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Article

Nutritional Value of Extruded Dog Food with Mechanically Separated Chicken Meat or Meat By-products

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Simple Summary: Fresh meat, such as mechanically separated chicken meat (MSCM), is usually listed first as an ingredient on pet food labels when selected by industry as a protein component. This commercial strategy may influence consumer choice in the comparison among pet food products. Since most dry pet foods are produced by food extrusion, it is important to compare the nutritional value of a dog diet based on MSCM with one based on meat by-products (MBP) to determine whether the "fresh meat" is really the better choice.

Abstract: The objectives of this study were: (a) to evaluate the effect of inclusion of mechanically separated chicken meat (MSCM) in dry dog food on fatty acid profile, *in vivo* and *in vitro* digestibility, and palatability as compared with dry dog food containing meat by-products (MBP); (b) to determine, whether or not, the inclusion of the one or the other ingredient changes the microbiology and the storage quality of the two food products; (c) to propose a new system (Daisy^{II} Incubator) to measure the *in vitro* digestibility of the two products. Their similar chemical composition notwithstanding, the MSCM product had lower palatability but better nutritional quality (with higher polyunsaturated fatty acid [PUFA] content and lower saturated fatty acid [SFA] content) than the MBP product. Microbiological risk assessment showed no microbiological hazards for either product. After 6 months storage, polyamine values were found to be higher in the MSCM than in the MBP. Finally, the Daisy^{II} Incubator proved a valid instrument for the study of *in vitro* digestibility also for dogs; since it provided data simply, quickly with less variability and cost than obtained with *in vivo* trials, it could represent the future for pet food digestibility studies. Our results indicate that inclusion of MSCM or MBP as the main protein ingredient in extruded pet food may be used advantageously in product formulations.

Keywords: dog food; fatty acid; palatability; digestibility; conservation quality; Daisy^{II} Incubator

1. Introduction

Overwhelmingly, pet owners consider their pets to be family members (63% of pet owners in the United States and more than 71% in Italy) [1,2]. Anthropomorphism of dogs and cats has driven pet owner preference for pet foods containing ingredients that they find in their own diet and processed so as to maintain the nutritional integrity of the ingredients and ensure food safety, high palatability, and digestibility. To meet these high expectations, the pet food industry is keen to increasingly include such ingredients, especially fresh and unprocessed meat, in dry dog food

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products. Behind this are commercial strategies, as well as various concerns about the meaning, content, and source of "by-products" from meats or poultry.

Consumer uncertainty about what passes as "by-products" may lead pet owners to perceive them as poor-quality ingredients, however [3]. Rendered protein meals are, in fact, widely used in the pet food industry and provide an excellent source of protein, energy, and minerals [4,5], however, misperceptions about their origin and content make fresh meat the more desirable ingredient. The Association of American Feed Control Officials [6] defines meat by-products as the edible parts and organs, but not the hair or skin, horns or hooves, intestinal contents, or feathers from poultry.

Fresh meat, on the other hand, refers to meat that has not undergone any treatment except having been maintained in the cold chain. Other treatments (e.g. cooking, drying, freezing, hydrolysis or addition of preservatives) exclude the meat ingredient from being called "fresh" [7]. These characteristics, together with the fact that fresh meat is usually listed as the first ingredient on pet food labels, could influence consumer choice among pet food products.

Including meat by-products or fresh meat in pet food can also have nutritional and technological implications. Compared with fresh meat alone, by-products provide more essential nutrients. For example, fresh meat lacks calcium and vitamin A, which are provided in by-products from the bones and liver [3]. Furthermore, rendering conditions, as well as the source and handling of raw materials, can greatly influence the nutritional quality and digestibility of the protein meals [8]. Furthermore, thermal treatment can interfere with these aspects as well.

Most dry foods are produced by extrusion. Correct extrusion conditions favor higher retention of amino acids, high protein and starch digestibility, less lipid oxidation, and higher retention of vitamins [9]. In addition, the extrusion process denatures undesirable enzymes such as anti-nutritional factors (trypsin inhibitors, hemagglutinins, tannins, and phytates) and sterilizes the finished product [9]. Although overcooking can diminish the nutritional quality of foods, the relatively high moisture content, moderate temperatures, and short cooking duration all help to maintain the nutritional quality of extruded foods [10-12].

As regards microbiological issues, there have been several recalls of commercial pet foods and treats in the United States because of contamination with *Salmonella* spp., *Escherichia coli*, and other foodborne pathogens [13]. Contamination not only poses the risk that pets ingesting these food items can become clinically ill or may become carriers of the pathogens but it also represents a public health concern for pet owners who handle food products and interact with their pets [14,15].

Finally, the use of animals in research elicits a diverse range of attitudes and emotions, with some people demanding the abolition of research on animals and others expressing strong support. Typically, opponents of animal research cite besides animal welfare and suffering also the uselessness of digestibility and palatability trials [16].

Given the above, the trend is to prefer the use of *in vitro* enzymatic analysis [17] over *in vivo* studies, which are more costly, laborious, and require animals anyway. In farm animal nutritional studies, *in vitro* digestibility is largely estimated using a Daisy^{II} Incubator (Ankom Technology Co., Fairport, NY, USA); this closed-system fermentation apparatus has been previously used in digestibility studies in ruminants [18,19] and monogastric animals [20-22]. Initially developed for multiple analysis of feeds, the incubator reduces labor demands, improves precision, and could offer an alternative system to traditional *in vitro* methods for pet food digestibility studies.

