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ABSTRACT

The digestibility (in vitro), toxicity and metabolic effects of rapeseed (RPH) and sunflower (SPH) protein hydrolysates have been evaluated in a murine animal model. The enzyme Alcalase® was employed to obtain a mild enzymatic hydrolysis of rapeseed and sunflower defatted seed meals (DSM) protein isolates. Both hydrolysates showed higher in vitro digestibility than the respective DSM, presumably as a consequence of the hydrolysis process that they had undergone. In vivo, RPH and SPH were well tolerated. Body and organ weights, biochemical blood parameters from treated male mice were comparable to controls. Food intake was regular in RPH and SPH animals, suggesting a good palatability of the hydrolysates. Not relevant perturbations of the principal hepatic and renal drug metabolism enzymes were observed in RPH or SPH mice. In conclusion, protein hydrolysates from sunflower and rapeseed DSM did not determine relevant toxicological effects; therefore, they could be considered as alternative protein sources and/or food ingredients.

Introduction

In the context of renewable energy, biofuel supply chains offer undeniable advantages that have led the European Union to stimulate their spread since 2003. Among these, the best known is the biodiesel chain, derived by a simple fatty acid methylation starting from renewable vegetable oils as those obtained from rapeseed, sunflower and other seeds. Nevertheless, the biodiesel penetration and diffusion in the market is still weak due to the low oil yields and insufficient valorisation of the related by-products, as glycerine and defatted seed meals (DSM), for developing industrial, agricultural and veterinary added value products.

One of the multi-applications of DSM from rapeseed and sunflower seeds is the production of protein hydrolysates to be used as an alternative source of protein for the food industry and especially for pet food. This need arises from the growing demand of products, in particular for dogs and cats, suffering from adverse food reactions (AFRs) (Kang et al. 2014; Willis-Mahn et al. 2014; Suto et al. 2015).

Since such disorders are mostly due to the food proteins, the treatment of these diseases involves the replacement of standard proteins with novel protein sources. In this regard, the home-prepared diet is considered the gold standard elimination diet (Scott et al. 2001; Biourge et al. 2004) but is time-consuming and requires a great deal of cooperation from the owner over a period of several weeks (Tapp et al. 2002). Moreover, home-made diets may also not be adequate for maintenance after diagnosis or even for diagnosis in young growing animals (Roudebush & Cowell 1992; Streiff et al. 2002).

More recently, hydrolysed veterinary diets have been proposed for the diagnosis of canine AFRs (Olivry & Bizikova 2010; Gaschen & Merchant 2011). During hydrolysis, protein sources are enzymatically broken down to polypeptides, changing and reducing the allergenic properties of the molecules (Boumans 2001; Cave & Guilford 2004). Consequently, hydrolysed proteins result less allergenic and more digestible (Sá et al. 2013). In this regard, in a study by

Jackson et al. ([2003](#)), the majority of dogs with confirmed adverse reactions to soy and corn did not show a flare-up of their skin disease when fed a soy hydrolysate and corn starch diet. Differences in the degree of hydrolysation and protein source can influence the clinical response as shown in experimentally sensitised dogs (Olson et al. [2000](#)). The protein hydrolysates currently used for AFRs treatment mainly derive from animal by-products (including chicken, poultry liver, casein) and non-EU seeds as soybeans, implying an expensive economic and environmental effort. Trying to overcome this problem, EU rapeseed and sunflower DSM (derived from the biodiesel chain), were investigated for their possible application in this context, also thanks to their high-protein content (Lomascolo et al. [2012](#)). In addition, the rapeseed protein isolate was considered safe by the Europeans Food Safety Authority (EFSA NDA Panel [2013](#)).

In the present study, a mild enzymatic hydrolysis of DSM protein isolates, by food-grade proteases, as Alcalase® enzyme, was performed in order to obtain a product rich in small peptides, that could be more easily absorbed by the animal intestine than the original proteins, and free of non-protein anti-nutritional compounds and fibres (Parrado et al. [1993](#); Conde et al. [2005](#); Chabanon et al. [2007](#)).

After rapeseed (RPH) and sunflower (SPH) protein hydrolysates production, for the first time, an investigation only focussed on their safety was conducted as a prerequisite for determining the suitability of these foodstuffs to be potentially added to pet diets. Therefore, the aim of this study was the evaluation of digestibility (*in vitro*), toxicity and metabolic effects (on phase I and II enzymes) of the diets containing RPH and SPH, in mice.

Materials and methods

Chemicals

For the enzymatic hydrolysis, Alcalase® (endopeptidase from *Bacillus licheniformis*, 2.4L) was purchased from Sigma-Aldrich (St. Louis, MO).

For the determination of the *in vitro* digestibility, the following chemicals were used: gastric lipase, Amano Lipase F-AP15 (Amano Enzyme Inc., Nagoya, Japan); bile salts, cholic acid–deoxycholic acid sodium salt mixture (Fluka, Milwaukee, WI); pancreatin from porcine pancreas and pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO).

