FEED-A-GENE

Adapting the feed, the animal and the feeding techniques to improve the efficiency and sustainability of monogastric livestock production systems

Deliverable D1.5

New enzymatic cocktails for novel feed ingredients

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1. Summary

Objectives

The objective of Task 1.5 is to study the impact of enzyme cocktails on the nutritional value of conventional and upgraded rapeseed meals (for details see deliverable 1.1), and of green biomass residues produced in Task 1.2 in combination with enzymes selected by DuPont.

Rationale:

The majority of protein-rich feed ressources for livestock production in Europe is imported. Compared to soybean meal (SBM), many of the locally produced protein sources (e.g., beans, pulses, and legumes) in Europe are inferior in nutritive value due to the presence of antinutritional compounds. It is therefore of relevance to study the potential to improve the nutritional value of European grown proteins by the use of novel enzymes. The use of feed enzymes is a proven technology to improve the nutritional value of feeds for broilers and pigs. Conventional European rapeseed meal (RSM) was used for the selection of novel proteases and NSPases to assess their potential and to optimize the dosing. *In vitro* response surface methodology was used to determine the potential of enzymes to solubilise protein from the substrate. The enzymes and doses obtained in *in vitro* experiments were used to optimize the dosing for *in vivo* experiments with broiler chickens and growing pigs. Performance, gastrointestinal parameters and nutrient digestibility were used to evaluate the effects of the enzymes. For the extraction of protein from green biomass (Deliverable 1.2) cell wall degrading enzymes were used to aid the extraction of protein. The fibre-rich pulp after the extraction of protein from green biomass without and with treatment of cell wall degrading enzymes was studied in experiments with growing rabbits.

Teams involved:

DuPont, UNEW, IRTA

Delivery of material from IFIP, Hamlet Protein, and Dupont Industrial Biosciences

Species and production systems considered:

Conventional pig, broiler, and rabbit production systems are considered.

2. Abbreviations used

AA :	amino acids
ADF :	acid detergent fibre
ADFI :	average daily feed intake
ADWG :	average daily weight gain
AME :	apparent metabolizable energy
Ara :	arabinose
avP :	available phosphorus
BW :	bodyweight
CF :	crude fibre
CP :	crude protein
dCa :	digestible calcium
digP :	digestible phosphorus
DM :	dry matter
EE :	ether extract (fat)
EU :	European Union
FCR :	feed conversion ratio
Gal :	galactose
GIT :	gastro-intestinal tract
GLM :	general linear models
Glu :	glucose
Man :	mannose
NDF :	neutral detergent fibre
NFE :	nitrogen-free extract
NSP :	non-starch polysaccharides
Pr :	protease
Proc :	procedure
RSM :	rapeseed meal
SAS :	Statistical analysis systems
SBM :	soyabean meal
SEM :	standard error of the mean
TTAD :	total tract apparent digestibility
Xyl :	xylose

3. Introduction

Soybean meal (SBM) represents 61% of the protein sources used to feed livestock but the European Union (EU) has only a self-sufficiency of 3% for its SBM needs. This is considered unsustainable for a variety of reasons (Leinonen *et al.*, 2013; Zander *et al*., 2016). The increased demand for imported soybean, especially by the non-ruminant feed industry, is attributed to its high crude protein (CP) content, high amino acid (AA) digestibility, and an AA profile complementary to that of cereals (de Visser *et al*., 2014). Increasing the EU production of domestic protein sources (e.g., beans, pulses, and legumes) and processing these at medium-sized plants from non-genetically modified crops may contribute to the reduction of dependency on imported SBM. The use of cost-effective protein sources can reduce the demand of protein imports in EU and increase the profitability of livestock farming.

Rapeseeds (*Brassica napus* and *Brassica juncea*) contain high protein content and have a well-balanced AA profile. Rapeseed meal (RSM), a by-product of oil industry after seed crushing, has a high protein content (approximately 35% CP) and may be considered as an alternative to SBM in diets for growing-finishing pigs. The EU was the world's largest producer of rapeseed in 2016/2017, with 20.5 million metric tons, which makes this feedstuff highly recommendable to satisfy the protein demand of the animal feed industry in Europe. Despite the high protein content, RSM also contains antinutritional factors, such as glucosinolates, sinapine, phytate, and it has a high fiber content that limits its use in animal feed (Ndou *et al*., 2018). The high fiber and phytate phosphorus content can reduce the digestibility of CP, AA ,and minerals (Mejicanos *et al*., 2018; Hansen *et al*., 2017). The high fiber content in RSM is partly caused by the high hull content in rapeseed expeller and a dehulling process can enhance its nutritive value. In terms of improvement of nutritional values of RSM, dehulling process is the most studied and disclosed method, although it is currently an economically unfeasible procedure for the oil industry due to the loss of oil present in the hulls (Mejicanos *et al*., 2018). Under non-disclosed methods, some companies (Hamlet Protein A/S, Denmark; Bühler AG, Uzwil, Switzerland) have produced low-glucosinolates RSM with a higher crude protein content and lower contents of fiber and other antinutritional factors.

The use of feed enzymes has been a proven technology to improve nutrient efficiency, growth performance, gut health while reducing feed cost and the environmental impact. Vegetable protein meals contain a wide variety of complex cell wall polyssacharides, which have a low nutritional value and can be considered as anti-nutritional compounds by their effects in the gastro-intestinal tract. There is therefore a potential to improve the nutritional value of EU protein concentrates by the use of novel and targeted enzymes.

With high yields of dry matter (DM) and CP per hectare at temperate climates, green crops like grasses and leguminous plants (*Fabaceae*) have the potential as an alternative source of protein for monogastric animals. To be used, the protein needs to be extracted from the forages, generating lzrge quantities of fiber-rich residues, which can be valorized by herbivorous animals.

4. Results

4.1 Selection of proteases and NSPases (DuPont)

An *in vitro* model simulating the conditions of the digestive tract has been developed at DuPont and was used to screen for the best protease candidates to solubilize protein from substrates (EU grown RSM or soya by-products). The potential of non-starch polysaccharide degrading enzymes (NSPases) to increase the nutrient release from substrates was also evaluated *in vitro*. The optimal dosing obtained *in vitro* and experiments with feed enzymes carried out by DuPont was used to estimate the optimal dosing for *in vivo* experiments with broiler chickens and growing pigs. Six different proteases were tested to solubilise RSM (Figure 1). Based on these results, Protease 1 was selected for further *in vitro* trials.