There are few studies to date that evaluate the effect of raw or fresh ingredients on pet food nutritional profile, conservation quality, palatability, and digestibility. The objectives of the present study were: (a) to evaluate the effect of mechanically separated chicken meat (MSCM) inclusion in dry dog food on nutritional characteristics, digestibility, and palatability as compared with dry dog food containing only meat by-products (MBP); (b) to determine whether or not there were differences in microbiology and pet food conservation quality; (c) to propose a new system using the Daisy^{II} Incubator to measure *in vitro* the digestibility of pet food.

2. *Materials and Methods*

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2.1. *Diet formulation and extrusion parameters*

Two extruded diets were formulated according to the European Pet Food Industry Federation [23] guidelines for adult dogs. The one was formulated using MSCM as the main protein source, while the other was formulated with poultry MBP as the main protein source. Tables 1 and 2 present the mean chemical composition, inorganic mineral, vitamin, and amino acid content of the two feed materials and of the two diets, respectively.

Table 1. Chemical composition (g/kg, as-fed basis), mineral content (g/kg, as-fed basis), vitamin content and amino acid content (g/100 g, as-fed basis) of the two types of feed materials (mean±SD).

Items	Mechanically Separated Chicken Meat	Meat By-products
DM (g/kg FM)	328.2±8.7	950.6±11.2
Crude protein	118.7±2.4	649.2±10.0
Ether extract	118.3±2.1	122.9±3.5
Nitrogen free extract	3.6±1.6	2.2±0.7
Cellulose	10.3±0.2	10.9±2.5
Ash	77.3±7.1	165.5±8.6
Ca	23.3±3.1	46.3±4.1
P	13.0±1.8	25.4±1.9
Mg	0.63±0.10	1.83±0.42
Zn	0.03±0.01	0.10±0.01
Fe	0.01±0.00	0.12±0.02
Vitamin B_1 (mg/kg)	0.23±0.01	1.11±0.1
Vitamin B_2 (mg/100g)	n.d.[1]	0.56±0.02
Vitamin B_{12} (µg/kg)	n.d.	0.04±0.02
Vitamin A (U.I/kg)	n.d.	n.d.
Vitamin D_3 (U.I/kg)	n.d.	n.d.
Vitamin E (mg/kg)	n.d.	n.d.
Aspartic acid	0.79±0.01	5.07±0.14
Threonine	0.34±0.02	2.40±0.14
Serine	0.33±0.01	2.53±0.09
Glutamic acid	1.24±0.01	8.29±0.20
Proline	0.97±0.04	3.78±0.19
Glycine	1.80±0.04	6.42±0.23
Alanine	1.02±0.01	4.31±0.11
Valine	0.47±0.01	2.88±0.05
Methionine	0.08±0.01	1.24±0.01
Isoleucine	0.33±0.01	2.24±0.05
Leucine	0.58±0.02	4.21±0.12
Tyrosine	0.18±0.01	1.70±0.05
Phenylalanine	0.34±0.01	2.39±0.08
Lysine	0.52±0.02	4.10±0.24
Histidine	0.16±0.01	1.34±0.05
Arginine	0.85±0.01	4.85±0.08
Cysteine	0.04±0.01	0.41±0.04
Tryptophan	n.d.	0.31±0.00

[1] n.d. = not detected.

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Table 2. Chemical composition (g/kg, as-fed basis), mineral content (g/kg, as-fed basis), vitamin content and amino acid content (g/100 g, as-fed basis) of the two diets (MSCM = Mechanically Separated Chicken Meat diet and MBP = Meat By-Product diet; mean±SD).

Items	MSCM[1]	MBP[1]
DM (g/kg FM)	940.5±0.5	930.1±0.3
Crude protein	263.1±0.2	264.7±1.0
Ether extract	176.8±0.3	187.0±2.7
Nitrogen free extract	401.4±0.8	373.8±4.8
Cellulose	26.6±0.9	24.1±0.6
Ash	72.6±0.9	80.6±1.5
Ca	15.6±0.8	18.4±4.7
P	10.7±0.4	12.4±3.0
Mg	1.41±0.07	1.41±0.34
Zn	0.28±0.01	0.27±0.01
Fe	0.29±0.01	0.26±0.03
Vitamin B_1 (mg/kg)	12.1±0.1	10.1±0.2
Vitamin B_2 (mg/100g)	1.47±0.01	1.28±0.02
Vitamin B_{12} (µg/kg)	0.11±0.01	0.09±0.00
Vitamin A (U.I/kg)	30267±896	31000±854
Vitamin D_3 (U.I/kg)	2153±693	2280±324
Vitamin E (mg/kg)	194±17	189±7
Aspartic acid	2.30±0.03	2.29±0.03
Threonine	1.07±0.02	1.08±0.02
Serine	1.22±0.02	1.16±0.02
Glutamic acid	3.62±0.05	3.50±0.07
Proline	1.64±0.09	1.56±0.04
Glycine	2.32±0.04	2.45±0.05
Alanine	1.74±0.02	1.78±0.05
Valine	1.30±0.01	1.36±0.04
Methionine	0.67±0.02	0.54±0.02
Isoleucine	1.04±0.06	1.04±0.06
Leucine	2.11±0.01	1.99±0.03
Tyrosine	0.82±0.03	0.66±0.07
Phenylalanine	1.14±0.02	1.15±0.01
Lysine	1.50±0.06	1.60±0.04
Histidine	0.56±0.02	0.56±0.01
Arginine	1.80±0.03	1.84±0.04
Cysteine	0.26±0.01	0.24±0.02
Tryptophan	0.15±0.01	0.13±0.00

[1]Diet ingredients: chicken meat or meal, rice, potato protein concentrate, animal fat, maize, beet pulp, brewer's yeast, hydrolyzed animal protein, *Spirulina platensis*, *Yucca schidigera*, hydrolyzed cartilage, hydrolyzed crustaceans, methyl sulfonyl methane, *Echinacea* root, oregano, garlic, vitamin and mineral mix.

