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SOLUBLE PROTEIN CONTENT ASSESSMENT IN DRY PET FOOD RAW MATERIALS: COMPARISON BETWEEN FRESH MEAT AND MEAT MEAL FORMULATIONS

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Abstract

Long shelf life and ease of use make dry pet food a popular choice among pet owners, inasmuch as it represents the majority of pet food on the market today. Two kinds of raw materials are commonly employed for the production of dry pet food, namely fresh meats (FMs) and particularly meat meals (MMs). These raw materials, before coming onto the market as dry pet food, undergo production processes, transportation, and, when it comes to MMs, industrial transformations, which may result in unwanted modifications of such ingredients, especially as far as their protein content is concerned. The goal of this study is to analyze the protein content of the raw materials regularly used in the production of dry pet food. Different formulations of white, red, and fish FMs and MMs have been prepared and analyzed. The protein concentration of both FM and MM mixes was assessed by the Bradford assay, with the aim being to evaluate the soluble protein content, which represent also a convenient digestibility index. Subsequently, the quality of proteins was evaluated through the characterization of the electrophoretic profile assessed by SDS-PAGE followed by staining with Coomassie Blue dye. The results proved that the formulations made of FMs, compared to the ones based on MMs, have a higher soluble protein content and a better-defined protein profile, thus making the former the best choice as raw materials for dry pet food production.

Key words*: Digestibility, Dry Pet Food, Fresh Meats & Meat Meals, Raw Ingredients, Soluble Protein Content.*

INTRODUCTION

The rate of growth of the dry pet food market is continuously increasing, and new formulations are always proposed. The need for a thorough evaluation of the quality of the raw materials used in the production process thus becomes urgent (Montegiove et al., 2021; Zicker, 2008).
Most dry pet foods found on the market today are made of two different types of raw materials, which differ in their protein content. They consist of fresh meats (FMs) and, in particular, meat meals (MMs) (Montegiove et al., 2021; Montegiove et al., 2020a; Thompson, 2008). FMs derive from wastes of meat intended for human consumption, whereas MMs are meat by-products obtained during meat processing. FMs mainly consist of animal parts that are not suitable for human consumption but have shown no signs of disease that can be transmitted to humans. MMs, on the other hand, according to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21/10/2009, may also include other parts of the animals, such as bristles, feathers, hooves, and horns. These MMs are largely used by pet food suppliers to obtain complete feedstuffs, by enhancing the protein and amino acid (AA) content in pet kibbles; on the other hand, MMs undergo intensive industrial processes which may determine unfavorable effects on their

digestibility (Montegiove et al., 2021; Murray et al., 1997). Further to this, the onset of oxidation processes and the partial degradation of MM raw materials can lead to the loss of protein content bioavailability (Montegiove et al., 2021; Ribeiro et al., 2019; van Rooijen et al., 2013). The handling processes also play a key role in maintaining the organoleptic properties of proteins, as during the transport and storage of raw materials the protein component could be altered by microorganisms, whose proliferation can lead to the decarboxylation of some AAs with the formation of biogenic amines, responsible for numerous toxic effects on the body (Brozić et al., 2019; Carter et al., 2014; Learey et al., 2018; Montegiove et al., 2021; Montegiove et al., 2020b; Piergiovanni & Limbo, 2010).

Therefore, the quality of the final product is strictly dependent on the initial choice of the raw materials used, which thus becomes a crucial point for the manufacturing companies in the dry food production process for dogs and cats. Hence, this study aims at carefully analyzing the protein component of the raw materials typically employed in dry pet food production. Mixes of white (*i.e.*, chicken, turkey, and rabbit), red (*i.e.*, pork, beef, and lamb), and fish (*i.e.*, salmon, tuna, and anchovy) FMs and MMs were investigated in this study as representative examples of different kinds of animal protein sources that are commonly found in the pet food industry (Figure 1) (Aldrich, 2006; Thompson, 2008; Yathavamoorthi et al., 2020).

Protein content analysis was carried out using the Bradford assay (Bradford, 1976) in order to quantify the soluble protein content, which represents a convenient digestibility index and, at the same time, give an estimate of the total protein content as highlighted by a recent study (Montegiove et al., 2021).

Protein quality was evaluated through the electrophoretic protein profile assessed by SDS-PAGE followed by Coomassie Blue staining.

