most likely caused the linear decrease in ATTD of OM, GE, CP, and CHO + L observed in the pigs fed increasing levels of RSM. This negative relation between fiber level and nutrient and energy digestibility has been extensively reported in pigs (e.g. Jørgensen *et al.*, 1996, Jørgensen *et al.*, 2007). Fiber type may also affect digestive processes. Because fiber in RSM is more lignified and insoluble than in SBM (Bach Knudsen, 2014),

the fiber fraction in our RSM diets was more resistant to degradation than in the control diet. Although RSM inclusion did not affect ATTD of T-NSP, the ATTD of DF was linearly decreased, because DF includes the indigestible lignin which increased with increasing RSM inclusion. Pérez de Nanclares *et al.* (2017) recently reported reduced apparent ileal digestibility and ATTD of most nutrients, including fiber, and energy in weanling pigs fed high-fiber RS co-products compared with SBM. The reduced digestibility of energy and nutrients may be caused by inhibition of enzyme activity, lack of enzyme-substrate interaction resulting from entrapment of nutrients in the complex fiber matrix of the RS diets, and/or by interference with other ANF in RSM. Similarly, the reduction in ATTD of DF may result from lack of microbiota-substrate interaction due to the complexity of the fiber matrix in the RS diets. In the present study, the lower degradation of the RSM diets resulted in increased bulking of feces (g DM/day) with increasing inclusion of RSM. This may have increased passage rate and reduced mean retention time of the digesta in the gastrointestinal tract (**GIT**)**,** thus decreasing the time available for enzymatic digestion and microbial fermentation. This further explained the reduced ATTD of nutrients and energy.

The diets were formulated on the basis of NE and SID CP and AA values, and adjusted to be similar across treatments. Chemical analyses of diets showed no major differences in the concentrations of GE, CP, and AA. In addition, intake of N and DE or ME was similar among pigs fed the different diets. Therefore, it can be assumed that any effect observed on protein and energy metabolism could be attributed to the replacement of SBM with RSM and not to unbalanced diets or differences in N and/or energy intake. Despite less N being available for absorption because of the reduced ATTD of CP and lower DN with increasing inclusion of RSM, retained N was similar across dietary groups, also when expressed in proportion of IN and DN. This is in agreement with previous research where inclusion of fiber in pig diets did not affect RN:IN (Zervas and Zijlstra, 2002; Hansen *et al.*, 2006) or RN:DN (Len *et al.*, 2007). Morgan and Whittemore (1988) suggested that unaffected or even higher RN with high-fiber diets is due to a higher N utilization, which is supported by the improved efficiency of utilization of DN for retention (RN:DN) found by Hansen *et al.* (2006) when including fiber in pig diets. However, our results and those of Len *et al.* (2007) could not support this suggestion, as RN:DN was not affected by increasing DF. Total N excretion was similar across dietary treatments. However, there was a repartition of N from urine to feces, as showed by the linear decrease in UN excretion coupled with a linear increase in FN as dietary RSM inclusion increased, and the consequent linear decrease of UN:FN. This repartition

probably reflects the increasing DF concentrations in the RSM diets. It is well established that high DF induces a shift in N excretion from urine to feces in pigs, partly due to a higher N excretion in the form of bacterial protein (reviewed by Bindelle *et al.*, 2009) and partly due to N associated to the fiber matrix. For RSM, in particular, the latter can account for a large part as RS hulls constitute 28-30% of the DM in oil-free RSM and they have a low digestibility of DM and N (Jensen *et al.*, 1990).