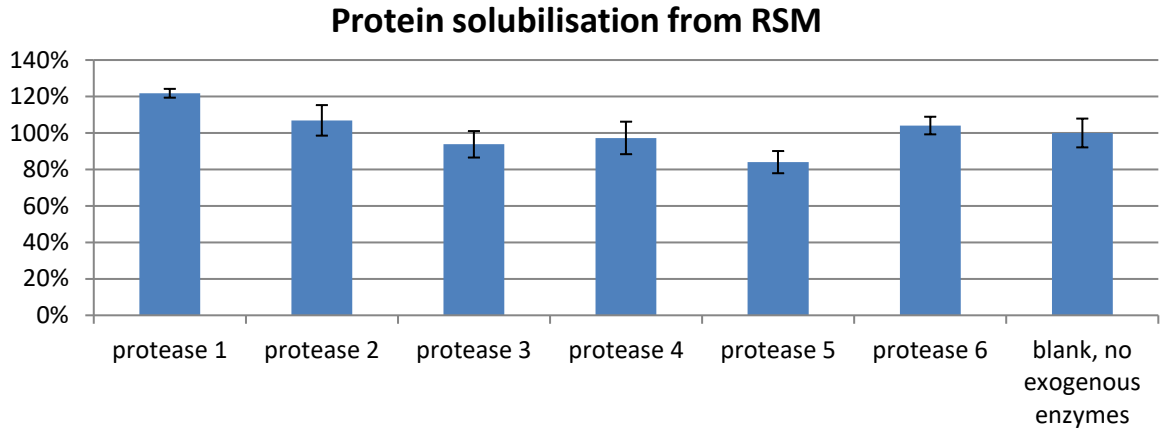

Figure 2. Protein solubilisation from RSM by candidate proteases.

The protease 1 was tested in combination with four different NSPase mixes to determine the release of glucose and total reducing sugars, and to confirm protein solubility from RSM (Figure 2). Based on the results of this test, NSPase mix 4 was selected for further *in vitro* trials. Finally, protease1 and NSPase mix 4 were used together to determine the optimal dose based on nutrient release and reduction of viscosity (Figure 3). The results observed in the *in vitro* trials allowed to select protease 1 and NSPase mix 4 for the *in vivo* experiments with RSM. Furthermore, based on current and other *in vivo* experiments with proteases, DuPont decided to use protease 1 at 10 ppm (w/w protease) and NSPase mix 4 at 0.025% (w/w product mix). The amount of these enzymes needed for the *in vivo* studies at UNEW and IRTA was then prepared, frozen, and shipped to the two partners to be used in the feed manufacturing.

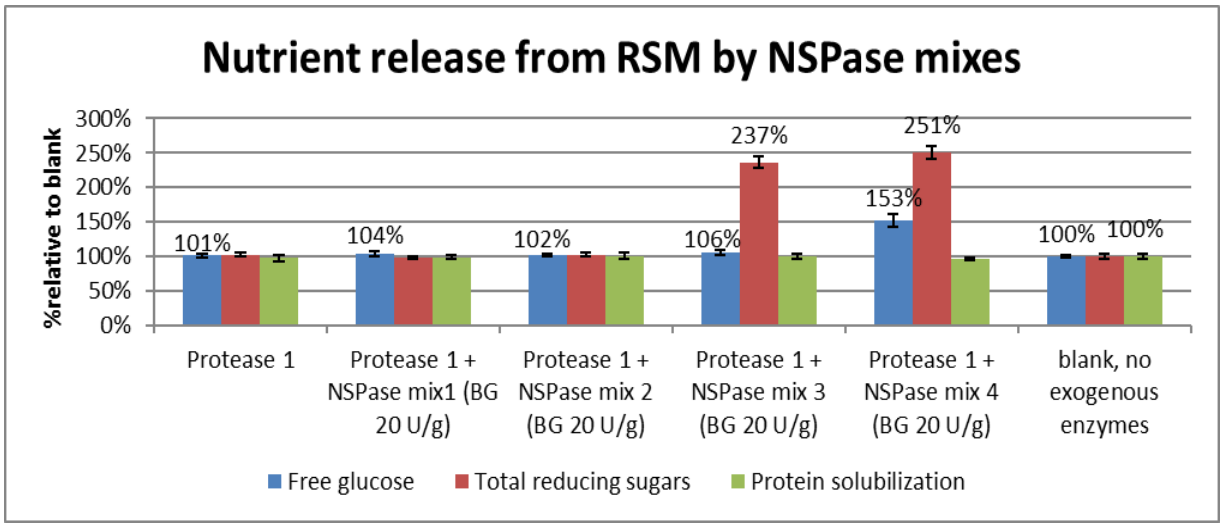

Figure 2. Release of glucose and total reducing sugars from RSM by NSPase mixtures.

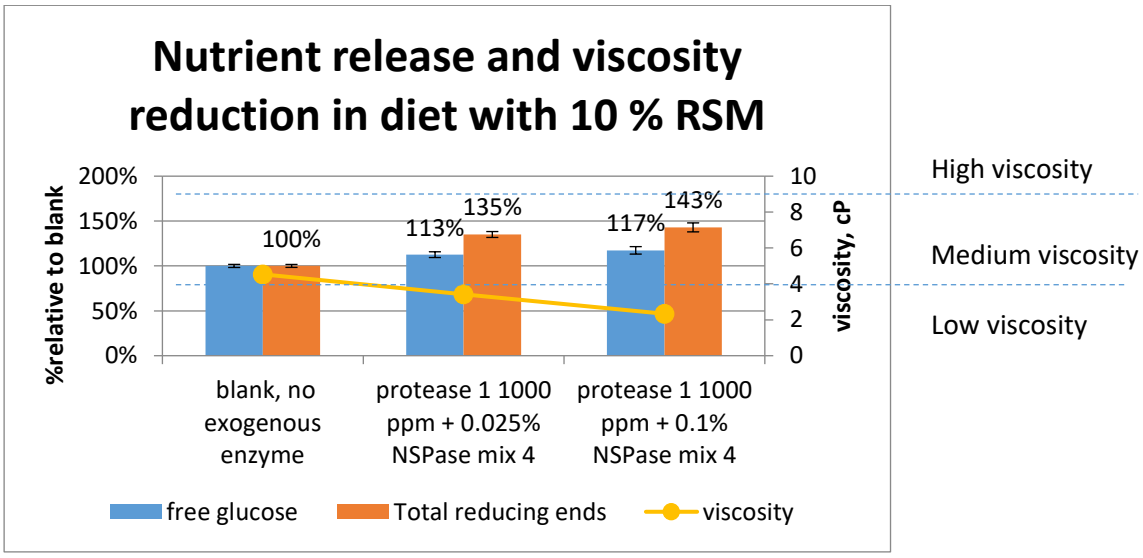

Figure 3. Nutrient release and viscosity reduction by NSPase mix 4 at two different concentrations.

4.2 Nutritive value of rapeseed meal in broilers fed without and with protease and NSPases (UNEW)

A European RMS selected by TerresInnovia in Task 1.1 was used in a study with 480 male Ross 308 day-old chicks housed in 48 pens of 1.7 m^2 with *ad libitum* access to feed and water throughout the trial. Six starter (d0-10) and grower (d11-24) mash diets were formulated deriving from the two raw materials (RSM and SBM) and three enzyme treatments: no enzymes, protease, and protease plus NSPases (see Annex 1, Table 8). Synthetic AAs were added to cover limiting EAA requirements at the same level on a digestible AA basis for starter or grower diets. Differences in apparent metabolizable energy of the RSM treatments were accounted for by supplementation of soy oil. Each treatment was replicated in 8 pens with 10 birds per pen. Performance was measured by pen bodyweight (BW) measured at arrival and pen BW and average daily feed intake (ADFI) at the end of the starter and grower period. For collection of intestinal content, three randomly selected chickens with a BW close

to the pen average were individually weighed at 10 and 24 days of age. They were euthanized and intestinal tissue collected from one of the three birds. From the birds killed at d24, a subsample of pooled ileal digesta from the upper third of the ileum was also collected for determination of intestinal viscosity.