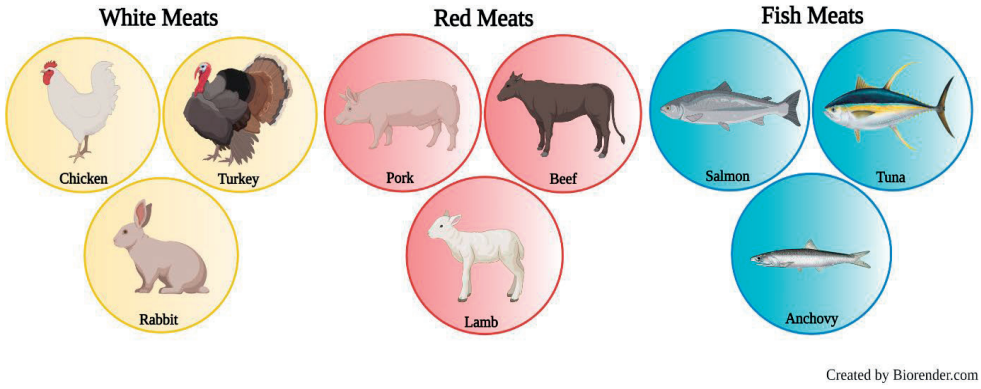

Figure 1. Protein sources for pet food raw materials analyzed in this study

MATERIALS AND METHODS

Raw Materials

The raw materials analyzed in this study are listed in Table 1 and they consist of a mix of white fresh meats (WFMs), white meat meals (WMMs), red fresh meats (RFMs), red meat meals (RMMs), fish fresh meats (FFMs) and fish meat meals (FMMs). Each formulation was prepared by mixing in equal parts each of the three animal raw materials considered for white, red, and fish FMs and MMs. All raw materials were provided by an Italian pet food company.

Determination of Moisture content

The AOAC's official method for animal feed moisture analysis was used to measure the moisture content of raw material formulations (Latimer, 2016).

An exact amount of each raw material mix (2 g) was uniformly distributed on a dish and dried at 135°C for 2 hours in an oven (Termaks TS 8136, Bergen, Norway).

The samples were weighed using an OHAUS Pioneer™ Analytical Balance (OHAUS Corporation, Parsippany, NJ, USA), after cooling at room temperature in a desiccator containing silica gel, until a constant and stable weight was achieved. The difference between the initial and final weight was used to measure the water content.

Table 1. List of raw materials used in this study

Raw Materials		
White Meats	Chicken	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Turkey	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Rabbit	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
Red Meats	Pork	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Beef	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Lamb	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
Fish Meats	Salmon	3 batches of FMs from Norwegian farms
		3 batches of MMs from European manufacturers
	Tuna	3 batches of FMs fished in the Pacific Ocean
		3 batches of MMs from European manufacturers
	Anchovy	3 batches of FMs fished in the Mediterranean Sea
		3 batches of MMs from European manufacturers

Protein Solubilization

The sample preparation was performed according to the protocol described by Montegiove et al. (2021). Raw material mixes were homogenized for 90 seconds at 4 °C in a hypotonic solution (10 mM Tris-HCl pH 7.5) at the concentration of 30 g/L (w/v) using ULTRA-TURRAX T25 (IKA®-Werke GmbH & Co. KG, Staufen, Germany).

In order to promote protein release from the organic matrix, 0.1% (v/v) IGEPAL® CA-630 (Sigma-Aldrich, Saint Louis, MO, USA), a non-denaturing detergent for satisfactory solubilisation of membrane protein complexes, was then applied. After that, with the purpose of removing the insoluble material, samples were sonicated for 30 seconds at 4°C with an ultrasonic disintegrator (Soniprep 150, MSE, Heathfield, East Sussex, UK) and centrifuged at 10,000 × g for 5 minutes at 4°C (5804 R, Eppendorf, Hamburg, Germany). The soluble protein fraction was recovered for the Bradford assay and SDS-PAGE coupled with Coomassie Blue staining.

Determination of Soluble Proteins

Soluble protein content in the three formulations was determined with the Bradford assay (Bradford, 1976) using Quick Start™ Bradford 1× Dye Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The Coomassie Brilliant Blue G-250 dye (Bio-Rad, Hercules, CA, USA), which has an absorption peak at 595 nm in the protein-bound form, was employed for the quantitative analysis. A Shimadzu UV-160A UV-Visible Recording Spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) was used to measure the absorbance at 595 nm. The soluble protein concentration of

the samples was calculated from the absorbance values using a calibration curve previously prepared with known concentrations of bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA). Data were normalized taking into account the different water content of the samples and expressed as g of soluble protein per 100 g of dry sample.