For the determination of phase I and II enzymatic activities, the following chemicals were used: Nicotinamide adenine dinucleotide phosphate,

oxidised and reduced form ($NADP^+$ and NADPH), 7-ethoxyresorufin, *p*-nitrophenol, aminopyrine, ethoxycoumarin, pentoxyresorufin, methoxyresorufin, resorufin, ethoxycoumarin, bovin serum albumin, L-glutathione reduced, Folin–Ciocalteu reagent, 1-chloro-2,4-dinitrobenzene, 1-naphtol were purchased from Sigma Chemical Co. (St. Louis, MO); glucose 6-phosphate, glucose 6-phosphate dehydrogenase and cytochrome c were from Boehringer-Mannheim (Mannheim, Germany). All other chemicals and solvent used were of the highest available commercially purity.

Enzymatic hydrolysis

Rapeseed and sunflower DSM were produced by industrial mechanical extraction based on a hexane process, and provided by Italcol S.p.A Castelfiorentino (Florence, Italy). The two hydrolysates SPH and RPH were prepared as follows: protein isolates were obtained from DSM by alkaline extraction (pH 10.5) and isoelectric precipitation (at pH 4.3 for sunflower and pH 5.0 for rapeseed proteins) as described by Villanueva et al. ([1999](#)) and Vioque et al. ([1999](#)), with the difference that the sedimentation/flotation step was skipped. Hydrolysis was performed on the obtained protein isolates, suspended in distilled water (10%, w/v), adding Alcalase® (endopeptidase from *Bacillus licheniformis*, 2.4L), $0.2 \, AU \, g^{-1}$ (Alcalase–meal protein ratio). The process was followed by the pH-stat technique (Mettler Toledo DL 50 Graphix), for 60 min, at pH 8 and 50 °C and stopped by adjusting the pH to 6.0 with citric acid. The final hydrolysates were then recovered by centrifugation (30,000*g*, 30 min, 10 °C), freeze-dried, and stored at room temperature.

The composition of the two hydrolysates SPH and RPH was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels in Tris-Glycine SDS running buffer (25 mM Tris-HCl pH 8.3, 192 mM Glycine, 0.1% SDS) and Coomassie blue staining technique. Hydrolysate peptide molecular weights were estimated by following the GE Healthcare protocols and by using the low molecular weight calibration kit for SDS electrophoresis (GE Healthcare, 14–94 kDa).

The SPH, RPH and protein isolates, obtained from rapeseed and sunflower DSM, were analysed by size gel filtration chromatography using an AKTA fast protein liquid chromatography (FPLC) system equipped with a Superose 12 HR 10/30 column (Amersham Biosciences) for peptide profile determination (Ugolini et al. [2015](#)). The FPLC chromatograms of RPH and SPH hydrolysates were compared with the respective

Table 1. Chemical composition of ingredients and experimental diets used in the *in vitro* digestibility trial (% of dry matter).

	Crude protein	Ether extract	Crude ash	NDF	Starch
Rapeseed DSM	32.7 ± 0.1	3.0 ± 0.1	7.0 ± 0.1	36.1 ± 0.8	14.4 ± 0.1
Sunflower DSM	29.8 ± 0.4	0.8 ± 0.1	6.7 ± 0.1	47.2 ± 1.1	3.8 ± 0.5
Rapeseed hydrolysate (RPH)	70.2 ± 1.2	7.4 ± 0.1	13.8 ± 1.2	0.4 ± 0.2	2.0 ± 0.8
Sunflower hydrolysate (SPH)	83.2 ± 1.8	1.1 ± 0.1	14.4 ± 0.8	1.1 ± 1.2	1.9 ± 0.6
Cereal mix (CM)	9.7 ± 0.1	5.1 ± 0.1	2.7 ± 0.1	12.3 ± 0.5	66.0 ± 0.3
CM + Rapeseed DSM[a]	20.5	4.1	4.7	25.1	41.7
CM + Sunflower DSM[a]	19.6	3.0	4.7	29.5	35.4
CM + RPH[a]	26.0	5.9	6.2	8.5	45.1
CM + SPH[a]	28.0	4.0	5.6	9.7	48.2

NDF: neutral detergent fibre; DSM: defatted seed meals; CM + rapeseed DSM, 5 g cereal mix + 4.45 g rapeseed meal; CM + sunflower DSM, 5 g cereal mix + 4.85 g sunflower; CM + RPH, 5 g cereal mix + 2.43 g rapeseed hydrolysate; CM + SPH, 5 g cereal mix + 1.92 g sunflower hydrolysate.
[a]Calculated values.

peptide profile of the protein isolates. Elution was monitored at 280 nm with a UV UCP-900 monitor (Amersham Biosciences, Amersham, UK). Molecular weights were determined with a calibration curve made with blue dextran (2000 kDa), catalase (240 kDa), aldolase (158 kDa) and chymotripsinogen A (25 kDa) as standards.

Free amino acids were determined by reversed phase HPLC analysis and automated precolumn derivatization with *o*-phtalaldehyde-3-mercaptopropionic acid (OPA) and 9-fluorenylmethylchloroformate (FMOC), as described in Ugolini et al. (2015). A Hewlett-Packard Model 1100 system, coupled with a diode array detector, and a Thermo Scientific Syncronicis-C18 column (3 × 250 mm, 5 μm) was used.

In vitro *digestibility*

In vitro digestibility of diets containing DSM or protein hydrolysates was determined using the two-step procedure proposed by Biagi et al. (2016), consisting of an incubation of the samples at 39 °C for 2 h with HCl and pepsin followed by 4 h with pancreatin; gastric lipase (10 g/L) was added to the HCl-pepsin solution and bile salts (25 g/L) were added to the pancreatin solution.