The linear increase of liver index with increasing RSM inclusion is in agreement with earlier findings (Fandrejewski *et al.*, 1994, Choi *et al.*, 2015, Parr *et al.*, 2015). Increased liver weight was associated with the presence of glucosinolates in the RS diets. In contrast, other studies have reported no effect of RSM-feeding on liver weight (Busato *et al.*, 1991; Pérez de Nanclares *et al.*, 2017). Despite the enlargement of this high-metabolic-rate organ, similar HE was observed across dietary treatments. Consequently, RE was similar for pigs fed the different diets, also when expressed in proportion to ME, which agrees with the observations of Hansen *et al.* (2006). It may be that, as argued in the latter study, the difference in DF content in their diets (48-111 g/kg DM) and ours (170-201 g/kg) was not large enough to induce an effect on HE:ME and RE:ME. Greater difference in DF content (59 vs. 268 g/kg DM) has been reported to increase HE:ME and consequently reduce RE:ME (Jørgensen *et al.*, 1996). The effect of glucosinolates in RSM on heat production in pigs was investigated by Buchmann and Wenk (1989) and Fandrejewski *et al.* (1994). In agreement with our results, these authors did not observe major changes in HE:ME even when including 30 or 25%, respectively, of a high-glucosinolate RSM (24.3 μmol/g DM in Fandrejewski *et al.* (1994) vs. 11.3 μmol/g DM in the present experiment). Despite no effect on HE:ME, Fandrejewski *et al.* (1994) also observed increased liver, thyroid, and kidney weights, which could be expected to result in an increased heat production, while Buchmann and Wenk (1989) reported a decrease in triiodothyronine (T_3) and thyroxine (T_4), which could be expected to reduce heat production through a decrease in the basal metabolic rate. Results from the previous and present study indicate that the causes for a change in heat production when feeding RSM could have opposite effects.

It could be expected that higher DF would lead to higher fermentation and consequently higher CH_4 production. However, increasing levels of RSM did not affect CH_4 production, and the values found made up 0.3-0.4% of DE, which are similar to those of Hansen *et al.* (2006). This is in line with the unaffected ATTD of T-NSP after replacing SBM with RSM, and probably a consequence of the DF fraction in the RSM diets being hard to degrade by the

young pigs, whose GIT and fermentation capacity were not fully developed. The numerically higher OXCHO:HE in pigs fed RSM0 and RSM10 and higher OXF:HE in pigs fed RSM20 and RSM30 may reflect the higher starch content in the two first diets and the higher fat content in the two latter ones.

In the present investigation, we could not document differences in any traits related to metabolic efficiency between dietary treatments. The ME_m requirement of 825 kJ/kg$^{0.60}$ stated by NRC (2012) is almost intermediate to our two estimates (700 vs. 900 kJ/kg$^{0.60}$), but values found in the literature vary to a large extent (e.g. 850 kJ/kg$^{0.60}$, van Milgen *et al.*, 2001; 1050 kJ/kg$^{0.60}$, Noblet *et al.*, 1989). The estimate of the efficiency of utilization of ME for retention, k_g, was lower (0.70) for the low estimate of ME_m than for the high estimate (0.80), as could be expected, and as demonstrated by the relation between estimates of ME_m and partial efficiencies in the studies by Noblet *et al.* (1989) and van Milgen *et al.* (2001). Both our estimates were within the range (0.69 to 0.82) reported by Thorbek *et al.* (1984).

Taken together, the results from the present experiment indicate that replacing SBM with up to 30% RSM as an alternative protein source in diets for young pigs reduced the digestive efficiency of the animals but did not compromise protein and energy metabolism or the efficiency of utilization of DN or ME for retention. Developing robust pig genotypes with greater capacity to digest recalcitrant fibers and processing efforts to increase RS fiber degradability in future studies are therefore key factors for achieving high feed efficiency when feeding RSM diets to pigs.

Acknowledgments

We thank T.J. Olsen, P.B. Lund, B. Thim, and B. Ditlevsen for their practical help at the farm, M. Stubgaard and L. Ørbæk for their assistance with the chemical analyses, and A. Ali for the operation of the respiration units. This research was financially supported by FeedMileage-Efficient use of feed resources for a sustainable Norwegian food production (the Research Council of Norway; grant no. 233685/E50), and by Foods of Norway, a Centre for Research-based Innovation (the Research Council of Norway; grant no. 237841/030).