Table 3 briefly describes the extrusion process. The croquettes are hot-air dried on a moving conveyor belt: the inside temperature is kept between 70 and 160 °C: for processing fresh meat products (minimum drying temperature 85 °C). Depending on the type of product, it takes, on average, 20 to 30 minutes to complete the process. The humidity of the output product is maintained at around 6.5-7.0% for the croquettes for the canine market. Besides guaranteeing gelatinization of starches and their digestibility, the extrusion process increases the kibble size from 50% to 75% [24,25].

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Table 3. Production of MSCM (Mechanically Separated Chicken Meat) diet and MBP (Meat By-Products) diet and relative processing temperature.

Phase	Duration (min)	MSCM Temperature (°C)	MBP Temperature (°C)
Preparation of the main ingredient mixture	-	Room temperature	Room temperature
Pre-grinding storage	-	Room temperature	Room temperature
Grinding	60	40 °	40 °
Grinding mixture storage	180	Room temperature	Room temperature
Pre-conditioning	1	From 25° to 60°	From 25° to 60°
Extrusion	0.5	From 85° to 110°	From 70° to 110°
Drying	30	From 85° to 160°	From 50° to 95°

2.2. *Fatty acid analysis*

To determine fatty acid composition of the diets, lipid extraction and gas chromatography (GC) were performed according to Peiretti and Meineri [26]. All analyses were performed in triplicate.

2.3. *Microbiological analyses and conservation quality*

Microbiological analysis was conducted to determine the microbiological quality of ingredients, intermediate feed, throughout the production stages (where possible contamination may occur), and feed at the end of production and at 6 months of storage under controlled conditions in samples from a box of the same batch. The production stages were suggested by the producer. All microbiological methods were ISO [27] or taken from the literature: ISO 6579:2002 for Salmonella also referred to pet food and ISO 4833-1991/AFNOR V08-054 for the total mesophilic bacterial count. All media were Oxoid (Basingtoke, UK), except for Chromocult® (Merck, Germany). Microbial counts are expressed as a logarithm (log) of colony-forming units (cfu) per gram of sample. Quantitative analysis was carried out in duplicate.

Briefly the methods:

- Total mesophilic bacterial count: plate count agar at 30 °C for 72 h;
- Enterobacteriaceae and *E. coli* counts: violet bile glucose agar and Chromocult® (Merck, Germany), respectively. Plates were incubated at 37 ° C for 24-48 h;
- Salmonella count: 50 g were suspended in buffered peptone water for pre-enrichment at 37 °C for 18 h, Rappaport-Vassiliadis medium with soya (RVS broth) at 41.5 °C for 24 h and Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth) at 37 °C for 24 h for selective enrichment, then plated the third day on xylose lysine dextrose agar (XLD) and brilliant green agar (BGA) incubated at 37 °C for 24 h; phenotipical identification was carried out on Kligler iron agar and genotypical identification was performed according to Rahn *et al*. [28];
- sulfide reductase clostridium: SPS medium at 37 ° for 48 h under strict anaerobic conditions [29].

Biogenic amines were determined according to Paulsen *et al.* [30] on a high performance liquid chromatography visible UV detector (HPLC UV/Vis). Peroxide determination was performed according to the EU official method [31]. Analyses were performed in triplicate.

2.4. *Palatability trial*

To evaluate the palatability of MSCM and MBP, the two-bowl trial method was used. Briefly: MSCM and MBP were placed in their respective bowls and presented simultaneously to a panel that judged food palatability according to three sensory characteristics: aroma, texture, and macronutrient profile [32]. The panel was composed of 40 adult dogs of any breed and size randomly divided by sex and individually caged at feeding time; for the remainder of the day they were housed in kennels in groups of 20. The trial lasted 24 h during which each dog was presented once, for 30 minutes, with

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the two bowls and had to choose from which to eat first. The bowls contained 500 g of MSCM or MBP. The quantities of each product consumed (intake) and left (outtake) were recorded for each dog. At the end of the trial, the quantities of MSCM and MBP consumed by the whole panel were summed to determine which of the two diets was consumed more. Data are expressed as the total amount of food consumed for each diet, intake ratio (IR), preference, and food selected as a first choice. Water was supplied *ad libitum*.

2.5. *In vivo digestibility trial*

The digestibility protocol followed the guidelines published in the official method of the Association of American Feed Control Officials [6]. The *in vivo* digestibility (Vd) was tested in 10 adult dogs (male and female) weighing between 8 and 16 kg. The animals were individually housed during the trial. In order to test the digestibility of MSCM and MBP, the same dogs were employed for both trials but with different schedules according to the official guidelines [6]. Each Vd trial consisted of 3 days of adjustment to the new diet, followed by a 4-day collection period during which the weight of food offered and refused and feces were recorded daily. A total of 500 g/day was offered as fed, while water was supplied *ad libitum*. The food was presented in the morning for 30 min. The total individual daily feces were weighed and then kept at -18 °C pending analysis. The feces samples of each dog were dried; the 4-day cumulative samples were pooled from the daily samples. Apparent digestibility is expressed as g/kg. The total tract apparent digestibility coefficients of DM was calculated for each diet according to Villaverde *et al.* [33] using the formula:

$$\text{Vd (g/kg)} = [(\text{X intake g} - \text{X outtake g})/ \text{X intake g}] \times 1000 \quad (1)$$

Housing and treatment protocols (Registration Number 612.623.82) adhered to European norms for animal welfare [34] at the Kennel "De Morgenstond" (Dussen, The Netherlands). The facility was maintained according to Dutch regulations [35].