There were four replicates (flasks) for each of the following four treatments:

1. 5 g cereal mix for dogs + 4.45 g rapeseed DSM (CM + rapeseed DSM)
2. 5 g cereal mix for dogs + 4.85 g sunflower DSM (CM + sunflower DSM)
3. 5 g cereal mix for dogs + 2.43 g RPH (CM + RPH)
4. 5 g cereal mix for dogs + 1.92 g SPH (CM + SPH)

After *in vitro* digestion, the undigested fraction of each bottle was dried at 70 °C until a constant dry weight was obtained. Chemical composition of diets

and undigested residues was determined according to AOAC standard methods (AOAC 2000; Method 954.01 for crude protein, Method 920.39 for ether extract, Method 942.05 for crude ash, Method 920.40 for starch) and neutral detergent fibre (NDF) was assayed accordingly to the procedure described by Van Soest et al. (1991), with a heat stable amylase and expressed inclusive of residual ash. Experimental diets were used in different amounts to ensure that each flask contained the same protein amount. The cereal mix was a commercial mix of cereals and vegetables (COOP Italia, Italy). Chemical composition of the ingredients and experimental diets is reported in Table 1.

In vivo *trial with mice*

All the experimental procedures were carried out in conformity with protocols endorsed by the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Bologna (Permit Number: 10-71-2012). All efforts were made to minimise suffering. All animal studies comply with the ARRIVE guidelines.

Twenty-four male Swiss-Albino CD1 mice (Harlan Laboratories S.R.L., Udine, Italy), 5 weeks old, weighing 39.7 ± 3.4 g, were housed under controlled conditions (12 h light–dark cycle, 22 °C, 50% humidity). All animals were examined for clinical signs on receipt and none had abnormalities suggestive of ill health. After one week of acclimatisation, mice were randomly assigned to three experimental groups, 4 animals per cage: i) Control (fed a standard rodent chow, Mucedola s.r.l. Milan, Italy), ii) RPH diet (fed a diet made of standard rodent chow + 10% RPH, Mucedola s.r.l.), iii) SPH diet (fed a diet made of standard rodent chow + 10% of SPH, Mucedola s.r.l. Milan, Italy). Both RPH and SPH were used at 10% and provided to mice 70 and 83 g

of protein per kg of diet, respectively. Food and tap water were offered *ad libitum*. The animals received the experimental diets for 28 consecutive days.

Body weight and food intake

Body weight was recorded at pre-test and thereafter weekly. Food intake was estimated by subtracting the amount of food left in the cages from the total amount provided every two days.

Signs and symptoms

During the study period, each mouse was visually inspected daily for the development of any physical appearance abnormalities of cutaneous apparatus, respiratory, cardiovascular and gastrointestinal systems.

Blood biochemistry parameters

Blood samples (one sample for mouse) were collected from the tail vein immediately before the sacrifice, transferred into heparinised tubes, and centrifuged for 15 min at 1500g to obtain plasma. Routinely measured biochemical parameters (glucose, creatinine, ALT, fructosamine, NEFA, total cholesterol, triglycerides, plasmatic total protein, albumin, albumin/globulins) were analysed in plasma samples by the Department of Veterinary Medical Sciences, University of Bologna. In particular, NEFA were measured using a commercial kit based on an *in vitro* enzymatic colorimetric method (NEFA-HR, Wako Chemicals GmbH, Neuss, Germany). The other blood parameters were assayed by colorimetric methods in automated chemistry analyser (AU 400, Olympus/Beckman Coulter, Brea CA).

Phase I and II enzymatic activities

In mice, the modulation of the main hepatic and renal enzymes of drug metabolism was assessed, in order to eventually highlight the associated implications (as drug–food interactions).

Tissue collection and preparation of subcellular fractions

Mice were fasted 16 h prior to being sacrificed. Animals were killed in accordance with approved Ministerial procedures appropriate to the species. Liver and kidney were immediately removed from each mouse, weighed and processed separately. The S9 fraction and the post-mitochondrial supernatant were prepared as recently described by Canistro et al. (2016). The fractions were immediately frozen in liquid

nitrogen, stored at $-80\,^\circ$C and used within a week for enzymatic analyses. In this short period, the activities measured in conditions of V_{max} and linearity of protein contents were stable.

Phase I enzymatic activities

Aminopyrine N-demethylase (APND) Activity was determined by the quantification of CH_2O release, according to Mazel (1971). The total incubation volume was 3 ml, composed of 0.5 ml of water solution of 50 mM aminopyrine and 25 mM $MgCl_2$, 1.48 ml of 0.60 mM $NADP^+$, 3.33 mM G6P in 50 mM Tris-HCl buffer (pH 7.4), 0.02 ml G6PDH (0.93 U/ml) and 0.125 ml of sample. After 5 minutes of incubation at $37\,^\circ$C, the yellow colour developed by the reaction of the released of CH_2O with the Nash reagent was read at 412 nm, and the molar absorptivity of 8,000 used for calculation (Nash 1953; Barillari et al. 2007).

p-Nitrophenol hydroxylase (p-NPH) Activity was determined in a final volume of 2 ml: 2 mM p-nitrophenol in 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and a NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, 0.2 U of isocitrate dehydrogenase and 1.5 mg of proteins. After 10 minutes of incubation at $37\,^\circ$C, the reaction was terminated by addition of 0.5 ml of 0.6 N perchloric acid. Precipitated proteins were removed by centrifugation and 1 ml of the resultant supernatant was mixed with 1 ml of 10 N NaOH. Absorbance at 546 nm was immediately recorded and 4-nitrocathecol determined ($\varepsilon = 10.28\,mM^{-1}\,cm^{-1}$) (Reinke & Mayer 1985; Vivarelli et al. 2016a).