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Tables

Table 1 *Ingredient composition of the experimental diets*

	Diets[1]			
Ingredient (g/kg as fed basis)	RSM0	RSM10	RSM20	RSM30
Barley[2]	345	345	345	345
Wheat[2]	215	176	140	103
Soybean meal[2]	206	145	81	17
Oats[2]	140	140	140	140
Rapeseed meal[2]	-	100	200	300
Tallow	48	49	49	50
Monocalcium phosphate	19	19	19	19
Limestone	13.5	13.5	13.5	13.5
HCl L-Lysine	2.70	2.90	3.12	3.40
DL-Methionine	1.10	0.50	-	-
L-Threonine	1.50	1.30	1.20	1.05
L-Tryptophan	0.3	0.3	0.3	0.3
Sodium chloride	4.0	4.0	4.0	4.0
Vitamin-trace mineral premix[3]	3.31	3.31	3.31	3.31
Attractant (Maxarome)	0.5	0.5	0.5	0.5
Calculated content NE (MJ/kg)	9.5	9.5	9.5	9.5

[1]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.
[2]Chemical composition in Supplementary Material S1
[3]Provided per kilogram of diet: 96 mg Zn (ZnO); 96 mg Fe ($FeSO_4$); 48 mg Mn (MnO); 20.8 mg Cu ($CuSO_4$); 0.48 mg I ($Ca(IO_3)_2$); 5700 IU vitamin A; 1200 IU cholecalciferol; 100.7 mg dl-α-tocopheryl acetate; 3.57 mg menadione; 2.4 mg thiamin; 9.0 mg riboflavin; 36.0 mg D-pantothenic acid; 12.0 µg cyanocobalamine; 12.0 mg niacin; 0.24 mg biotin; and 1.8 mg folic acid.

548 **Table 2** *Chemical composition of the experimental diets (as fed basis)*[1]

	Diets[2]			
Analyzed contents (g/kg)	RSM0	RSM10	RSM20	RSM30
GE (MJ/kg)	17 ± 0.1	17 ± 0.1	18 ± 0.1	18 ± 0.3
DM	895 ± 4	895 ± 4	896 ± 3	899 ± 3
CP	186 ± 3	179 ± 1	182 ± 4	181 ± 3
Fat	73 ± 1	81 ± 1	89 ± 1.5	99 ± 3
Ash	57 ± 0.4	59 ± 1	60 ± 1	61 ± 1
Lysine	10.7	10.4	10.5	10.8
Methionine	3.3	2.8	2.6	2.8
Cysteine	2.9	3.0	3.4	3.7
Threonine	8.5	8.0	8.3	8.3
Tryptophan	2.1	2.4	2.5	2.6
Total CHO[3]	580 ± 4	576 ± 4	565 ± 6	557 ± 7
Starch	380 ± 10	377 ± 5	350 ± 12	316 ± 5
Total NSP	148 ± 4	147 ± 6	151 ± 7	157 ± 2
Soluble NSP	36 ± 13	48 ± 8	35 ± 8	45 ± 3
Cellulose	36 ± 3	37 ± 3	39 ± 4	39 ± 3
Total NCP	112 ± 4	110 ± 3	112 ± 3	118 ± 3
Rhamnose[4]	0.7 (0.4)	0.8 (0.4)	0.9 (0.4)	1.1 (0.5)
Fucose	0.8 (0.4)	0.7 (0.3)	0.7 (0.2)	0.8 (0.2)
Arabinose	22 (5)	22 (7)	24 (7)	27 (9)
Xylose	37 (7)	34 (8)	34 (5)	33 (7)
Mannose	5.3 (1.4)	4.6 (1.5)	4.2 (1.2)	4.1 (1.2)
Galactose	12 (5)	10 (5)	9.4 (3.8)	8.7 (3.2)
Glucose	26 (13)	27 (21)	25 (13)	27 (18)
Uronic acids	8.8 (3.7)	10 (4)	13 (5)	16 (3)
Klason lignin	21 ± 1	28 ± 1	36 ± 6	44 ± 3
Fiber content				
DF	170 ± 5	175 ± 5	187 ±11	201 ± 1
NDF	115	124	135	154
Total glucosinolates[5]	-	1.1	2.3	3.4