2.6. *In vitro digestibility trials*

In vitro digestibility was estimated using a Daisy^{II} Incubator. The incubator has four rotating digestion jars that are agitated at constant and uniform temperature inside a temperature-controlled chamber. Each jar can hold up to 23 filter bags with samples, one blank without sample, and an enzymatic solution.

The extruded diets were weighed (0.5 ± 0.01 g) in triplicate into Ankom F57 filter bags; heat sealed, put in the jar with the enzymes and buffer solution, and digested. To simulate digestion, two digestibility techniques differing in phosphate buffer, type and amount of enzymes, and digestion period were used. The one solution (HV-IVD), as proposed by Hervera *et al.* [36], and the second (BG-IVD) as proposed by Biagi *et al.* [37] were prepared (Table 4). The enzymes were: pepsin (P7125, Sigma Aldrich), pancreatin (P1500, Sigma Aldrich), and bile salt. Phosphate buffer prepared according to Hervera *et al.* [36] consisted of the acid (KH_2PO_4) and its conjugate base (K_2HPO_4) [38]. According to Biagi *et al.* [37], the phosphate buffer solution was prepared as described in Martillotti *et al.* [39]. Throughout digestion, the jars were agitated at a constant temperature of 39 °C. At the end of incubation, the bags were washed, *in vitro* dry matter disappearance was measured, and *in vitro* digestibility (IVD) was calculated as follows:

$$\text{IVD (g/kg)} = [(DM_{\text{ante incubation}} - DM_{\text{post incubation}})/ DM_{\text{ante incubation}}] \times 1000 \quad (2)$$

Table 4. *In vitro* digestibility using the Daisy^{II} Incubator and according to the method described in Hervera *et al.* [36] (HV-IVD) and in Biagi *et al.* [37] (BG-IVD).

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Step	HV-IVD	BG-IVD
	0.5 ± 0.01 g of sample[1]	0.5 ± 0.01 g of sample
1 Gastric digestion	1200 ml phosphate buffer (0.1M, pH 6) 480 ml HCl (0.2M) 480 mg pepsin 24 ml cloramphenicol solution (0.5g/100 ml ethanol) pH 2, 39 °C, 2 h	1440 ml of pepsin-lipase-HCl solution (HCl 0.075N; pepsin 2g/L; gastric lipase 1g/L) 39 °C, 2 h
2 Post-gastric digestion	480 ml phosphate buffer (0.2 M, pH 6.8) 240 ml NaOH (0.6 M) 4.8 g pancreatin pH 6.8, 39 °C, 4 h	1440 ml -pancreatin-bile salt-phosphate buffer solution (10g/L pancreatin 25 g/L; bile salt) pH 7.5, 39 °C, 4 h
3 Collection of undigested fraction	F57 washed, twiced with ethanol (96%) and twice with acetone (99%), dried overnight at 70 °C, analysed for CP, EE, DM, OM	F57 washed, dried overnight at 65 °C, analysed for CP, EE, DM, OM

[1] Quantities for each jar and twenty-four F57 filter bags (twenty-three replicate samples and one blank).

Table 5. Fatty acid profile (mean±SD; g/100 g of total fatty acid) of the two diets (MSCM = Mechanically Separated Chicken Meat; MBP = Meat By-Products) and the significance between them.

Compound	MSCM	MBP	*P*
Capric acid (C10:0)	n.d.[1]	0.02±0.03	-
Lauric acid (C12:0)	0.12±0.01	0.42±0.01	0.000
Myristic acid (C14:0)	1.16±0.02	1.21±0.01	0.025
Myristoleic acid (C14:1)	0.22±0.03	0.23±0.01	0.692
Pentadecanoic acid (C15:0)	0.16±0.01	0.15±0.00	0.016
Palmitic acid (C16:0)	20.69±0.03	20.78±0.03	0.020
Palmitoleic acid (C16:1n7)	4.85±0.03	4.80±0.03	0.123
Margaric acid (C17:0)	0.27±0.01	0.24±0.00	0.001
Heptadecanoic acid (C17:1)	0.24±0.01	0.22±0.01	0.013
Stearic acid (C18:0)	6.24±0.10	6.12±0.04	0.108
Oleic acid (C18:1n9)	40.64±0.15	40.58±0.11	0.592
Elaidic acid (C18:1n9, trans)	0.54±0.02	0.41±0.01	0.000
Linoleic acid (C18:2, cis-cis)	22.60±0.14	22.23±0.02	0.012
Linoelaidic acid (C18:2, trans-trans)	0.13±0.03	0.11±0.10	0.706
α-Linolenic acid (C18:3n3)	1.96±0.02	1.33±1.15	0.403
Arachidic acid (C20:0)	0.15±0.01	0.15±0.01	1.000
Behenic acid (C22:0)	n.d.	0.02±0.03	-
Eicosenoic acid (C20:1)	0.49±0.04	0.61±0.10	0.374
Lignoceric acid C24:0)	0.13±0.02	0.14±0.01	0.275
SFA[2]	28.91±0.09	29.23±0.02	0.004
MUFA[3]	46.45±0.10	46.44±0.02	0.878
PUFA[4]	24.55±0.16	24.25±0.05	0.036

[1] n.d. = not detected. [2]SFA = saturated fatty acid. [3]MUFA = monounsaturated fatty acid. [4]PUFA= polyunsaturated fatty acid.