4.2.1 Broiler trial (UNEW)

Animals and sampling: All procedures were approved by the Animal Welfare and Ethical Review Body (AWERB) of Newcastle University. Four-hundred and eighty male Ross 308 day-old chicks were obtained from a commercial hatchery and were housed in a windowless, thermostatically controlled building in 48 pens of 1.7 m^2. Each pen was equipped with a tube feeder and a bell-drinker, and wood shavings were used as litter to a depth of 5 cm. Birds had *ad libitum* access to feed and water throughout the trial. Temperature at pen level was monitored daily and maintained to meet Aviagen recommendations (Aviagen, 2014), starting at 34°C at chick placement and gradually reduced to 20°C by 25 days of age. Light intensity at pen level ranged from 80 to 100 lux, whilst a lighting schedule of 23L:1D was applied for the first 7 days of age and switched to 18L:6D for the remainder of the trial.
Feed analyses: All diets were analysed at a UKAS accredited commercial laboratory to the internationally recognised standard for competence ISO/IEC 17025:2005 (Sciantec Analytical Services, Cawood, UK). Briefly, DM was measured by determining the loss in weight of the feed sample after heating at 103 - 105°C for 3 hours. Crude fat was determined by extraction with petroleum ether and total fat by acid hydrolysis followed by petroleum ether extraction. Crude protein was determined with the Dumas method on a LECO FP-528 Nitrogen Analyser (Leco Instruments UK, Cheshire) and crude fibre and acid detergent fibre (ADF) using an Ankom 220 Analyser (Ankom, Technology, Fairport, NY, USA). Ash was determined gravimetrically after incinerating the sample at 510°C for 4 hours. TiO_2 analysis in feed was according to the method described by Short *et al*. (1996). Neutral detergent fibre (NDF) was estimated by enzymatic gravimetry.

Sampling: Three randomly selected chickens were weighed at d10 and d24 of age and removed from their home pens. Following euthanasia with a lethal injection of sodium pentobarbitone (Euthatal®, Merial, Harlow, United Kingdom), the ileum was excised and intestinal contents were collected from the lower two-thirds of the ileum until 2 cm from the ileocecal junction with gentle finger stripping. The contents were pooled for each pen. Moreover, a subsample of pooled ileal digesta was collected and immediately stored at -80 °C pending viscosity determination. On d25 of age after overnight fasting, three birds per pen were selected and their gastrointestinal tract (GIT) and organs were removed for obtaining liver, pancreas, empty gizzard and proventriculus weight as well as length and weight of the small intestine per segment.

Viscosity and chemical analyses of digesta: Frozen ileal digesta samples originating from the upper third of the ileum were thawed in a water bath at 40°C. After defrosting, the samples were centrifuged (with 12 000 *g* for 10 min), 0.5 ml of supernatant was then loaded to a Brookfield Digital Viscometer (Model LDVI+CP, Brookfield, Engineering Laboratories, Stoughton, MA) with a plate-plate geometry and a gap of 2 mm appropriate for small volumes to carry out digesta viscosity measurements. The temperature of the sample was maintained at 40°C during the measurements. The results are presented as solution viscosity in centipoise (CPs). The ileal digesta samples collected from the lower two-thirds of the ileum were freeze-dried overnight and along with samples of the diets, and were ground to pass through a 0.5-mm sieve and stored in airtight plastic containers at −20°C pending

chemical analyses. All samples were analysed for CP with the Dumas combustion method using Leco's CNS 2000 analyser (Leco Instruments UK, Cheshire), and TiO_2 according to the methodology of Short *et al*. (1996).

Calculations and statistical analysis: All statistical analyses were conducted in SAS 9.4 (SAS Institute, Cary, NC). For all statistical assessments pen was considered the experimental unit and data were analysed with PROC GLM with diets and enzymes inclusion as fixed factors and their interaction. Weight of organs and gastrointestinal tract (GIT) as well as length of small GIT segments, obtained at d24 of age were expressed as a ratio to the sampled empty carcass weight of the slaughtered bird. When significant differences were detected, treatment means were separated and compared by the Tukey's multiple comparison test. Significance was determined at $P < 0.05$. All values are expressed as model-predicted least square means with the SEM.

4.2.2 Growth performance and feed efficacy

Effects of diets and inclusion of protease and NSPase enzymes on performance variables are presented in Table 1. Final BW of broiler chickens was significantly reduced ($P < 0.001$) due to a reduction in average weight gain during the growing period (58.7 vs 63.5 g/d) and overall (43.7 vs 46.3 g/d) when RSM was used rather than SBM in diets. Also, feed intake was significantly reduced during the growing period and overall ($P < 0.01$) due to the source of protein included in the diets. As a consequence, feed efficiency was also significantly affected ($P < 0.01$) by protein sources. Neither Protease alone or Protease +NSPase improved BW, or the feed conversion ratio. Only a significant effect on feed intake ($P < 0.01$) during the starter period was detected. However, this effect disappeared during the grower period and overall.

Table 1. Effect of RSM meal and enzyme inclusion on performance variables[1] of broilers over the starter (d1-10) and grower periods (d11-24).

	Body weight, d			Feed intake, g/d			Feed conversion ratio		
Days of age	**0**	**10**	**24**	**0-10**	**11-24**	**0-24**	**0-10**	**11-24**	**0-24**
Source [1]									
RSM	38.1	263	1088a	26.0	81.0b	58.1b	1.16	1.42a	1.33a
SBM	38.0	261	1150b	25.7	84.5a	60.0a	1.16	1.37b	1.30b
Enzyme [1]									
Blank	38.1	256	1112	25.3b	83.0	58.9	1.16	1.39	1.32
Protease	38.0	263	1127	25.9ab	83.0	59.2	1.16	1.39	1.30
Prot+NSPase	38.1	267	1118	26.4a	82.4	59.0	1.16	1.41	1.32
Source x Enzyme									
RSM+ Blank	37.9	263	1083	25.9	80.8	57.9	1.15	1.42	1.33
RSM+ Protease	38.2	261	1073	25.6	79.8	57.2	1.17	1.41	1.32
RSM+Pr+NSPase	38.3	264	1107	26.4	82.6	59.2	1.17	1.42	1.33
SBM+ Blank	38.2	248	1140	24.6	85.1	59.9	1.17	1.36	1.31
SBM+Protease	37.9	266	1180	26.2	86.2	61.2	1.15	1.36	1.29
SBM+Pr+NSPase	37.9	269	1128	26.4	82.2	58.9	1.15	1.39	1.30
P-values									
Source	0.494	0.688	0.001	0.414	0.001	0.014	0.629	0.001	0.002
Enzyme	0.994	0.092	0.680	0.009	0.900	0.954	0.918	0.238	0.428
Source x Enzyme	0.216	0.106	0.052	0.026	0.058	0.077	0.303	0.567	0.783

[a,b] Means within a row with different letters differ significantly ($P < 0.05$).

[1] SBM, soybean meal; RSM, Rapeseed meal; Pr, protease; Pr+NSPase, protease plus NSPases.