SDS-PAGE and Coomassie Blue Staining Method

The electrophoretic profile of the samples was evaluated according to Laemmli's protocol (Laemmli, 1970). An exact quantity of soluble protein extract was mixed with sample buffer (0.1 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and 25 mM dithiothreitol; Sigma-Aldrich, Saint Louis, MO, USA). Samples were boiled for 5 minutes and electrophoresed on 10% acrylamide gel (Mini-PROTEAN® 3 Cell, Bio-Rad, Hercules, CA, USA) at 40 mA. Gels were then stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data shown in this study are reported as mean values of the three analyzed formulations ± standard error of the mean (SEM). Student's t-test was used to assess the significance of the differences between the means of the protein content of each type of FM and its relative MM formulation (WFM vs. WMM; RFM vs. RMM; FFM vs. FMM) evaluated by the Bradford assay. The level of significance for the data was set at $p < 0.05$. All statistical tests were performed using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSIONS

Raw material soluble protein content, which, as highlighted by a recent study, represents also a convenient digestibility index and gives an estimate of the total protein content (Montegiove et al., 2021), was evaluated through the Bradford assay (Bradford, 1976), a fast, reproducible and cheap method for the quantification of proteins based on the use of the Quick Start™ Bradford 1x Dye Reagent. This method indeed turns out to be a simple and quick way to estimate the bioavailable protein content compared to the official methods (*e.g.*, the Kjeldahl and Dumas methods) usually used for this type of analysis (Latimer, 2016; Montegiove et al., 2021; Nielsen, 2017). These official methods, on one hand, take into account all the protein content, but, on the other hand, are much more time consuming, potentially hazardous for the workers, because of the reagents and high temperatures required, and more expensive for the manufacturing companies (Conklin-Brittain et al., 1999; Sáez-Plaza et al., 2013a; Sáez-Plaza et al., 2013b). Ultimately, these kinds of methods, estimating the nitrogen content, could overestimate the real protein content (Liu et al., 2015; Mariotti et al., 2008; Mæhre et al., 2018; Peng et al., 2014). However, as previously mentioned, a recent study showed how a method that assessed the soluble protein content can also give an estimate of the total raw material protein content allowing the problematics correlated to the traditional and official methods to be overcome (Montegiove et al., 2021).

Before assessing the soluble protein content by means of the Bradford assay, each raw material mix was investigated as for its moisture level. Figure 2 reports the water content in the different formulations.

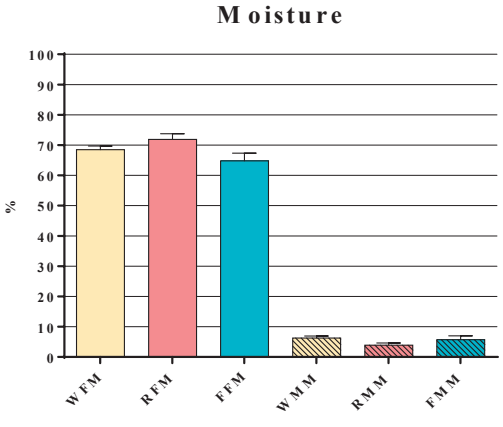

Figure 2. Water content in white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes. Data are reported as mean ± SEM, n = 3

It becomes clear that FM formulations feature much higher water contents compared to MM formulations. The moisture levels in the FM formulations span from about 65% in the case of FFMs to 72% in the case of RFMs; while all MM formulations exhibit a water content level lower than 10%. This feature is the result of the high-temperature treatment and dry processes used for the preparation of MMs through the rendering process (Montegiove et al., 2021; Murray et al., 1997).

Taking into account the different moisture level in the FM and MM mixes, the soluble protein content was subsequently evaluated by performing the Bradford assay. As it is apparent from the results shown in Figure 3, the soluble protein content is almost halved in the case of MM formulations with respect to the concentration found in all FM formulations. WFM mix has a content in soluble proteins about 1.9 times higher (16.2 g/100 g of dry sample) compared to the WMM mix, similar to what observed for the FMM mix, where the content is about 1.7 times higher (13.8 g/100 g of dry sample); while the RFM mix has a soluble protein content about 2.2 higher (15.3 g/100 g of dry sample) compared to the relative RMM mix.

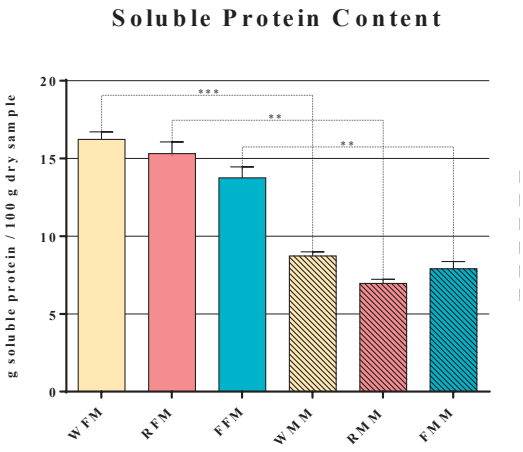

Figure 3. The soluble protein content of white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes determined by the Bradford assay. Data are reported as mean ± SEM, n = 3, ** $p < 0.01$, *** $p < 0.001$

These findings could be justified by the fact that MMs are also composed of bristles, feathers, hooves, and horns, containing large amounts of collagen, elastin, and keratin, which are fibrous proteins known to be insoluble or poorly soluble. In fact, globular proteins are fairly soluble and exhibit relatively high digestibility features, whereas fibrous proteins tend to be resistant to digestion (Kies, 1981; Liu et al., 2015).