2.7. Statistical analysis

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Fatty acid content and data from the digestibility trials were analyzed using SPSS software (version 11.5.1 for Windows, SPSS Inc., USA) by the general linear model using one-way ANOVA with diet as the main factor; conservation quality parameters were analyzed by ANOVA for multifactorial analysis of variance for the two main factors (diet and conservation time) to identify differences. Time effect (month 0 and 6), diet effect (MSCM vs. MBP) and the time×diet interaction were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Fatty acid profile

Table 5 presents the fatty acid composition of the two diets. MSCM was richer than MBP in polyunsaturated fatty acids (PUFAs), whereas MBP was higher in saturated fatty acids (SFAs) than MSCM, because MSCM had higher values for linoleic acid and lower values for some SFAs (capric, lauric, myristic, and palmitic acid). MSCM also had higher values for other SFAs (pentadecanoic and margaric acid) and MUFAs (heptadecanoic and elaidic acid).

3.2. Microbiological profile and conservation quality

Microbiology results are shown in Table 6 (ingredients) and Table 7 (after extrusion, after drying, and final feed formula at time 0 and after 6 months of storage under controlled conditions). All ingredients had a variable and high microbiological count for mesophilic bacteria and Enterobacteriaceae (range from 1.48 log CFU/g to 5.73 log CFU/g). *Clostridium* spores and *E. coli* were not detected in any samples.

Salmonella spp. was detected in meat by-product before extrusion, but never after extrusion. Biomolecular analysis confirmed the identification after culture of *Salmonella enterica*. After 6 months of storage under controlled conditions, the microbiological profile was confirmed: the total mesophilic bacterial count ranged between 1.77 log CFU/g and 2.09 log CFU/g feed. Enterobacteriaceae, *Clostidium*, and *E. coli* were under the detection level and Salmonella was never detected.

Table 6. Mean counts (mean±SD, log CFU/g) of the main microbial groups detected in the ingredients.

Ingredient	Total Mesophilic Bacterial Count	Enterobacteriaceae	*E. coli*	*Clostridium* Sulfite Reductase	Salmonella[1]
Chicken meat	5.28±2.45	3.20±1.84	1.95±0.62	n.d.[2]	n.d.
Meat by-products	5.23±2.32	2.97±1.75	n.d.	n.d.	Positive
Rice	5.73±2.49	3.11±1.84	n.d.	n.d.	n.d.
Potatoes	5.43±0.01	n.d.	n.d.	n.d.	n.d.
Chicken Fat	1.48±0.84	n.d.	n.d.	n.d.	n.d.
Maize	4.59±1.83	2.58±1.32	n.d.	n.d.	n.d.
Beet Pulp	4.20±1.70	2.51±1.19	n.d.	n.d.	n.d.
HAP3	3.15±2.05	3.70±1.35	n.d.	n.d.	n.d.
Spiruline	3.66±1.62	n.d.	n.d.	n.d.	n.d.

[1] Determined on 50 g. [2] n.d. = not detected. [3] HAP = hydrolyzed animal protein.

Table 7. Mean counts (mean±SD log CFU/g) of the main microbial groups) in the two diets (MSCM = Mechanically Separated Chicken Meat diet and MBP = Meat By-Products diet) at different phases.

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Diet	Phase[1]	Total Mesophilic Bacterial Count	Enterobacteriaceae	*E. coli*	*Clostridium* Sulfite Reductase	Salmonella[2]
MSCM	1	2.08±1.19	n.d.[3]	n.d.	n.d.	n.d.
MSCM	2	2.15±1.05	n.d.	n.d.	n.d.	n.d.
MSCM	3	2.08±0.75	n.d.	n.d.	n.d.	n.d.
MSCM	4	2.10±1.19	n.d.	n.d.	n.d.	n.d.
MBP	1	1.85±0.62	n.d.	n.d.	n.d.	n.d.
MBP	2	1.48±0.90	n.d.	n.d.	n.d.	n.d.
MBP	3	1.70±0.92	n.d.	n.d.	n.d.	n.d.
MBP	4	1.78±0.92	n.d.	n.d.	n.d.	n.d.

[1] Phase 1: after extrusion, Phase 2: after drying, Phase 3: at start of conservation period, Phase 4: after 6 months of conservation. [2] Determined on 50 g. [3] n.d. = not detected.

The microbiological risk assessment showed no microbiological hazards for the use of MSCM or MBP.

Table 8. Natural polyamines (mean±SD, mg/kg, as-fed basis) of the two types of meat (fresh and meal).

Compound	Mechanically Separated Chicken Meat	Meat By-Products
Putrescine	1.5±0.1	78.6±96.7
Cadaverine	n.d.[1]	164±214
Tryptamine	n.d.	n.d.
Phenylethylamine	n.d.	6.3±0.2
Spermidine	17.2±0.5	35.0±35.7
Spermine	32.7±7.5	60.1±75.5
Histamine	n.d.	14.1±24.5
Tyramine	n.d.	61.2±69.7

[1] n.d. = not detected.