4.2.3 Ileal digestibility and digesta viscosity

Effects of protein source or enzymes on apparent ileal DM and CP digestibility, and on ileal viscosity are presented in Table 2. The DM and CP digestibility were both significantly reduced ($P < 0.01$) due to RSM inclusion in diets. Inclusion of protease seemed to improve ($P < 0.05$) DM digestibility relatively to diets without enzymes or with inclusion of proteases and NSPases at day 24. No effect of either protein source or enzymes on viscosity of the gastro-intestinal content were observed.

Table 2. Effect of rapeseed meal and enzyme inclusion on the coefficient of apparent ileal dry matter and crude protein digestibility, and on ileal viscosity at the end of the starter (d10) and grower period (d24) .

	Dry matter, %		Crude protein, %		Viscosity, Cps	
Days of age	**10**	**24**	**10**	**24**	**10**	**24**
Source [1]						
RSM	74.1	72.7	85.8	83.5	2.07	2.13
SBM	74.6	78.1	86.9	88.6	2.16	2.14
Enzyme [1]						
Blank	74.5	75.7	86.2	86.4	2.13	2.11
Protease	74.5	76.0	86.4	86.2	1.98	2.02
Prot+NSPase	74.1	74.3	86.5	85.5	2.25	2.28
Source x Enzyme						
RSM+ Blank	74.0	72.5	85.5	83.3	2.12	2.18
RSM+ Protease	75.3	73.1	86.2	83.6	1.86	1.90
RSM+Pr+NSPase	72.9	72.5	85.7	83.6	2.25	2.32
SBM+ Blank	75.0	79.0	86.8	89.5	2.14	2.03
SBM+Protease	73.7	79.0	86.7	88.8	2.09	2.14
SBM+Pr+NSPase	75.1	76.2	87.2	87.4	2.25	2.23
P-values						
Source	0.529	0.0001	0.123	0.0001	0.454	0.989
Enzyme	0.861	0.048	0.934	0.165	0.177	0.255
Source x Enzyme	0.151	0.126	0.835	0.047	0.683	0.408

[a,b] Means within a row with different letters differ significantly ($P < 0.05$).

[1] SBM, soybean meal; RSM, Rapeseed meal; Pr, protease; Pr+NSPase, protease plus NSPases.

4.2.4 Gastrointestinal tract development

The effects of protein source and enzyme inclusion on GIT and organ weight and on small intestinal segment length are presented in Table 3. Inclusion of RSM significantly increased liver, gizzard, and gastrointestinal weight ($P < 0.05$), as well as it increased jejunum and ileum length ($P < 0.01$). Inclusion of enzymes did not show any effect on gastro-intestinal organs.

Table 3. Effect of rapeseed meal and enzyme inclusion on intestinal segment length and weight relative to eviscerated carcass weight at the end of the grower period (d24).

Days of age	Liver g/kg	Pancreas g/kg	Gizzard g/kg	Jejunum cm/kg	Ileum cm/kg	GIT weight, g/kg
***Source*[1]**						
RSM	28.3	2.89	20.2	60.4	53.0	61.8
SBM	26.6	2.98	19.1	56.6	49.9	56.1
***Enzyme*[1]**						
Blank	27.6	2.96	20.2	57.2	51.1	58.8
Protease	27.1	2.93	19.7	58.9	51.5	59.5
Prot+NSPase	27.6	2.92	19.2	59.4	51.8	58.5
Source x Enzyme						
RSM+ Blank	28.9	3.00	21.5	59.5	53.7	61.7
RSM+ Protease	27.9	2.86	19.6	61.0	53.7	63.6
RSM+Pr+NSPase	28.1	2.81	19.6	60.6	51.7	60.0
SBM+ Blank	26.3	2.92	18.9	55.0	48.6	55.9
SBM+Protease	26.4	3.00	19.8	56.7	49.3	55.5
SBM+Pr+NSPase	27.1	3.03	18.7	58.2	51.2	56.9
P-values						
Source	0.016	0.394	0.038	0.002	0.0001	0.0001
Enzyme	0.81	0.951	0.244	0.263	0.923	0.684
Source x Enzyme	0.589	0.515	0.088	0.723	0.077	0.129

[a,b] Means within a row with different letters differ significantly ($P < 0.05$).

[1] SBM, soybean meal; RSM, Rapeseed meal; Pr, protease; Pr+NSPase, protease plus NSPases; GIT, gastro-intestinal tract.

4.3 Nutritive value of upgraded rapeseed meal fed without and with protease and NSPases in pigs (IRTA)

A trial was conducted to compare the efficacy of conventional RSM grown in the EU and the same RSM upgraded by bioprocessing at Hamlet Protein to increase its crude protein content in combination with exogenous enzymes for growing pigs. Growth performance (ADWG), feed intake (ADFI), feed conversion ratio (FCR), and total tract apparent digestibility (TTAD) of major nutrients were used as response criteria. The trial was conducted following a 2x3 factorial arrangement with six experimental treatments. These corresponded to diets based on conventional RSM or upgraded by Hamlet Protein in combination with protease or protease plus NSPase enzymes. The enzymes were sprayed over the pellets at the feed mill. Cereal-based diets with 24% inclusion of conventional RSM were formulated according to the nutrient requirements of growing pigs. The total amount of RSM was replaced by 22.0% of upgraded RSM on a basis of similar CP content and adjusted by supplying synthetic AA supply to meet AA requirements. Consequently, CP, lysine, and other AA contents were similar for all diets, regardless of the source of RSM used. Pigs were fed the experimental diets *ad libitum* during the whole trial, which lasted 39 days. One-hundred forty-four crossed Pietrain x (Large White x Landrace) pigs initially weighing 27.6 kg were allocated at two pigs per pen for a total of 12 pens per treatment. One male and one female were penned together so that 72 males and 72 females were used. They were grouped in 12 blocks of live weight at the beginning of the trial. During the last week of trial, fresh faeces were collected early in morning from the pigs and frozen, before being processed.

4.3.1. Trials with growing pigs

IRTA has an Integrated Quality Policy and the Institute is ISO 9001:2015 certified for their R+D+T projects, contracts, and services. The experimental procedures used in this study were in accordance with ISO 9001:2015 quality criteria, and under the global SOPs PG-75 (Conducting Research studies) and IT-0602-F-011 (Quality Control of feed mill).

Experimental feeds: Feeds were produced at IRTA's feed mill, according to a multi-step manufacturing schedule. Major feed ingredients were ground through a 25 HP hammermill (Rosal VR-30) until the particles passed through a 3 mm sieve and then sent to a 500 kg horizontal mixer (Rosal) and mixed during 5 min. For each batch of feed, a 10-kg sample SBM containing 48% protein was used and premixed with the corresponding experimental product and additives (mineral and vitamin premix, amino acids, macro-minerals). Titanium dioxide as indigestible marker was included in the diets. The mixture was then mixed and homogenised, before the addition of fat/oil. Mash feed was sent to the pelleting system: conditioner, pelleting press, and vertical cooler (Mabrik PV-30). Steam flux and feed entry were regulated automatically by the system (Mipps 210, Mangra SA). Temperatures observed during the pelleting/conditioning process were registered. Pelleted feed was sent to the packaging system. At the feed mill, the feedbags were identified with date of mixing, treatment code, bag number, and net weight. Feeds were stored at the trial site under dry conditions at room temperature. Representative samples of each batch of feed were taken in the feed manufacturing plant following the preparation of the mixtures. Per production batch, a minimum of five subsamples of approximately 500 g each were taken at regular intervals throughout bagging ansd thoroughly homogenised. Final feed samples were bagged in ziplock bags and labelled following protocol instructions. One set of feed samples from each trial was analysed for the main nutrients (i.e., DM, ash, gross energy, CP, fat, crude fibre (CF) and non-starch polysaccharides (NSPs)).