Pentoxyresorufin O-dealkylase (PROD), ethoxyresorufin O-deethylase, (EROD) and methoxyresorufin O-demethylase (MROD) Reaction mixture (PROD) consisted of 0.025 mM $MgCl_2$, 200 mM pentoxyresorufin, 0.32 mg of proteins and 130 mM NADPH in 2.0 ml 0.05 M Tris-HCl buffer (pH 7.4). Resorufin formation at $37\,^\circ$C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin (excitation 563 nm, emission 586 nm) (Lubet et al. 1985). EROD and MROD activities were measured exactly in the same manner as described for the pentoxyresorufin assay, except that substrate concentration was 1.7 mM for ethoxyresorufin and 5 mM for methoxyresorufin (Burke et al. 1985; Melega et al. 2013).

Ethoxycoumarin O-deethylase (ECOD) ECOD was determined by the quantification of umbelliferone

formation, according to Aitio (1978). Incubation mixture was consisted of 2.6 ml, composed of 1 mM ethoxycoumarin, 5mM $MgCl_2$, NADPH-generating system (see aminopyrine assay) and 0.25 ml of sample. After 5 minutes of incubation at 37 °C, reaction was stopped by the addiction of 0.85 ml of trichloroacetic acid (TCA) 0.31 M. The pH of the mixture was brought to about 10 by adding 0.65 ml of 1.6 M NaOH-glycine buffer (pH 10.3); the amount of umbelliferone was measured fluorimetrically (excitation 390 nm; emission 440 nm) (Canistro et al. 2009).

Phase II enzymatic activities

Glutathione S-transferase (GST) The incubation mixture for measuring overall GST activity contained 1 mM glutathione +1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol +0.025 ml of sample in a final volume of 2.5 ml 0.1 M phosphate Na^+/K^+ buffer (pH 6.5). The product of the reaction of the thiol group of glutathione with the electrophilic group of CDNB was read at 340 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$) (Habig et al. 1974; Sapone et al. 2016).

UDP-glucuronosyl transferase (UDP-GT) UDP-GT was determined kinetically using 1-naphtol as substrate (final concentration, 50 mM) by the continuous fluorimetric (excitation 390 nm; emission 440 nm) monitoring of 1-naphtholglucuronide production in the presence of 1 mM uridine-5′-diphosphoglucuronic acid (Mackenzie & Hänninen 1980). Experiments were performed in the presence or absence of Triton X-100 (0.2%) as a detergent, in order to improve the assay sensitivity (Vivarelli et al. 2016b).

Protein concentration Protein concentration was determined according to the method described in Lowry et al. (1951) and revised in Bailey (1967), using bovine serum albumin as standard and diluting samples 200 times to provide a suitable protein concentration.

Statistical analysis

Statistical analysis of digestibility data was performed by one-way ANOVA, with the Newman–Keuls test as the post test. Differences were considered statistically significant at $p < .05$. Statistical analysis of the results derived from the animal treatment was performed using Wilcoxon's rank method as reported by Box and Hunter (1978).

Results

Enzymatic hydrolysis

As a result of the preliminary protein isolation by the isoelectric precipitation procedure, final SPH and RPH had a high protein content of 83.2 and 70.2%, respectively (Table 1) and their peptide molecular weight distribution was evaluated by gel filtration chromatography and SDS-PAGE. The SDS-PAGE analysis showed the presence of peptides with a molecular weight lower than 25 KDa and 14 KDa for SPH (Figure 1) and RPH (Figure 2), respectively. The gel filtration chromatography profile clearly indicated the shift towards lower molecular size protein-peptide in SPH and RPH, if compared with the protein isolates, and showed the
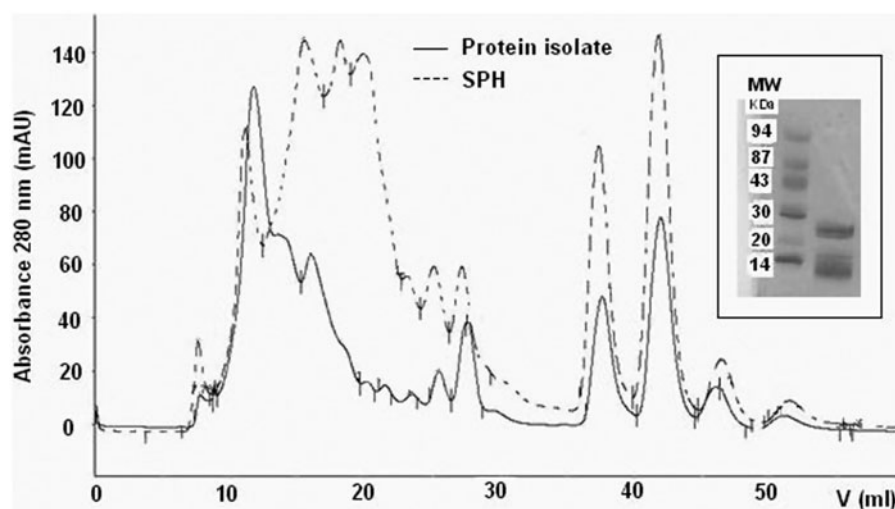