Table 9. Polyamine (mean±SD mg/kg, as-fed basis) and peroxide content (meq/kg, as-fed basis) of the two diets (MSCM = Mechanically Separated Chicken Meat diet and MBP = Meat By-Products diet) at the start and the end of the conservation period.

Diet	MSCM		MBP		Diet	Time	Diet x Time
Time (month)	0	6	0	6	*P*	*P*	*P*
Putrescine	56.9±2.3	94.5±14.2	58.3±3.7	74.2±11.2	0.137	0.001	0.092
Cadaverine	88.4±3.9	112.9±17.1	89.8±6.3	87.4±13.3	0.187	0.306	0.139
Tryptamine	n.d.[1]	15.7±2.5	n.d.	n.d.	-	-	-
Phenylethylamine	4.3±0.5	7.1±1.3	3.9±1.0	4.2±1.0	0.032	0.047	0.088
Spermidine	18.1±2.5	19.6±3.0	25.4±2.8	18.6±2.9	0.055	0.171	0.055
Spermine	7.3±2.1	3.5±0.6	13.8±5.4	2.1±0.5	0.101	0.013	0.034
Histamine	10.3±0.6	28.5±4.4	7.7±0.3	16.7±2.6	0.002	0.001	0.126
Tyramine	52.9±2.6	93.7±14.3	36.5±3.6	67.3±10.3	0.003	0.001	0.310
Peroxide value	1.9±0.2	6.0±0.8	2.5±0.4	6.0±0.8	0.213	0.001	0.709

[1] n.d. = not detected.

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Table 8 reports the polyamine content of the two types of meat, with lower content of putrescine, spermidine, and spermine in MSCM than MBP, that had cadaverine, histamine, and tyramine. Table 9 shows that among the polyamines evaluated, only phenylethylamine, histamine and tyramine contents were greater in MSCM than MBP; putrescine, phenylethylamine, histamine, tyramine, and peroxide value were significantly increased, while spermine was decreased after 6 months of storage in both diets

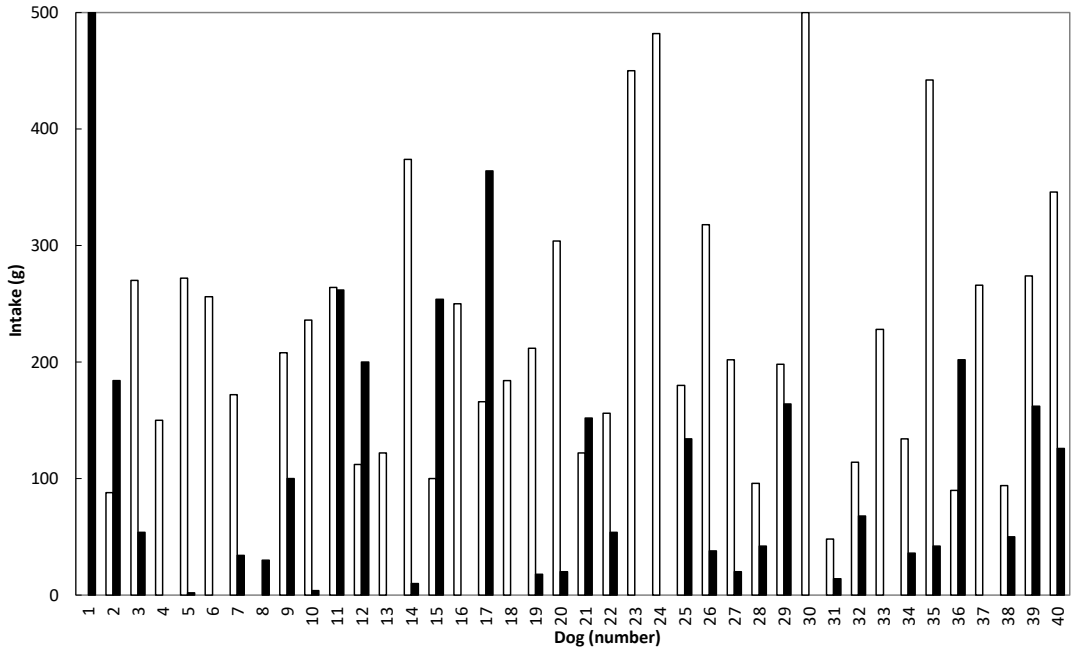

Figure 1. Summarized food intake of the two diets [MSCM = Mechanically Separated Chicken Meat (black columns) and MBP = Meat By-Products (white columns)].