Chemical analyses: Faeces were oven-dried at 65°C for 72 h and ground (<0.5 mm) before analysis. Feeds and faeces were analysed for DM, GE, CP, starch, ash, fat, CF, mineral, and NSPs contents. All the methods and procedures followed AOAC (2000). Dry matter content was determined on a stove at 103 °C until reaching a constant weight, ash by incineration for 4 h on a oven at 550 °C, fat by a Buchi Extraction System B-811 (Buchi Labortechnik AG, Flewil, Switzerland), crude protein by the Dumas procedure by a Nitrogen/protein FP-528 analyzer (LECO corp., St Joseph, Mo, USA), and CF by an ANKOM 200/220 Fiber Analyzer. Gross energy was determined in an adiabatic calorimeter (IKA C-4000, IKA® - Werke GmbH, Staufen, Germany) and metabolizable energy contents was calculated as 0.79 of the gross energy value determined by calorimetry according to INRA (2002). Total starch was analyzed by colorimetry, as a result of the glucose release after enzymatic hydrolysis as described by Willamil *et al*. (2012). Total NSPs were extracted from samples according to Englyst *et al*. (1994). Calcium, P and Ti contents from ash samples previously obtained were analysed after acid digestion by inductively-coupled plasma mass spectrometry (ICP-MS).

Calculations and statistical analysis: TTAD of major nutrient and individual NSPs was calculated according to the following equation:

$$TTAD = (1-(X_{faeces}/ X_{diet}) \times (M_{diet}/M_{faeces})) \times 100$$

where X_{faeces} and X_{diet} are the nutrient concentrations in faeces and the diet, respectively, and M_{diet} and M_{faeces} are the marker (Ti) concentrations in diet and faeces, respectively.

The pig trial was set up as a 2x3 factorial design with six dietary treatments, 12 blocks of liveweight or 12 replicates per treatment. A factorial model with RSM source (Conventional vs upgraded), enzyme addition (without, with proteases, and proteases plus NSPases), and interaction between all factors as main effects was used. The statistical analysis was performed using the GLM procedure of SAS and means were compared using Student-Newman-Keuls test.

4.3.2 Performance and feed efficiency

The effects of RSM and enzymes on growth performance are presented in Table 4. On average, pigs gained 768 g/d, ingested 1.62 kg/d, and had a feed:gain ratio of 2.12. No significant differences were observed on ADWG due to the type of RSM ($P > 0.05$) and type of enzymes ($P > 0.05$). No interactions ($P > 0.05$) were observed between the type of RSM and enzyme supplementation on growth performance or feed efficiency. However, a highly significant effect of the type of RSM on ADFI ($P < 0.01$) and FCR ($P < 0.002$) was observed. Pigs fed upgraded RSM diets had a 70 g/d lower ADFI improving their FCR by about 100 g relatively to pigs fed conventional RSM diets.

Table 4. Effect of conventional (RSM-) and upgraded (RSM+) rapeseed meal supplemented with exogenous enzymes on growth performance of growing pigs.

	Rapeseed meal		Enzymes			Statistical analysis[2]			
Treatment[1]	RSM-	RSM+	No	Prt	Prt+NSP	RSM	ENZ	R*EZ	RSD
BW, kg	38.9	38.9	38.8	38.0	39.1	0.97	0.75	0.62	1.71
ADFI, kg	1.66b	1.59a	1.619	1.631	1.615	0.01	0.89	0.71	0.12
ADWG, g	767	769	757	779	768	0.92	0.60	0.97	78.1
FCR	2.17b	2.07a	2.15	2.10	2.11	0.002	0.36	0.79	0.12
BW (39d), kg	65.7	65.8	65.2	66.8	66.0	0.93	0.67	0.88	3.54

[1] RSM-: diet based on conventional unprocessed rapeseed meal with 35% of crude protein. RSM+: diet based on RSM- processed by Hamlet Protein to improve crude protein (40% of CP). No: RSM type without enzyme inclusion. Prt: RSM type plus protease. Prt+NSP: RSM type plus protease and NSPase.

[2] ENZ: dietary enzyme inclusion; R*EZ: Interaction between RSM type and enzymes; RSD: residual standard deviation.

a,b Means within the same row and category name (rapeseed meal or enzyme) with different superscript differ ($P < 0.05$).

4.3.3 Total tract apparent digestibility

The effects of RSM used, and enzymes on total tract digestibility of major nutrients are presented in Table 5. The TTAD of DM, energy, CP, and NSP was not affected by the type of RSM used or the inclusion of protease or NSPase enzymes ($P > 0.05$). Instead, TTAD of fat, ash, Ca, and P was significantly improved ($P < 0.05$) by the type of RSM, particularly in diets without enzymes inclusion, which explains the significant interaction observed ($P < 0.05$). In general, enzyme supplementation did not affect TTAD of any of the parameters evaluated. The absence of an effect of proteases on CP digestibility may be explained by the formulation of diets with similar digestible AA contents. Furthermore, growing-finishing pigs have a well developed digestive system and the effects of exogenous enzymes are less than when enzymes are applied in diets for younger pigs. The efficiency of exogenous carbohydrases (NSPases) depends on the structure of the fiber and the degree of lignification of the fiber (Bach Knudsen, 2014). The TTAD of fat, Ca, and P is greater for RSM+ diets compared with RSM- in absence of enzyme supplementation.

Table 5. Effect of conventional rape seed meal (RSM), high protein RSM and enzyme inclusion (protease and NSPase) on TTAD (%) of major nutrient components for growing pigs.

	Dietary treatments[1]						Statistical analysis[3]			
Item[2]	T1	T2	T3	T4	T5	T6	RSM	Enz	RS*Enz	RSD
DM	83.6	84.4	84.6	83.7	84.2	84.1	0.92	0.90	0.08	1.29
OM	85.9	86.6	86.8	85.9	86.5	86.3	0.71	0.90	0.10	1.19
GE	84.8	85.5	85.8	84.7	85.4	85.1	0.57	0.94	0.08	1.30
CP	82.1	82.6	83.8	81.7	83.0	82.7	0.25	0.75	0.12	2.11
EE	78.7	82.4	80.9	81.0	80.9	81.2	0.02	0.84	0.03	2.39
CF	40.3	40.0	41.3	37.7	39.2	39.9	0.57	0.92	0.35	5.18
NFE	91.4	91.8	91.8	91.4	91.8	91.6	0.95	0.90	0.15	0.76
Ash	43.1	48.7	46.6	46.3	46.1	46.0	0.04	0.72	0.01	3.37
P	40.0	49.3	43.8	46.3	43.0	46.5	0.001	0.91	0.02	4.23
Ca	42.3	49.9	48.5	47.0	48.8	47.4	0.17	0.36	0.01	5.13
NSPs	65.6	65.6	64.9	63.5	64.9	63.7	0.29	0.62	0.53	3.86
Ara	73.7	74.3	74.0	72.9	74.3	73.2	0.04	0.38	0.54	2.90
Xyl	53.8	56.9	53.7	54.3	55.2	55.4	0.63	0.72	0.70	5.66
Man	87.4	88.1	87.9	87.0	87.4	84.1	0.48	0.49	0.33	1.66
Gal	70.0	72.6	70.8	69.4	71.5	70.5	0.79	0.47	0.14	3.41
Glu	58.2	58.6	58.7	54.2	58.0	55.9	0.15	0.53	0.22	4.69