549 [1]Values are means ± standard deviation (SD) from the chemical analysis of representative samples from all diets
550 from each period (n = 4), except for amino acids and NDF, which were analyzed in pooled samples of each diet
551 from the four periods (n = 1).
552 [2]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental
553 diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.
554 [3]The total carbohydrate content was calculated as CHO = DM – ash – CP – fat.
555 [4]Values in parenthesis are soluble NSP.
556 [5]The total glucosinolate content is given in mmol/kg diet and was calculated based on the analyzed glucosinolate
557 content of the rapeseed meal and the inclusion level in the diet.

Table 3 *Effects of replacing soybean meal with increasing levels of rapeseed meal (RSM) on apparent total tract digestibility (ATTD, %) of main nutrients, energy and individual fiber fractions in young pigs*[1]

	Diets[2]					*P*-value		
Chemical constituent	RSM0	RSM10	RSM20	RSM30	SEM	Diet	Period	Linear
OM	82.1^{a}	80.0^{ab}	79.4^{b}	77.2^{c}	0.7	<0.001	0.383	<0.001
GE	82.1^{a}	79.6^{b}	79.7^{b}	77.7^{b}	0.8	0.004	0.418	<0.001
CP (N x 6.25)	82.2^{a}	79.0^{bc}	79.5^{ab}	76.5^{c}	1.0	0.003	0.0215	<0.001
Fat	76.1^{ab}	73.8^{b}	77.9^{ab}	79.5^{a}	1.6	0.089	0.913	0.038
CHO	85.8^{a}	84.5^{ab}	83.0^{b}	80.7^{c}	0.5	<0.001	0.282	<0.001
T-NSP	59.4	57.7	56.9	56.6	1.4	0.482	0.188	0.149
Cellulose	31.2	29.8	31.9	33.3	2.9	0.869	0.047	0.504
T-NCP								
Rhamnose	18.8	19.8	27.4	27.4	4.5	0.371	0.401	0.103
Fucose	67.0^{a}	62.0^{ab}	58.5^{bc}	53.5^{c}	2.3	0.003	0.057	<0.001
Arabinose	67.4^{b}	68.2^{b}	70.5^{ab}	72.5^{a}	1.1	0.013	0.432	0.001
Xylose	46.2	42.4	41.2	39.7	2.4	0.267	0.436	0.063
Mannose	91.4^{a}	89.0^{a}	87.1^{ab}	82.7^{c}	1.5	0.004	0.824	<0.001
Galactose	79.6	79.8	79.3	74.9	2.4	0.439	0.044	0.186
Glucose	85.9^{a}	79.9^{a}	72.1^{b}	67.1^{b}	2.0	<0.001	<0.001	<0.001
Uronic acids	73.0^{a}	70.8^{a}	63.4^{b}	59.1^{c}	1.4	<0.001	0.217	<0.001
DF	42.2^{a}	38.9^{ab}	36.9^{ab}	35.6^{b}	2.2	0.178	0.274	0.034

[1]Values are least-squares means and pooled standard error of the mean (SEM), n = 8 except for the RSM10 group where n = 6.