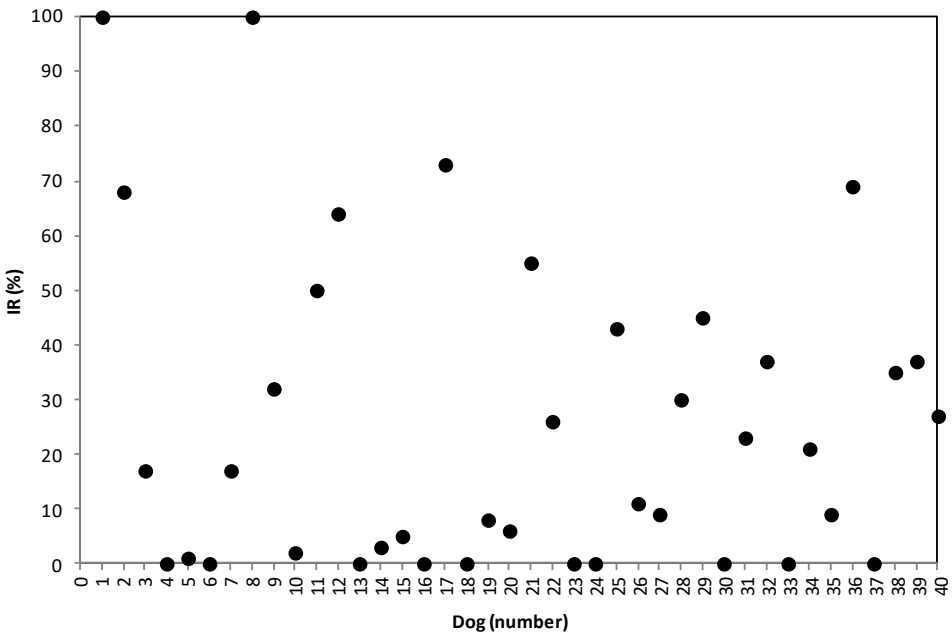

Figure 2. Intake Ratio [IR% = MSCM/(MSCM + MBP) x 100] of the MSCM diet (based on Mechanically Separated Chicken Meat) as compared with the MBP diet (based on Meat By-Products).

3.4. Palatability

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The palatability trial showed higher intake of MBP than MSCM (Figure 1). The MSCM diet alone was consumed by a single subject and was preferred by only 4 out of the 40 dogs. MBP was appreciated by all the remaining dogs and in 6 subjects exclusively. Figure 2 shows the Intake Ratio of the MSCM diet corrected for total intake per subject. This was done to eliminate the difference related to breed and food intake.

3.3. Digestibility

Table 10 presents the results of the *in vivo* and *in vitro* trials of diet digestibility. The *in vivo* method revealed no significant differences in digestibility between the two feeds. As measured by the *in vitro* method, HV-IVD revealed a significant difference between diets, whereas BG-IVD did not. It can be observed, however, that, as compared with the *in vivo* digestibility value, the *in vitro* method slightly overestimated the digestibility coefficients for both diets (Table 10).

Table 10. *In vitro* dry matter digestibility (g/kg) according to the Hervera *et al.* [36] method (HV-IVD) and according to the Biagi *et al.* [37] method (BG-IVD) and *in vivo* dry matter digestibility (*in vivo*) of the two diets (MSCM = Mechanically Separated Chicken Meat diet and MBP = Meat By-Products diet) and the significance between them.

Digestibility method	MSCM	MBP	*P*
In vivo	838.0±44.2	825.4±27.6	0.453
HV-IVD	888.8±0.5	877.9±0.1	0.021
BG-IVD	912.5±0.3	917.5±0.8	0.360

4. Discussion

4.1. Fatty acid profile

The PUFA n-6/PUFA n-3 ratio was 11.5 and 16.7 for the MSCM and the MBP diet, respectively. These ratios are within the range reported by Kearns *et al.* [40] who studied the effect of diets formulated to contain PUFA n-6/PUFA n-3 ratios of 5 or 25 on oxidative status and immune response of young and old dogs. They found that a diet with a PUFA n-6/PUFA n-3 ratio of 5 had a positive effect on the immune response of young or geriatric dogs. Hall *et al.* [41] compared three diets with a PUFA n-6/PUFA n-3 ratio of 1.4, 18, and 40, respectively, fed to geriatric dogs and concluded that the dose of PUFA n-3 administered determined the plasma PUFA n-3 composition, independent of the PUFA n-6/PUFA n-3 ratio.

4.2. Microbiological profile and conservation quality

The microbiology of pet food has been associated with certain zoonoses [42,43]. Our results clearly show high microbiological counts for all the ingredients. If we compare our data with the few published data, this is not surprising given the origin of the food [44,45]. Due to the combination of pressure and temperature, the extrusion process reduced the bacterial count by between 3 and 4 log in both formulations. This result is in agreement with previous studies [29,46].

The total mesophilic bacterial count was lower than previously reported [29,44]. While this parameter is relatively high for a final product with a long shelf-life, in our opinion and as stated by other authors the very low water activity (Aw of 0.40) can easily counteract bacterial growth [29]. The same behavior was noted for Enterobacteriaceae: their counts decreased from 3.70 log CFU/g to below the detection limit (1 log CFU/g) in both formulations. These results evidence a better situation for this batch than reported by other authors [29,44]. Also, as seen for other dry pet food formulations, the *E. coli* count was consistently under the detection limit [29].

Salmonella, a highly common zoonotic pathogen responsible for causing infection in pets and owners [42,43,46], was detected only in untreated raw ingredients, in agreement with Van Bree *et al.* [45]. Sampling at three different time points in the production chain, where contamination could be expected, gave negative results. Our results show that, for this batch, extrusion treatment was safe, as reported elsewhere in the literature [29, 46].

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Furthermore, after 6 months storage there was a slight increase in microbiological parameters in both formulas, as observed by other authors [29]. Our results show that MSCM appears more susceptible to degradation than MBP. Natural polyamines are organic compounds that originate from amino acids as a result of decarboxylation. This process can result from bacterial activity and can occur during food processing or storage. The most common monoamines, (histamine, tyramine, and tryptamine) and polyamines (putrescine and cadaverine) are generated, respectively, from the amino acids histidine, tyrosine, tryptophan, ornithine, and lysine; whereas, spermidine and spermine derive from putrescine. Polyamines are found in many protein foods and their amount is an important indicator of the degree of freshness and storage of products [47].

