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# BIOGENIC AMINE ANALYSIS IN FRESH MEATS AND MEAT MEALS USED AS RAW MATERIALS FOR DRY PET FOOD PRODUCTION

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***Abstract***

*Fresh meats and meat meals are important components for the production of dry pet food. Both of these raw materials are by-products of meat processing generated during the production of food for human consumption. Being by-products, they can be more subject to contamination and proliferation of microorganisms which degrade the organic component and lead to the development of degradation products such as biogenic amines. Biogenic amines are nitrogen compounds produced by microbial decarboxylation of amino acids, thus being very present in foods rich in certain amino acids. The ingestion of foods containing a large amount of biogenic amines can cause intoxication and harmful consequences for the body. The increase in the presence of biogenic amines in food can be attributed to direct contamination by microorganisms or to inappropriate storage conditions of the food. In fact, to prevent the formation of biogenic amines, it is needed to respect the proper times and methods for the conservation of the raw materials used.*
*This study analyses the possible presence of biogenic amines in the raw materials used for dry pet food production through the use of mass spectrometry, capable of identifying molecules present in small quantities.*
*The results show how meat meals have higher concentration of biogenic amines compared to fresh meats, suggesting that the proliferation of microorganisms and the consequent formation of these nitrogen compounds in meat meals is probably due to the low quality of the raw materials used and to their inadequate storage conditions.*

***Key words***: *biogenic amines, pet food, fresh meats & meat meals, toxicological aspects, raw material quality.*

## INTRODUCTION

Amine compounds are molecules naturally present in living organisms and therefore in food as a consequence of metabolic processes. This kind of amines are chemicals that occur naturally, generated by bacteria degrading the amino acid component. They are classified in three categories, according to their chemical structure: Aromatic amines (Histamine, 2-Phenethylamine, Tryptamine and Tyramine), Aliphatic amines (Cadaverine and Putrescine) and Aliphatic polyamines (Agmatine, Spermidine and Spermine) (Figure 1).
These compounds can be labelled as "biogenic amines" (Bardózc, 1995; Smith, 1980), and are formed by the decarboxylation of amino acids or by the amination or transamination of aldehydes and ketones by specific microbial enzymatic pathways (Learey et al., 2018; Shalaby, 1996; Suzzi & Torriani, 2015).
The formation of biogenic amines is strictly dependent on the content of proteins and free amino acids in the food. In the case of pet food, whose ingredients are mainly represented by meat processing by-products and therefore particularly rich in protein, they are often characterized by the presence of high concentrations of biogenic amines (den Brinker et al., 2003; Learey et al., 2018).
In fact, if on the one hand, the presence of biogenic amines depends on the type of microorganisms present in food and their growth, on the other hand, it is also strictly dependent on factors associated with raw materials such as meat composition, pH and

handling conditions; all these factors influence the availability of free amino acids subject to the microbial decarboxylation reaction (Ruiz-Capillas & Jimenez-Colmenero, 2005).

Biogenic amines can then represent a valid indicator for evaluating the freshness of pet food and the possible microbial contamination it can undergo (Learey et al., 2018). The raw materials used for the production of dry pet food are mostly composed of animal by-products that can be subject to the formation of biogenic amines due to transport and handling processes, during which a series of reactions mediated by proteolytic enzymes of microbial derivation can lead to the formation of free amino acids possibly undergoing decarboxylation reactions. This process continues until the ingredients are subject to high temperature stages, such as extrusion, which deactivate the proteolytic enzymes. Heat treatments cease the action of enzymes but do not destroy biogenic amines, which are instead stable to heat. Hence, the concentration of formed amines will not be reduced during processing and may even increase during storage phases, due to further microbial contaminations (Radosevich, 2006; ten Brink et al., 1990). The majority of dry pet food nowadays on the market is produced starting from two different kinds of raw materials: meat meals and fresh meats (Thompson, 2008). Fresh meats are obtained as waste of the meat intended for human consumption, while meat meals derive from meat by-product processing according to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21/10/2009. These meals are mainly used by pet food manufacturers to supply protein sources in order to prepare pet kibbles; however, the intensive industrial process they undergo may cause the onset of raw material degradation which could foster microbial processes leading to the formation of biogenic amines (Camire et al., 1990; Lankhorst et al., 2007; Piergiovanni & Limbo, 2010; Rokey, 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006).