[1] T1: Conventional RSM (RSM-)/no enzymes; T2: High protein RSM (RSM+)/no enzymes; T3: RSM-/protease; T4: RSM+/protease; T5: RSM-/protease/NSPase; T6: RSM+/protease/NSPase);

[2] DM: dry matter. OM: organic matter. GE: gross energy. CP: crude protein. EE: ether extract. CF: crude fiber. NFE: nitrogen-free extract. ME: metabolizable energy. NSPs: Total non-starch polysaccharides. Ara: arabinose. Xyl: xylose. Man: mannose. Gal: galactose. Glu: glucose.

[3] ENZ: dietary enzyme inclusion; R*EZ: Interaction between RSM type and enzymes; RSD: residual standard deviation.

4.4 Nutritive value of residual green biomass pulp extracted without and with cell wall degrading enzymes for rabbits (IRTA)

A growing rabbit trial was conducted to compare the efficacy of residual biomass pulp obtained after extraction of protein from green biomass as described in deliverable D1.2. Growth performance, feed intake, feed conversion ratio (FCR), and total tract apparent digestibility (TTAD) of major nutrients were used as response criteria.

4.4.1 Trials with rabbits

Growing rabbits trial: Experimental treatments (5) corresponded to a control alfalfa-based (30%) diet or diets where green biomass pulp replaced (w/w) 10 or 20% of alfalfa. Two types of green biomass pulp (obtained without or with enzymes) were tested. The diets were formulated according to Spanish nutrient recommendations for growing rabbits. Two hundred rabbits from INCO herd (female F1 IRTA X male HyPlus PS40; 100 entire males and 100 females) of approximately 5 weeks of age and 0.85 kg BW were used in a 4-week growth trial. Rabbits were housed in 50 pens of 4 animals each. All cages contained animals of both sexes. At start of the trial, rabbits were randomly distributed by initial weight in 10 blocks of 5 consecutive cages. All five treatments were randomly distributed among the five pens (1 pen per treatment) within the block. Each cage (0.80 x 0.40 = 0.32 m^2) had a fully slatted floor, was provided with a nipple drinker, and feed was distributed *ad libitum* using a feeding hopper. Room environment was controlled with automatic heating and forced ventilation. Artificial lighting was provided to ensure a minimum of 12 h of light/day. During the last week of the trial, fresh faeces were collected early in morning pens and frozen, before being processed.

Calculations and statistical analysis: The trial was a randomized complete block design with five treatments, 10 blocks, and 10 replicate treatment. A statistical model with treatment and block was used, and orthogonal contrasts were performed to discriminate between levels of inclusion and type of green biomass pulp.

4.4.2 Growth performance and feed efficiency

The effect green biomass pulp on growth performance is presented in Table 6. Following the recommendations to reduce in-feed antimicrobials usage in animal production, no antibiotics were included in diets. This is presumably the reason why the mortality was high (22.5%) during the first weeks of trial due to an *E.coli* outbreak. As a consequence, rabbits were submitted to an oral colistin treatment for 2 weeks *via* drinking water according to the veterinary prescription.

Table 6. Effect of residual green biomass pulp with/without enzymes on growth performance of growing rabbits.

Treatment	**T1**	**T2**	**T3**	**T4**	**T5**	**Statistical analysis[1]**			
Green Pulp, %	**0**	**10**	**10**	**20**	**20**	**Contrast**			
Enzymes	**No**	**No**	**Yes**	**No**	**Yes**	**Trtm**	**Dose**	**Enz**	**RSD**
Initial BW, kg	1.026	1.007	1.004	1.000	1.049	0.48	0.37	0.34	0.067
Final BW, kg	2.221	2.187	2.136	2.120	2.176	0.71	0.95	0.79	0.171
Mortality, %	20.0	25.0	30.0	17.5	20.0	--	--	--	--
Weight gain, kg	1.195	1.180	1.132	1.122	1.127	0.73	0.74	0.47	0.150
ADWG, g/d	41.2	40.7	39.0	38.7	38.9	0.73	0.74	0.46	5.17
ADFI, g/d	130	128	135	128	137	0.36	0.89	0.05	13.6
FCR	3.208	3.320	3.420	3.312	3.420	0.45	0.96	0.27	0.280

[1] Trtm: dietary treatment; Enz: enzyme inclusion during the process of protein extraction; RSD: residual standard deviation.

On average, rabbits gained 1.152 kg corresponding to an ADG of 39.7 g/d. Feed consummation was 131 g/d and feed:gain ratio 3.352. No significant differences and no interactions ($P > 0.05$) were observed on performance due to the type or levels of green biomass pulp included. However, by contrast analysis, significant effect of the type of pulp used was detected ($P < 0.05$). Rabbits fed diets containing residual pulp after extraction with enzymes had a higher feed intake than the others. As no effect was observed on ADWG, a trend to increase numerically FCR was observed. This effect could be due to a lower energy content of these diets which could explain the increase in feed intake.

4.4.3 Total tract apparent digestibility

The effect green biomass pulp on digestibility of major nutrient are presented in Table 7. TTAD of DM, OM energy, CP and fiber fractions like NDF, ADF or hemicelluloses was statistically affected by dietary treatments (P<0.01) whereas the CP digestibility was not affected by the diet. Inclusion of green biomass pulp reduced TTAD of main nutrients, as well as of fiber fractions, and this effect was stronger with the highest inclusion level (P<0.01). Utilization of enzymes during extraction of green protein also lead to reduced nutrient TTAD digestibility (P<0.10) when the residual pulp is included in diets. From these results it is clear that residual green biomass has a negative impact on nutrient digestibility. Pulp replaced alfalfa on a weight by weight basis, without taking into account any correction of its dietary nutritional value indicating that the nutritive value of the green biomass pulp extracted without and with the use of enzymes is lower than that of alfalfa.

Table 7. Effect of residual green biomass pulp with/without enzymes on nutrient TTAD digestibility of growing rabbits.

Treatment	T1	T2	T3	T4	T5	Statistical analysis[1]			
Green Pulp, %	0	10	10	20	20	Contrast			
Enzyme	No	No	Yes	No	Yes	Trtm	Dose	Enz	RSD
Dry matter	57.0ab	58.1a	56.2ab	55.5b	55.3b	0.01	0.006	0.09	1.87
Energy	59.3a	58.3a	55.4ab	56.5b	54.5b	0.002	0.002	0.06	1,89
Organic matter	62.1a	60.0ab	57.3abc	58.1bc	56.6c	0.003	0.005	0.03	1.83
Crude protein	68.4	68.6	65.7	66.6	65.b5	0.31	0.89	0.56	2.54
Ash	36.7a	37.1	32.4 ab	30.6b	30.7b	0.15	0.31	0.08	4.53
Crude fiber	21.2a	17.3ab	14.2b	16.5b	19.2ab	0.008	0.002	0.07	3.68
NDF	32.5a	30.8ab	28.9ab	27.6b	30.6ab	0.001	0.001	0.08	3.25
ADF	19.6a	19.6a	11.9b	14.7b	12.7b	0.001	0.001	0.12	4.10
Hemicellulose	50.1ab	46.7cb	49.8 ab	44.7c	51.4a	0.001	0.001	0.51	3.89

[1] Trtm: dietary treatment; Enz: enzyme inclusion during the process of protein extraction; RSD: residual standard deviation.
a,b Means within the same row and category name (rapeseed meal or enzyme) with different superscript differ ($P < 0.05$).