Figure 1. Gel filtration chromatography of the sunflower protein isolate (—) and the final sunflower protein hydrolysate (- - -) on Superose 12 HR 10/30. Inset: SDS-PAGE of sunflower protein hydrolysates (SPHs); MW, molecular weight markers (kDa).
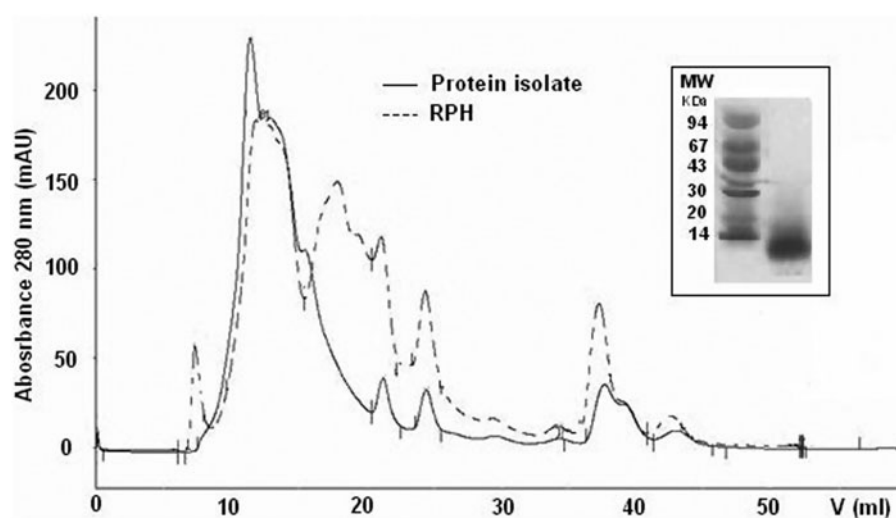
Figure 2. Gel filtration chromatography of the rapeseed protein isolate (—) and the final rapeseed protein hydrolysate (- - -) on Superose 12 HR 10/30. Inset: SDS-PAGE of rapeseed protein hydrolysates (RPHs); MW, molecular weight markers (kDa).

Table 2. *In vitro* digestibility (%) of diets containing different sources of protein.

Diet	Dry matter	Organic matter	Crude protein	Ether extract	Crude ash
CM + Rapeseed DSM	72.5b	74.1b	87.1a	95.4b	35.8a
CM + Sunflower DSM	63.4a	64.6a	89.1b	93.8a	37.4b
CM + RPH	90.4c	90.4c	96.1c	97.8d	89.7c
CM + SPH	90.1c	90.2c	96.1c	95.8c	89.0c
Pooled SEM	0.26	0.22	0.06	0.07	0.50

There were four replicates for each treatment. CM + rapessd DSM, 5 g cereal mix +4.45 g rapeseed meal; CM + sunflower DSM, 5 g cereal mix +4.85 g sunflower meal; CM + RPH, 5 g cereal mix +2.43 g rapeseed protein hydrolysate; CM + SPH, 5 g cereal mix +1.92 g sunflower protein hydrolysate. Experimental diets were used in different amounts to ensure that each flask contained the same amount of protein. Within a row, different letters indicate significant differences ($p < .05$) among diets. CM: cereal mix; DSM: defatted seed meals; RPH: rapeseed hydrolysate; SPH: sunflower hydrolysate.

presence of new-formed low molecular weight compounds, represented by peaks with elution volume higher than 15 ml, probably represented most by small peptides (as showed by SDS-PAGE) and low percentage of amino acids (Figures 1 and 2). The use of Alcalase alone, in fact, permitted a mild protein hydrolysis and the SPH and RPH hydrolysate content of total free amino acids determined by HPLC analysis following OPA-FMOC derivatization (Ugolini et al. 2015) was limited to 0.6–1.0% w/w.

In vitro digestibility

Results regarding the *in vitro* digestibility of diets containing DSM or protein hydrolysates are reported in Table 2. Compared with diets containing rapeseed and sunflower DSM, dry matter, organic matter, crude protein, ether extract and crude ash digestibility was significantly higher in diets containing the hydrolysates ($p < .05$). Digestibility of dry matter, organic matter and crude protein did not differ between RPH and SPH diets ($p > .05$).

Table 3. Body weight and food intake in Swiss Albino male mice fed a standard chow diet (control) or a diet containing rapeseed or sunflower protein hydrolysates over 28 days period.

Parameters	Control	RPH	SPH
Body Weight, g			
Initial	38.9 ± 4.3	39.4 ± 3.4	40.6 ± 3.4
Final	41.6 ± 5.2	41.4 ± 4.2	43.9 ± 2.0
Weight gain, g	2.7 ± 2.5	2.0 ± 2.3	3.3 ± 1.5
Food intake, g/animal/day	5.9	7.1	6.7

Each value represents the mean ± SD of eight independent experiments on eight mice. Statistical analysis was applied only on body weight data. RPH: standard rodent chow +10% rapeseed protein hydrolysate; SPH: standard rodent chow +10% sunflower protein hydrolysate.