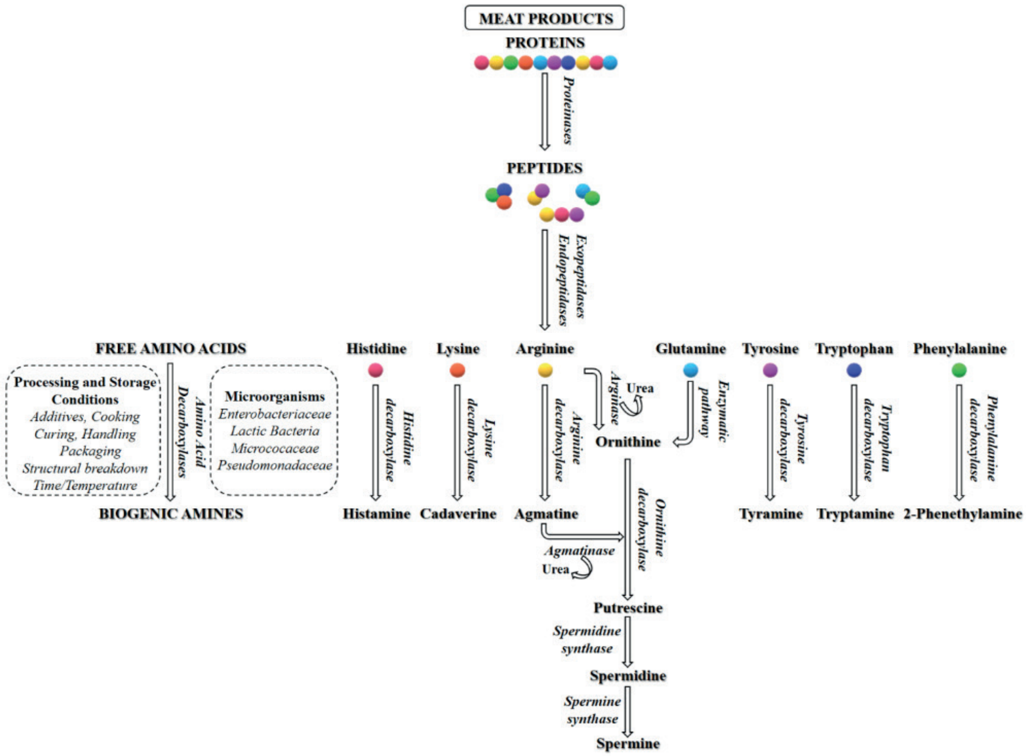


Figure 1. Representation of the factors influencing the formation of biogenic amines