5. Conclusions

An *in vitro* model simulating the conditions of the digestive tract was used to screen for the best protease candidates to solubilize protein from RSM and SBM. The potential of non-starch polysaccharide degrading enzymes (NSPases) to increase nutrient release from the substrates was evaluated *in vitro*.

The European RSM was included replacing 10 or 20% SBM in starter and growing period into diets in combination with inclusion of proteases or proteases plus NSPases enzymes. Replacing 10 or 20% SBM with RSM reduced growth performance and final bodyweight of chickens at the end of the trial. In addition, ileal DM and CP digestibilities were reduced, and relative digestive organ organ weight was increased. Therefore, current RSM cannot replace SBM for broiler chickens. Inclusion of protease or protease plus NSPase cocktail enzymes did not affect any of the parameters evaluated in the study.

The EU RSM upgraded by bioprocessing by Hamlet Protein to increase its protein content was used to replace conventional RSM (22 vs 24% inclusion) as the main protein source in diets for growing pigs. The inclusion of RSM was based on a similar protein content and adjustement of synthetic AA supply. Consequently, diets contained similar protein and essential AA, which could explain that growth performance was similar. On the opposite, upgraded RSM improved feed efficiency, which could be associated with an improvement in fat, Ca, and P digestibility. Exogenous enzymes provided as proteases and proteases plus NSPases were not able to improve the nutrient availability of RSM. Further studies should focus on the use of enzymes on RSM-based diets with reduced nutrient content, aiming at a compensatory effect of the enzymes to improve nutrient utilization. The improvement of feed efficiency with upgraded RSM seems to indicate a positive contribution to reduce EU protein imports for animal feeding.

The residual biomass pulp obtained after protein extraction (with or without enzymes) from green biomass (see deliverable D1.2 for details) was included in diets to replace alfalfa and evaluated in growing rabbits. Growth performance was not affected whilst nutrient TTAD digestibility was strongly reduced by the type and level of inclusion. Replacement of alfalfa was done on a weight basis assuming a similar nnutritional value due to a lack of characterization of its feeding value. This was the first study

of this type of raw material. Further studies should address the question of *in vitro* characterization before test it again in animal trials.

Although the *in vitro* studies indicated that the selected proteases and NSPases were able to solubilize proteins and release reducing sugars from the RSM, neither the study with broilers nor the study with pigs demonstrated an improvement in the nutritive value of RSM *in vivo*. It is well known that the fibre fraction in RSM has a very complex composition (Bach Knudsen, 2014), which most likely is the reason for the lack of effect. Regarding the pulp of green biomass extracted without and with the use of cell wall degrading enzymes, the results reported in deliverable D1.2 showed a lower carbohydrate content of the extracted protein fraction, but only little change in the fibre composition of the pulp. The lack of effect of the enzymes on the *in vivo* rabbit parameters and the lower nutritive value of the pulps compared to the Lucerne is probably a reflection of the removal of good quality protein and readily available carbohydrates."

6. Annexes

Annex 1: Product and diet compositions

Table 8. Analyzed composition (%) of conventional (RSM-) and upgraded (RSM+) rapeseed meals used.

Item	**RSM-**[1]	**RSM+**[2]
Dry matter	88.2	88.9
Ash	6.38	7.29
Crude protein	35.0	40.0
Crude fiber	11.96	12.90
Gross energy, Mcal/kg	4.21	4.34
Metabolizable energy, Kcal/Kg	3.32	3.43
Phytic Phosphorus	3.45	1.79
Glucosinolates, µmol/g	13.7	16.9

[1] RSM-: conventional unprocessed rapeseed meal with 35% of crude protein from Terres Inovia.

[2] RSM+: RSM- processed by Hamlet Protein to improve its crude protein (40% of CP).

Table 9. Composition of the diets produced through the combination of different processing methods, offered to broilers over the starter (0-14) and grower periods (15-28) (UNEW).

	Starter (d0-10)		Grower (d11-24)	
Diet	**SBM**	**RSM**	**SBM**	**RSM**
Ingredient (%)				
Wheat	55.03	50.09	58.05	47.85
Maize (Corn)	10.00	10.00	10.00	10.00
Rapeseed meal (EU)	0.00	10.00	0.00	20.00
Hipro Soy (USA)	29.00	22.00	25.00	12.00
Soy Oil	1.23	3.05	2.50	5.95
Limestone	0.83	0.74	0.76	0.59
Dicalcium Phosphate	0.70	0.65	0.65	0.50
Sodium bicarbonate	0.20	0.20	0.20	0.20
Salt	0.20	0.20	0.20	0.20
Lysine HCl	0.36	0.43	0.30	0.40
DL-Methionine	0.35	0.33	0.28	0.21
L-Threonine	0.20	0.23	0.16	0.19
L-tryptophan	0.00	0.00	0.00	0.01
Vitamins and mineral [1]	0.40	0.40	0.40	0.40
Phytase+Xylanases	0.02	0.02	0.02	0.02
TiO_2	0.50	0.50	0.50	0.50
Enzyme preparation	1.00	1.00	1.00	1.00
Calculated composition				
AMEp	12.34	12.34	12.75	12.75
CP	21.30	21.00	19.63	19.37
digCP	18.42	17.75	16.89	15.86
Crude Fibre	2.89	3.65	2.84	4.39
Digestible Lys	1.21	1.21	1.06	1.06
Digestible Met+Cys	0.90	0.91	0.80	0.79
Digestible Threonine	0.81	0.81	0.71	0.71
Digestible Tryptophan	0.21	0.21	0.19	0.19
Digestible Ca	0.9	0.9	0.85	0.85
Available P	0.43	0.43	0.41	0.41
Analysed composition				
DM	87.2	87.6	87.8	88.3
CP	21.0	20.9	19.4	19.2
Total Oil (b)	4.00	6.67	4.72	7.66
CF	2.3	3.9	2.2	4.3
NDF	9.2	11.3	9.0	12.3
ADF	2.72	5.07	2.73	7.66
Ash	5.7	6.0	4.9	5.1

[1] The premix supplied per kilogram of diet: antioxidant, 100 mg; biotin, 0.30 mg; cholecalciferol, 5000 IU/kg µg; folic acid, 2.2 mg; menadione, 3.2 mg; niacin, 60 mg; pyridoxine, 5.4 mg; trans-retinol, 13.000IU/kg; riboflavin, 8.6 mg; thiamine, 3.2 mg; dl-α-tocopheryl acetate 80IU/kg; choline chloride, 1700 mg; Cu, 16 mg; Fe, 20 mg; I, 1.25 mg; Mn, 120 mg; Mo, 0.5 mg; Se, 300 µg; Zn, 110 mg.