In vivo trial with mice

Body weight and food intake

No significant difference ($p > .05$) was observed in body weight gain among the different mice groups (Table 3). Furthermore, compared with control, food intake was slightly numerically increased in mice eating RPH and SPH (Table 3).

Signs and symptoms

No toxicological signs were recorded throughout the experiment through physical examination. During the whole study, mice were in good health and appeared to well tolerate the diets. No mortality occurred in any of the treatment groups. No abnormal behaviour was observed. No respiratory signs such as dyspnoea, cyanosis or apnoea, neither nervous signs as convulsions, piloerection or changes in motor function were shown. Gastrointestinal (i.e. diarrhoea, soft faeces) or skin (dermatitis, alopecia) toxicological signs were not observed.

Blood biochemistry parameters

Biochemical blood determinations serve as an indicator of toxicity of a test material (Schilter et al. 2003). As shown in Table 4, there were no statistically significant differences in clinical biochemical parameters among all the animal groups ($p > .05$). All blood biochemistry values fell into the normal physiological range (Schnell et al. 2002; Serfilippi et al. 2003).

Terminal organ weights

There were no significant ($p > .05$) differences in terminal organ weights among experimental groups (data not shown).

Phase I enzymatic activities

Table 5 reports various microsomal mixed function monooxygenases detected as single probe in the liver and kidney of mice.

In the liver, CYP 3A1/2-supported (APND) oxidase was induced by both RPH (up to 52.7%, $p < .05$) and SPH (up to 18.9%, $p < .05$) treatments. In mice fed with RPH, the CYP2E1-linked monooxygenase (pNPH) was significantly increased (up to 18.9%, $p < .01$). The main up-regulations were observed for CYP2B1/2 (PROD) and CYP1A2 (MROD) -supported oxidases in animals fed with SPH (respectively, up to 24.7%, $p < .01$ and 44.2%, $p < .01$). A slight down-regulation was recorded in RPH treatment for CYP1A1-linked oxidase (EROD) (12.1% loss, $p < .05$), while an up-regulation was observed, for the same isoform, in the SPH group (up to 21.4%, $p < .01$).

In the kidney, pNPH was significantly decreased by RPH (6.3% loss, $p < .05$) and SPH (24.0% loss, $p < .01$). The main down-regulations were observed for PROD in RPH (13.6% loss, $p < .01$) and SPH (49.4% loss, $p < .01$) treatments. Significant reductions were also recorded for both CYP1A2-supported (MROD) oxidase (29.5% loss, $p < .05$) and CYP1A1-supported (EROD) oxidase (46.0% loss, $p < .01$) in mice receiving SPH.

Table 4. Blood biochemistry parameters in Swiss Albino male mice fed a standard chow diet (control) or a diet containing rapeseed or sunflower protein hydrolysates.

Parameters	Control	RPH	SPH
ALT, U/L	158 ± 11	161 ± 13	152 ± 14
Creatinine, mg/dL	0.34 ± 0.02	0.34 ± 0.02	0.34 ± 0.03
Glucose, mg/dL	32 ± 6	41 ± 8	29 ± 7
Fructosamine, μmol/L	351 ± 23	358 ± 24	350 ± 22
Triglycerides, mg/dL	152 ± 20	159 ± 15	142 ± 12
NEFA, mmol/dL	2.09 ± 0.10	2.11 ± 0.15	2.00 ± 0.13
Total cholesterol, mg/dL	169 ± 12.1	163 ± 14.0	172 ± 16.2
Total protein, g/dL	5.73 ± 0.43	5.71 ± 0.49	5.69 ± 0.44
Albumine, g/dL	3.03 ± 0.02	3.02 ± 0.05	3.01 ± 0.04
Albumine/globulins ratio	1.12	1.12	1.12

Each value represents the mean ± SD of eight independent experiments on eight mice. RPH: standard rodent chow +10% rapeseed protein hydrolysate; SPH: standard rodent chow +10% sunflower protein hydrolysate.

Table 5. Phase I enzymatic activities in the liver and kidney of Swiss Albino male mice fed a standard chow diet (control) or a diet containing rapeseed or sunflower protein hydrolysates.

Parameters	Control		RPH		SPH	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Aminopyrine N-demethylase (CYP3A1/2), nmol mg^{-1} min^{-1}	16.876 ± 0.608	n.d.	25.761 ± 2.684*	n.d.	20.05 ± 0.725*	n.d.
p-Nitrophenol hydroxylase (CYP2E1), nmol mg^{-1} min^{-1}	1.109 ± 0.083	0.605 ± 0.030	1.319 ± 0.026**	0.567 ± 0.010*	1.042 ± 0.111	0.460 ± 0.008**
Ethoxycoumarin O-deethylase (CYP1A1/2, CYP2A, CYP2B, CYP2E1), pmol mg^{-1} min^{-1}	0.746 ± 0.071	n.d.	0.832 ± 0.112	n.d.	0.716 ± 0.065	n.d.
Pentoxyresorufin O-dealkylase (CYP2B1/2), pmol mg^{-1} min^{-1}	4.483 ± 0.275	0.081 ± 0.001	4.693 ± 0.353	0.070 ± 0.005**	5.590 ± 0.390**	0.041 ± 0.009**
Metoxyresorufin O-demethylase (CYP1A2), pmol mg^{-1} min^{-1}	43.030 ± 5.799	0.112 ± 0.023	44.221 ± 4.221	0.093 ± 0.014	62.038 ± 7.103**	0.079 ± 0.016*
Ethoxyresorufin O-deethylase (CYP1A1), pmol mg^{-1} min^{-1}	12.656 ± 0.580	0.037 ± 0.006	11.121 ± 0.478*	0.035 ± 0.008	15.361 ± 0.688**	0.020 ± 0.006**

Each value represents the mean ± SD of eight independent experiments on eight mice.
*Significant difference ($p < 0.05$) between treated groups and the control;.
**Significant difference ($p < 0.01$) between treated groups and the control (Wilcoxon's rank method).
RPH: standard rodent chow +10% rapeseed protein hydrolysate; SPH: standard rodent chow +10% sunflower protein hydrolysate; n.d.: not detectable for detection limits.