[2]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 4 *Nitrogen metabolism (g/kg$^{0.75}$ per day) in young pigs fed increasing levels of rapeseed meal (RSM) replacing soybean meal*[1]

	Diets					P-value		
	RSM0	RSM10	RSM20	RSM30	SEM	Diet	Period	Linear
Ingested nitrogen (IN)	2.60	2.52	2.50	2.49	0.05	0.329	<0.001	0.094
Digested nitrogen (DN)	2.14^{a}	1.96^{bc}	2.01^{b}	1.89^{c}	0.04	0.003	<0.001	0.001
Excreted nitrogen	1.16	1.17	1.14	1.16	0.04	0.961	0.005	0.872
Fecal nitrogen (FN)	0.48^{b}	0.52^{ab}	0.53^{ab}	0.60^{a}	0.03	0.003	<0.001	0.001
Urinary nitrogen (UN)	0.70^{a}	0.65^{ab}	0.61^{ab}	0.58^{b}	0.04	0.116	0.564	0.019
UN:FN	1.55^{a}	1.27^{ab}	1.18^{b}	1.05^{b}	0.09	0.006	<0.001	<0.001
Retained nitrogen (RN)	1.44	1.33	1.38	1.35	0.05	0.477	<0.001	0.326
RN:IN	0.55	0.53	0.55	0.53	0.02	0.560	0.003	0.544
RN:DN	0.67	0.67	0.69	0.69	0.02	0.627	<0.001	0.242

[1]Values are least-squares means and pooled standard error of the mean (SEM), n = 8 except for the RSM10 group where n = 6.
[2]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.
[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 5 *Energy metabolism (kJ/kg$^{0.75}$ per day) in young pigs fed increasing levels of rapeseed meal (RSM) replacing soybean meal*[1]

	Diets					*P*-value		
	RSM0	RSM10	RSM20	RSM30	SEM	Diet	Period	Linear
Digestible energy (DE)	1245	1204	1211	1179	26	0.317	<0.001	0.096
Metabolizable energy (ME)	1204	1166	1175	1144	26	0.386	<0.001	0.130
Heat production (HE)	757^{a}	677^{b}	723^{ab}	704^{ab}	21	0.082	<0.001	0.221
Retained energy (RE)	447	488	452	440	31	0.743	<0.001	0.675
RE:ME	0.37	0.40	0.38	0.38	0.02	0.745	0.007	0.954
Energy retained in protein (RPE)	217	199	207	198	8	0.256	<0.001	0.136
Energy retained in fat (RFE)	230	289	245	242	28	0.547	0.001	0.946
O_2 consumption (l/kg$^{0.75}$ per day)	36.3^{a}	32.0^{b}	34.9^{ab}	33.9^{ab}	1.2	0.142	0.002	0.413
CO_2 production (l/kg$^{0.75}$ per day)	34.3^{a}	31.8^{ab}	31.7^{b}	31.1^{b}	1.0	0.082	<0.001	0.023
CH_4 production (l/kg$^{0.75}$ per day)	0.11	0.11	0.10	0.11	0.02	0.955	0.318	0.982
Respiratory quotient (RQ)	0.95^{ab}	0.99^{a}	0.91^{b}	0.92^{b}	0.02	0.049	0.470	0.077

[1]Values are least-squares means and pooled standard error of the mean (SEM), n = 8 except for the RSM10 group where n = 6.

[2]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Figure captions

Figure 1 Liver index ($g/kg^{0.75}$) of young pigs fed increasing levels of rapeseed meal

Figure 2 Substrate oxidation in young pigs fed increasing levels of rapeseed meal (OXCHO = oxidized carbohydrate, OXF = oxidized fat, OXP = oxidized protein, HE = heat production)

Figure 3 Linear regression analysis of A) metabolized energy ($ME^{0.60}$) on retained energy ($RE^{0.60}$): $ME^{0.60} = 902 \pm 105.3 + 1.253 \pm 0.090 \times RE^{0.60}$; $R^2 = 0.87$, CV = 6.11 and B) $RE^{0.60}$ on $ME^{0.60}$: $RE^{0.60} = 0.695 \pm 0.050 \times ME^{0.60} - 485 \pm 117.6$; $R^2 = 0.87$, CV = 9.3.