The soluble protein content, assessed by the Bradford assay, was strongly in favor of FM formulations, regardless of the type of raw material taken into account, highlighting how these ingredients may be considered the best choice to be used in dry pet food production from a protein point of view, in that they have a greater quantity of soluble proteins which exhibit high digestibility and bioavailability features (Montegiove et al., 2021; Kies, 1981; Liu et al., 2015).

Afterward, in order to evaluate the quality of the protein content in the samples, the electrophoretic profile of both FM and MM raw material mixes was acquired and compared (Figure 4).

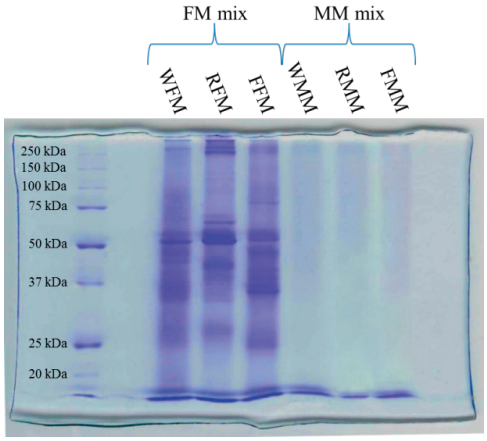

Figure 4. Protein profile of white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes evaluated by SDS-PAGE, followed by the Coomassie Blue staining method

The results shown in Figure 4 demonstrate how there is a substantial difference in the electrophoretic banding pattern between the two kinds of raw material formulations, suggesting a potential partial degradation of

proteins in all MM mixes analyzed. As a matter of fact, the lanes of MM mixes are characterized by smears rather than net bands, as instead expected in the presence of intact or slightly degraded proteins (Fischer, 1983). However, the smears disappear in the lower part of the gel, where small peptides would be found. This finding implies that the dimensions of the formed peptides are so small that they cannot be retained by the gel during the electrophoretic run. These results well correlate with the intensive rendering processes undergone by MMs, which may cause severe degradation and deterioration of the raw materials (Brozić et al., 2019; Carter et al., 2014; Learey et al., 2018; Montegiove et al., 2021; Montegiove et al., 2020b; Ribeiro et al., 2019; van Rooijen et al., 2013). *In vitro* and *in vivo* studies have indeed demonstrated how rendered raw materials, *i.e.* MMs, are more difficult to digest than meats (Montegiove et al., 2021; Murray et al., 1997). In addition, a degradation of the protein content, combined with inappropriate transport conditions could lead to the proliferation of some microorganisms as a result of decarboxylation processes would form biogenic amines, toxic compounds for the organism, which being heat-stable could be also found in the final product (Brozić et al., 2019; Carter et al., 2014; Einarsson et al., 2019; Learey et al., 2018; Montegiove et al., 2020b).

This study has thus shown how the various raw materials usually employed for dry pet food production effectively differ in their soluble protein content. These differences could significantly affect the quality of the final products, as the soluble protein content is closely related to the digestibility and bioavailability of the protein component (Montegiove et al., 2021; Kies, 1981; Liu et al., 2015).

These results could help the manufacturing companies to guide the choice toward the best raw materials to be used for the production of healthier dry foods for companion animals.

CONCLUSIONS

In conclusion, the reported investigation has demonstrated that the different kinds of raw materials generally used in the production of dry pet food, *i.e.*, FMs and MMs, have a quantitatively and qualitatively different protein composition.

As opposed to MM mixes, FM formulations appear to be the best kinds of raw materials that can be chosen when it comes to the production of dry food for pets, in terms of both protein bioavailability, as demonstrated by the higher soluble protein content evaluated through the Bradford assay, and better protein quality, as revealed by the electrophoretic analysis, which instead showed a marked degradation of proteins in MM formulations.

These findings can therefore provide a new approach in order to both produce better-quality pet food and assess the protein content in the starting raw materials. Companies could collect novel information on how to proceed in the formulation of new quality-improved products.

In light of these results, further and more in-depth studies may be carried out in order to correlate these preliminary results with the properties of the final products in terms of protein content, also evaluating the possibility of performing *in vitro* and *in vivo* tests.

Finally, this study has clearly shown how raw materials composed of FMs appear to be the best choice as ingredients for the production of dry food for dogs and cats.

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