Table 10. Ingredient and analyzed composition of the experimental diets used in growing pigs trial (IRTA).

Ingredients (%)	**RSM-**[1]	**RSM+**[2]
Barley	20.00	20.00
Wheat	13.64	21.34
Rice broken	14.00	14.00
Maize	13.01	8.58
RSM from TerresInnovia (RSM-)	24.00	---
RSM upgraded by HP (RSM+)	---	21.97
Soybean meal 48%	1.42	---
Starch	6.00	6.00
Fat 3/5 Grefacsa	4.21	4.31
Calcium carbonate	0.63	0.66
Monocalcium phosphate	0.85	0.89
Salt	0.18	0.12
Sodium bicarbonate	0.35	0.44
L-Lysine-HCl	0.47	0.47
DL-Methionine	0.03	0.02
L-Threonine	0.14	0.13
L-Tryptophan	0.03	0.02
L-Valine	0.01	0.02
Vitamins and minerals [3]	0.40	0.40
Noxyfeed	0.02	0.02
Post-pelleting solution	0.60	0.60
Nutrients		
Metabolizable energy, MJ/kg	9.912	9.945
Dry matter	89.70	90.40
Crude protein	15.40	16.00
Crude fiber	3.70	3.80
Ash	5.00	5.10
Calcium	0.72	0.77
Phosphorus	0.59	0.61
Non-starch polysaccharides (NSPs)	11.57	11.50
Arabinose	13.4	13.5
Xylose	19.8	20.2
Manose	13.8	15.1
Galactose	7.1	6.9
Glucose	45.8	44.2

[1] RSM--: diet based on conventional unprocessed rapeseed meal with 35% of crude protein.

[2] RSM++: diet based on RSM- processed by Hamlet Protein to improve its crude protein (40% of CP).

[3] Provides per kg feed: vitamin A (E-672) 5500 UI; vitamin D_3 (E-671) 1100 UI; vitamin E (alfa-tocopherol) 7 mg; vitamin B_1 0.5 mg; vitamin B_2 1.4 mg; vitamin B_6 1 mg; vitamin B_{12} 8 µg; vitamin K_3 0.5 mg; calcium panthotenate 5.6 mg; nicotinic acid 8 mg; choline 120 mg; Fe (E-1) (from $FeSO_4 \cdot 7H_2O$) 80 mg; I (E-2) (from $Ca(I_2O_3)_2$) 0.5 mg; Co (E-3) (from $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$) 0.4 mg; Cu (E-4) (from $CuSO_4 \cdot 5H_2O$) 5 mg; Cu (E-4) (from amino acids quelate) 5 mg; Mn (E-5) (from MnO) 40 mg; Zn (E-6) (from ZnO) 100 mg; Se (E-8) (from Na_2SeO_3) 0.25 mg.

Table 11. Chemical and nutrient composition of the experimental diets used on the growing rabbit trial (IRTA).

Ingredients (%)	**Ctrl**	**T-2**	**T-3**	**T-4**	**T-5**
Alfalfa	30.00	20.00	20.00	10.00	10.00
Green biomass	---	10.00	---	20.00	---
Green biomass + enzymes	---	---	10.00	---	20.00
Wheat midds	21.76	24.51	24.31	24.52	24.31
Barley	15.00	10.00	10.00	10.00	10.00
Sunflower meal	13.59	15.88	16.08	15.84	16.06
Sugarbeet pulp	10.00	10.00	10.00	10.00	10.00
Carob pulp	5.00	5.00	5.00	5.00	5.00
Molasses	1.50	1.50	1.50	1.50	1.50
Fat 3/5 Grefacsa	1.50	1.50	1.50	1.50	1.50
Sepiolite	0.75	0.75	0.75	0.75	0.75
Salt	0.28	0.29	0.29	0.29	0.29
Sodium bicarbonate	0.20	0.20	0.20	0.20	0.20
L-Lysine-HCl	0.08	0.07	0.06	0.08	0.08
DL-Methionine	0.05	0.03	0.03	0.04	0.04
Minerals & vitamins[1]	0.20	0.20	0.20	0.20	0.20
Copper sulphate	0.04	0.04	0.04	0.04	0.04
Noxyfeed	0.02	0.02	0.02	0.02	0.02
Diclazuril 0.5%	0.02	0.02	0.02	0.02	0.02
Nutrients					
Energy (Mcal DE/kg)	2.446	2.417	2.416	2.407	2.407
Energy (Mcal ME/kg)	2.289	2.256	2.255	2.248	2.247
Crude Protein (%)	16.36	16.41	16.39	15.81	15.75
Crude Fibre (%)	15.03	15.85	15.86	16.23	16.23
NDF (%)	32.41	35.78	35.64	38.46	38.17
ADF (%)	18.36	20.11	19.86	21.35	20.83
Starch (%)	13.90	12.06	12.00	12.06	12.00
Sugars (%)	6.70	6.79	6.79	6.73	6.73
Fat (%)	3.50	3.50	3.59	3.50	3.68
Ash (%)	7.22	7.09	6.97	6.75	6.51
Calcium (%)	0.84	0.83	0.83	0.82	0.82
Phosphorous (%)	0.45	0.48	0.48	0.48	0.48
Total Lysine (%)	0.75	0.75	0.75	0.75	0.75
Total Methionine (%)	0.33	0.32	0.32	0.33	0.33

[1] Provides per kg feed: vitamin A (E-672) 8000 UI; vitamin D_3 (E-671) 1500 UI; vitamin E (alfa-tocopherol) 25 mg; vitamin B_1 0.4 mg; vitamin B_2 2 mg; vitamin K_3 1 mg; calcium panthotenate 8 mg; nicotinic acid 20 mg; choline 200 mg; Fe (E-1) (from $FeSO_4 \cdot 7H_2O$) 30 mg; I (E-2) (from $Ca(I_2O_3)_2$) 0.5 mg; Co (E-3) (from $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$) 0.59 mg; Cu (E-4) (from $CuSO_4 \cdot 5H_2O$) 5 mg; Mn (E-5) (from MnO) 20 mg; Zn (E-6) (from ZnO) 40 mg; Se (E-8) (from Na_2SeO_3) 0.112 mg.

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Annex 3: Scientific output

Melo, A.D.B.; 2019. Evaluation of fedd technologies in rapeseed meal and their effects on growth performance and nutrient digestibility of growing pigs. PhD thesis dissertation, Pontifícia Universidade Católica do Paraná, Brasil, 55pp.

Melo A.D.B., Villca B., Esteve-García E., Lizardo R.; 2018. Rapeseed meal and enzyme supplementation on growth performance and nutrient digestibility in pigs. Book of abstracts nº24, 69th EAAP Annual Meeting, 27-31 of August, Dubrovnik, Croatia, 704pp, https://doi.org/10.3920/978-90-8686-871-1.

Melo A.D.B., Villca B., Esteve-García E., Lizardo R.; 2018. The influence of faeces drying method on nutrient digestibility of growing pigs. Book of abstracts nº24, 69th EAAP Annual Meeting, 27-31 of August, Dubrovnik, Croatia, 704pp, https://doi.org/10.3920/978-90-8686-871-1.