Table 6. Phase II enzymatic activities in the liver and kidney of Swiss Albino male mice fed a standard chow diet (control) or a diet containing rapeseed or sunflower protein hydrolysates.

Parameters	Control		RPH		SPH	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Glutatione S-transferase, nmol/mg per min	1.837 ± 0.087	0.546 ± 0.085	1.919 ± 0.114	0.544 ± 0.030	1.914 ± 0.155	0.487 ± 0.055
UDP-gluguronosyl transferase, nmol/mg per min	1.610 ± 0.108	0.286 ± 0.047	$1.291 \pm 0.174*$	0.286 ± 0.076	$1.160 \pm 0.148*$	0.274 ± 0.050

Each value represents the mean $\pm$ SD of eight independent experiments on eight mice. RPH: standard rodent chow $+10\%$ rapeseed protein hydrolysate; SPH: standard rodent chow $+10\%$ sunflower protein hydrolysate.
*Significant difference ($p < .05$) between treated groups and the control (Wilcoxon's rank method).

Phase II enzymatic activities

As reported in Table 6, down-regulations for both RPH and SPH treatments were observed in the liver for UDP-glucuronosyl-transeferase (respectively, 19.8% loss, $p < .05$, and 28.0% loss, $p < .01$). The RPH and SPH diets did not significantly affect post oxidative enzymes in the kidney of mice.

Discussion

The aim of this study was the evaluation of digestibility (*in vitro*), toxicity and metabolic effects (on phase I and II enzymes) of diets containing RPH and SPH, in mice, in order to evaluate the possibility to employ these protein hydrolysates as alternative protein sources in companion animals. Nowadays, the market of industrial food for pets, especially for those with a suspect or a confirmed diagnosis of AFRs, is currently growing. In this regard, protein hydrolysates have demonstrated to be particularly hypoallergenic, as their employment has shown to treat efficaciously food allergies even deriving from the corresponding whole protein source (Jackson et al. 2003).

The employment of European rapeseed and sunflower DSM (derived from the biodiesel production chain and characterised by a high protein content), easily useful and broadly available, could represent a valid alternative to this problem, reducing the costs.

The production of hydrolysates from rapeseed and sunflower DSM typically requires a first protein isolation step from the DSM that usually precedes the hydrolysis step. This procedure may decrease the protein extraction yield (Ugolini et al. 2015), but allows to obtain a product free of antinutritional compounds (namely clorogenic acid in sunflower DSM or fitic acid and glucosinolates in rapeseed DSM) and still rich in protein (Parrado et al. 1991; Parrado et al. 1993; Villanueva et al. 1999; Vioque et al. 1999; Chabanon et al. 2007). In this regard, chemical analysis confirmed a high-protein content of both RPH and SPH evaluated in the present study.

Digestibility of diets containing DSM or protein hydrolysates derived from rapeseed or sunflower were evaluated *in vitro*. In the present study, RPH and SPH were used as a complete replacement for the rapeseed and sunflower DSM. Compared with diets containing the hydrolysates, total dry matter digestibility was much lower in diets containing the intact DSM, presumably mainly due to their higher fibre content. In fact, fibre cannot be digested using the *in vitro* procedure that was performed, as it is aimed to simulate the enzymatic digestion that takes place in the stomach and small intestine of dogs. Also, the observed difference in dry matter digestibility between diets containing rapeseed and sunflower DSM may be explained by the higher fibre content of the latter. Furthermore, this nutrient may have exerted minerals binding-properties (well-known, accordingly to literature; Baye et al. 2017), as demonstrated by the lower crude ash digestibility in DSM-based diets. Probably, this effect contributed to influence dry matter digestibility results. Similarly, also organic matter resulted in more digestible in diets containing protein hydrolysates, probably because of the corresponding higher protein and ether extract digestibility values. Anyway, with regards to the digestibility of lipids, it can be supposed that the differences observed exerted only a limited influence on organic matter digestibility, considering the modest amounts of this nutrient in the diets evaluated. While the slightly different lipid digestibility percentages between DSM and protein hydrolysate-based diets are difficult to explain, it can be presumed that the higher protein digestibility of diets containing RPH and SPH was the consequence of the hydrolysis process. In fact, it has been shown that, in humans, the ingestion of a casein hydrolysate may result in improved protein digestion and absorption when compared with intact casein (Koopman et al. 2009). Similarly, nitrogen apparent digestibility was improved in Atlantic salmons fed increasing levels of fish protein hydrolysates (Refstie et al. 2004; Hevrøy et al. 2005). In another study, *in vitro* digestibility of casein and whey proteins was improved when they were hydrolysed to different degrees of hydrolysis (Sindayikengera & Xia 2006).