Figures

Figure 1

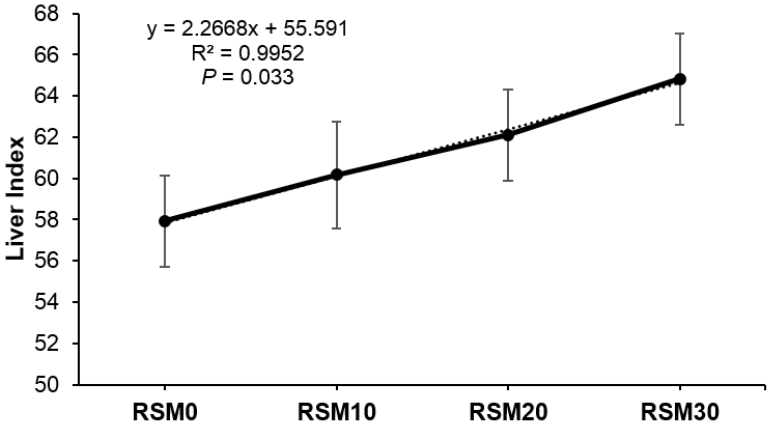


Figure 2

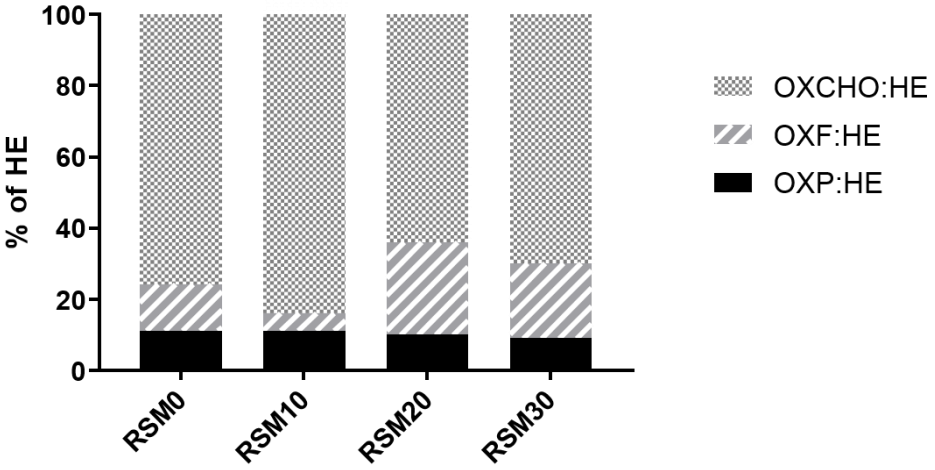
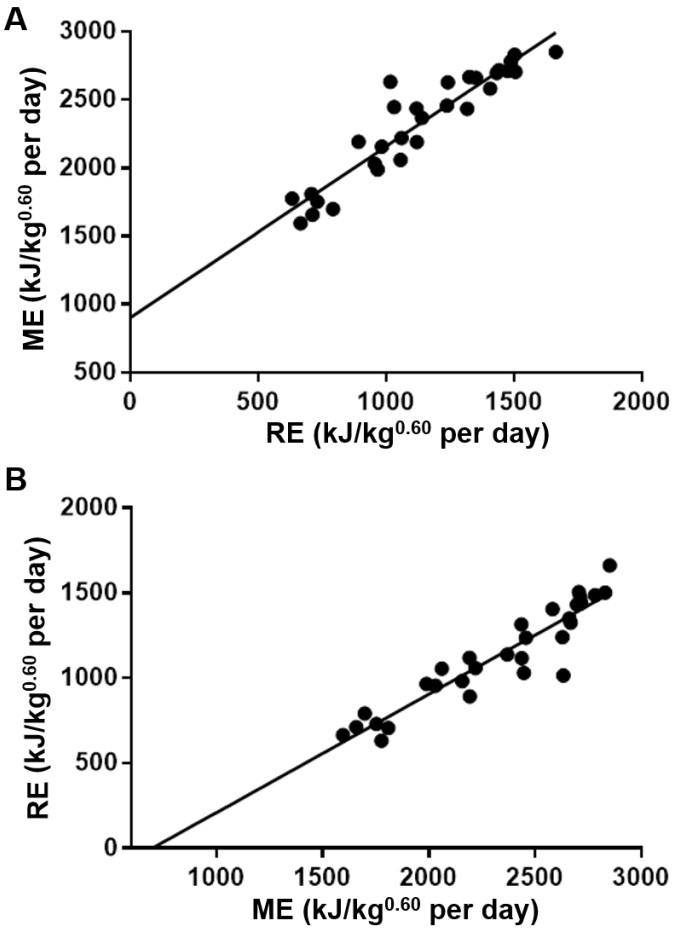