Studies on changes in polyamine content in meat products are inconsistent, however. Changes in polyamine content during meat storage result from bacterial activity. Ruiz-Capillas and Jiménez-Colmenero [48] reported that the polyamine level in minced meat products increased due to muscle fiber disintegration and increased microbial contamination. Putrescine levels in fresh meat are usually low, often near the limit of detection of the analytical procedures used [49]. The only amines present at significant levels in fresh meat are spermidine and spermine [50]. This is in agreement with the results obtained in our study (Table 8).

Regarding the storage quality of the product, after 6 months of storage, the polyamine levels were higher for MSCM than MBP (Table 9). Polyamine synthesis requires the availability of amino acid precursors that may be present in the food product. As shown in Table 4, MSCM contained more tyrosine and tryptophan than MBP, which are two of the amino acid precursors of tyramine and tryptamine, respectively. Some amines (e.g., tyramine, putrescine, and cadaverine) can form during the meat preservation [51]. Paulsen *et al.* [30] analyzed 55 samples of canned pet food and found that 75% of the levels of the biogenic amines varied between 5.4 and 21.9 mg/kg, depending on the type of amine, while it ranged from below the detection limit to 133 mg/kg for cadaverine, histamine, phenylethylamine, putrescine, and tyramine. They concluded that amine concentrations in non fish components are lower than in fish components.

4.3. Palatability

The difference in intake between the MBP and the MSCM diet could have been due to several different factors. First, the composition of the product: the MBP diet was fatter (200 g/kg DM) than the MSCM (188 g/kg DM). In fact, dogs prefer fat-rich diets [52] to high-protein or carbohydrate diets. Another factor is the extrusion process: during MSCM production the minimum temperature was higher than that used for MBP (85 °C vs. 50 °C), which reduced the humidity (6% MSCM vs. 7% MBP), and, because palatability enhancer is added immediately before drying phase, possibly also reduced the aromas in the dry pet food. The literature reports data similar to ours, except that reduced palatability may be attribute to polyamine content [53]. Our data indicated no correlation between polyamine levels and palatability, however.

3.3. Digestibility

The *in vitro* digestibility was very high for both diets, with values higher than reported by Brambillasca *et al.* [54] for dry food (average 76.9%). This could be explained by the positive effect of extrusion that allows gelatinization of starches and increases digestibility in comparison with the pelleting process [55]. However, Biagi *et al.* [37] reported an average of 70.4% for the digestibility of extruded diets for dogs, which contrasts with the average reported in the literature (82.2%) and reported in their article.

Moreover, our data show different results obtained from the two methods and use of the Daisy^{II} Incubator. According to HV-IVD, digestibility was higher for the MSCM diet than the MBP diet, (888.8±0.5 vs. 877.9±0.1 g/kg). Since the chemical composition of the MSCM diet had less ether extract - though cellulose content was higher (26.6 vs. 24.1 g/kg), it contained higher quantities of starch (274.7 vs. 248.3 g/kg). Increasing the fiber level in dog diet decreases its digestibility [54], which is negatively related (r=-0.86) to apparent digestibility of extruded food [56]. BG-IVD did not reveal significant differences between MSCM and MBP (912.5±0.3 g/kg vs. 917.5±0.8 g/kg). *In vitro* values

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were always higher than *in vivo* digestibility and less variable, particularly with the BG-IVD method. The standard deviation ranged between 0.1 and 0.8 g/kg *in vitro* and 27.6 and 44.2 g/kg *in vivo*. Both *in vitro* methods used in this study were carried out in three steps, but used different types and amounts of enzyme for different incubation times: BG-IVD employs higher quantities of enzymes and a longer incubation time than HV-IVD. We assume that the longer duration of digestion in BG-IVD contributed to overestimation of the results. Regmi *et al.* [57] found in pigs that *in vitro* digestibility was greater with longer digestion time, while the amount of enzymes was irrelevant for the digestibility.

Previous studies have shown that the ANKOM method produces digestibility values comparable to traditional procedures for many foods [58-62]. The *in vitro* method proposed by Hervera *et al.* [36] and utilized in our study yielded values closer to *in vivo* results, in line with Hervera *et al.* [63,64] who showed the highest accuracy approach of *in vivo* crude protein apparent digestibility (r=0.81) and *in vivo* digestible energy (r^2=0.94), respectively.

5. Conclusions

Their similar chemical compositions notwithstanding, the MSCM diet had lower palatability but better nutritional quality (with higher content of PUFAs and lower content of SFAs) than the MBP diet. Microbiological risk assessment revealed no microbiological hazards for the use of either MSCM or MBP. Evaluation of storage quality at 6 months showed higher polyamine levels for MSCM than MBP. The Daisy^{II} Incubator was found to be a valid instrument for studying *in vitro* digestibility also for dogs, providing data simply, quickly, with less variability and costs than *in vivo* trials. It could represent the future for digestibility studies in pet food. Our results indicate that MSCM or MBP as the main protein ingredient in extruded pet food may be advantageously used in pet food products.

Author Contributions: G.M. was responsible for planning the study, conducting chemical analysis, reviewing the manuscript. P.G.P. was responsible for planning the study, writing and editing the manuscript. S.T. was responsible for conducting the *in vitro* digestibility trials and reviewing the manuscript. A.C. was responsible for writing and editing the manuscript. E.L. was responsible for manufacturing the diets and conducting the *in vitro* digestibility trials. D.P. was responsible for conducting the microbiological analysis. N.R. was responsible for organizing the palatability trials, conducting the *in vivo* digestibility trials, and reviewing the manuscript. L.P. was responsible for planning the study, manufacturing the diets and conducting the *in vivo* digestibility trial.

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