A range of studies is used for hazard identification and characterisation of any new material for use in

food or food supplement. The mice Swiss Albino CD1 were fed for 28 consecutive days diets containing 10% of RPH or SPH with the aim of showing up both adverse toxicological effects in major vital systems and the modulation of drug metabolism enzymes.

The use of hydrolysates in the diets could reduce palatability of the feed for the presence of free amino acids and short peptides that can alter the flavour of the food making it bitterer. For this reason, food consumption and body weight were checked during the duration of the whole experiment. In contrast with the hypothesis, during the treatment, a regular food consumption was observed in treated animals compared with controls, suggesting a good palatability of the hydrolysates and the body weight gain was not affected by dietary treatment. In this regard, under various regulatory guidelines, body weight gain is an integral part of the conventional safety evaluation of a test material. Significant body weight loss is considered one of the most sensitive indicators of an animal's deteriorating health status (Schilter et al. 2003). Based on these evidences, it could be stated that all animals maintained good health conditions throughout the whole study. Furthermore, concerning data on food intake, mice fed with diets containing protein hydrolysates revealed numerically slightly higher amounts of food consumed. Lacking other parameters endorsing the increased food consumption in the treated groups (such as, e.g. a strong hyperactivity), it was concluded that the observed variation may be due to the consistency of the diets containing the hydrolysates, which appeared to be more friable than the control diet, leading to higher amounts of food wasted by the animals. These data not being very reliable, statistical analysis was not applied.

The experimental diets had no significant effect on animals' blood parameters (ALT, creatinine, glucose, fructosamine, triglycerides, NEFA, total cholesterol, total protein, albumin and albumin/globulin). The normal physiological ranges in which all the blood biochemistry values evaluated fell clearly indicate the absence of hematotoxic potential of the diets. In particular, the unchanged activity of plasmatic enzymes such as ALT (which indicates liver function), between controls and treated mice, suggests the absence of hepatic alterations attributable to diets containing protein hydrolysates. Finally, the measured parameters reveal a general metabolic profile not significantly altered in mice following RPH or SPH diet intake.

Diets containing protein hydrolysates could also set off adverse effects at enteric level, such as osmotic diarrhoea and soft faeces, as it is well known that low molecular weight compounds (such as aminoacids and small peptides) is able to increase osmolarity in the small intestine (Mahmoud 1994). However, during the treatment, animals did not show any gastrointestinal symptoms and food consumption was constant. In addition, there was no evidence of toxicological effects on respiratory tract, nervous system and skin in mice.

The modulation of the metabolic system was evaluated in different tissues. The observed perturbations of the oxidative and post-oxidative enzymes, although statistically significant, from a biological point of view, can be considered as not dangerous. A one-directional trend was not recorded, but a complex pattern of mild up and down-regulations that cannot affect the whole metabolism of the animal. These fluctuations, not involving the entire enzymatic patterns, do not compromise their important functions. It can be concluded that both protein hydrolysates had no important effects on drug metabolism enzymes. It is very important to evaluate the perturbation of these catalysts, because they play a predominant cellular role being implicated in endogenous and exogenous metabolism, and both their induction and/or suppression might have serious toxicological relevance.

It is well known that epigenetic mechanisms, as those linked to the positive and negative modulation of metabolising enzymes, can be associated with co-carcinogenesis, co-mutagenesis and promotion events. In fact, the increased bio-activation of ubiquitous pro-mutagens/pro-carcinogens saturating the enzymes involved in the 'error-free repair', can seriously pose DNA at structural damage risk. Furthermore, the up-regulation of phase I monooxygenases has been connected with the enhanced production of reactive oxygen species, a factor that may increase the incidence of several injuries (Paolini et al. 1994). Moreover, these enzymes not only metabolise exogenous substances, but also perform cellular and systemic functions (e.g. growth, differentiation, apoptosis, homeostasis and neuroendocrine regulation) due to their ability to recognise a wide range of endogenous substrates (steroids, corticosteroids, androgens, oestrogens, cholesterol, vitamin D, fatty acids, bile acids, thromboxanes, leukotrienes). Not least, nowadays pets are treated for various diseases (cardiovascular, cutaneous, neurological, cancers) also by multiple drugs administrations. For this reason, it is essential to know the potential modulations associated with these enzymes. In fact, the possible induction and/or inactivation of drug metabolism enzymes following the intake of xenobiotics, could affect the therapeutic efficacy and/or toxicity of co-administered drugs (pharmacokinetic drug–food interactions). As such, drug metabolism enzymes manipulation should be

interpreted with care, since, in many cases, it can also contribute to increased toxicological risks. Anyway, this was not the case in the present study, as both RPH and SPH proved to be completely safe at this level.

Conclusions

This is the first report on safety assessment of RPH and SPH and the observations in this study imply that mice well tolerated the RPH and SPH diet. In the toxicity study the hydrolysates failed to induce any significant alterations in parameters, indicating safety such as food intake, growth, organ weight, blood biochemical parameters. Moreover, RPH and SPH did not compromise the hepatic and renal drug metabolising enzymes functions, even if mild fluctuations of some oxidative and post-oxidative catalysts were recorded.

In conclusion, the present findings encourage further exploration of the protein hydrolysates from sunflower and rapeseed DSM as alternative protein sources in several applications, such as food for pets suffering from adverse food reactions.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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