100
80
60
40
20
0
% of HE
RSM0
RSM10
RSM20
RSM30
OXCHO:HE
OXF:HE
OXP:HE

Figure 3


A
3000
2500
2000
1500
1000
500
ME (kJ/kg0.60 per day)
0
500
1000
1500
2000
RE (kJ/kg0.60 per day)
B
2000
1500
1000
500
0
RE (kJ/kg0.60 per day)
1000
1500
2000
2500
3000
ME (kJ/kg0.60 per day)

Supplementary material for:

Increasing levels of rapeseed meal in diets for pigs: Effects on protein and energy metabolism

M. Pérez de Nanclares, C. Marcussen, A.-H. Tauson, J.Ø. Hansen, N.P.Kjos, L.T. Mydland, K.E. Bach Knudsen and M. Øverland

Material S1

Barley: 86.2% DM, 7.4% CP, 1.3% fat, 53.5% starch, 16.0% NDF, 5.1% ADF, 1.6% ash.

Whole wheat: 86.4% DM, 11.1% CP, 1.6% fat, 58.1% starch, 9.0% NDF, 2.2% ADF, 1.4% ash.

Soybean meal: 88.4% DM, 46.1% CP, 1.0% fat, 0.3% starch, 6.3% ash.

Oats: 86.8% DM, 7.6% CP, 5.5% fat, 26.2% NDF, 40.0% starch, 2.3% ash.

Rapeseed meal: 90.1% DM, 34.0% CP, 11.1% fat, 1.2% starch, 20.1% NDF, 6.0% ash, 6.97 µmol/g progroitin, 1.59 µmol/g 4-hydroxyglucobrassicin, 1.26 µmol/g glucobrassicanapin, 0.76 µmol/g glucobrassicin, 0.74 µmol/g gluconapin, 11.3 µmol/g total glucosinolates.

Table S1 *Effects of replacing soybean meal with increasing levels of rapeseed meal (RSM) on the general performance of young pigs*[1]

	Diets[2]					*P*-value			
	RSM0	RSM10	RSM20	RSM30	SEM	BW	Diet	Period	Linear
Body weight (kg)	28.6	27.9	28.5	28.3	1.6	-	0.990	<0.001	0.950
DM intake (g/day)	982	985	973	966	18	<0.001	0.876	<0.001	0.458
Daily weight gain (g/day)	504	520	536	492	29	0.002	0.680	<0.001	0.879
Feces dry weight (g/d)	173[b]	190[ab]	197[a]	217[a]	8.8	<0.001	0.011	0.016	0.001

[1]Values are least-squares means and pooled standard error of the mean (SEM), n = 8 except for the RSM10 group where n = 6.

[2]RSM0: Control diet based on grains and soybean meal and with no rapeseed meal; RSM10,20,30: experimental diets where wheat and soybean meal were partially replaced with 10, 20, and 30% of rapeseed meal, respectively.

ISBN: 978-82-575-1478-5
ISSN: 1894-6402

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