A possible way to limit the formation of biogenic amines in pet food could therefore be represented by a series of measures aimed at reducing microbial contamination and the subsequent degradation of the protein component during the storage and handling of raw materials used for pet food production. It has been shown that in sterile meat there are no biogenic amines, while their concentration increases proportionally in parallel with the development of microbial flora (Bardózc, 1995; Slemr & Beyermann, 1985). Although some biogenic amines, such as Putrescine and Cadaverine, are naturally present at low concentrations in meat products following some reactions of cellular metabolism, the pool of biogenic amines in the final product is very often far greater than the quantities naturally present. It is therefore necessary to control all those factors and processes that can promote the formation of free amino acids and microbial growth, in order to limit the presence of these amines in the final product. It is thus clear how the storage and handling of raw materials can greatly influence the concentration of biogenic amines. For instance, as reported above, it has been seen that heating processes of raw material can significantly reduce the concentration of biogenic amines, by determining the inactivation of the microbial decarboxylases; however, the presence of these substances in the final product is directly dependent on the quality of the different raw materials used which may have already developed a large amount of these amines. In fact, high concentration of biogenic amines in processed meats or meat by-products indicates low quality of the starting raw materials (Bover-Cid et al., 2001; Paulsen et al., 1997; Ruiz-Capillas & Jimenez-Colmenero, 2005). Among biogenic amines, utmost importance is placed on Histamine, responsible for allergic reaction (Kovacova-Hanuskova et al., 2015; Maintz & Novak, 2007; Taylor & Eitenmiller, 1986; White, 1990), Cadaverine, Tyramine, Tryptamine, 2-Phenethylamine, Putrescine, Spermidine and Spermine, which all can have toxic effects on the body in different ways (del Rio et al., 2019; Learey et al., 2018; Lewis, 1998; Til et al., 1997). To date, no guidelines have been drawn regarding the threshold levels for biogenic amines present in pet food, although numerous studies have been carried out showing that high concentrations of some biogenic amines, *e.g.* histamine, induce adverse effects in animals (Bjeldanes et al., 1978; Blonz & Olcott, 1978; Privitera et al., 1969). Adult animals are usually able to detoxify biogenic amines at low concentrations, while puppies may develop harmful effects following the daily intake of even small quantities of these substances (Radosevich, 2006).

The aim of this work is to evaluate the presence of biogenic amines in the raw materials used for dry pet food production. An analysis of the different samples by LC/MS–QTOF (Liquid Chromatography/Mass Spectrometry - Quadrupole Time Of Flight) was carried out in order to quantify the possible presence of biogenic amines.

## MATERIALS AND METHODS

### *Raw Materials*

The raw materials used in this study are listed in Table 1 and they consist of: chicken fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), chicken meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); pork fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), pork meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); salmon fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), salmon meat meal for companion animal food, 10 batches from pet food manufacturers (Italy).

Table 1. List of raw materials used in this study

| Raw Materials | | |
|---|---|---|
| Chicken | Fresh meat for companion animal food | 10 batches from pet food manufacturers |
| | Meat meal for companion animal food | 10 batches from pet food manufacturers |
| Pork | Fresh meat for companion animal food | 10 batches from pet food manufacturers |
| | Meat meal for companion animal food | 10 batches from pet food manufacturers |
| Salmon | Fresh meat for companion animal food | 10 batches from pet food manufacturers |
| | Meat meal for companion animal food | 10 batches from pet food manufacturers |

### *Determination of Moisture content*

Food moisture was calculated according to the method described by da Silva et al. (2018). Briefly, an exact amount of raw material (40 g) was dried in oven (Termaks TS 8136) at 90°C for 6 hours, then it was cooled down at room temperature in a desiccator containing silica gel. Samples were then weighed using OHAUS™ Analytical Balance (Pioneer™) until a stable weight was reached. Water content was calculated as the difference between initial and final weight.

***Sample preparation***

A quantity corresponding to 100 mg of each dry sample was carefully weighed in an Eppendorf tube and 1 mL of Methanol containing 2.5 μg/mL of Phenylglycine as Internal Standard was added. Tubes were shaken 20 minutes at 1500 rpm at room temperature in a Thermomixer (T-Shaker Thermomixers, EuroClone). The tubes were then centrifuged at 3300 × g for 10 minutes (Eppendorf™ 5415D Centrifuge) and the supernatant transferred into a vial. An amount corresponding to 0.5 μL of each sample was injected into the LC/MS-QTOF system (Agilent™ 1290/Agilent™ 6530).

***Determination of Biogenic Amines***

The Ion Pairing Chromatography (IPC) method was used to achieve a wide separation of polar metabolite classes with 150 × 2.1 mm, 3 μm ACME Amide C18 column (Phase Analytical Technology, LLC) thermostated at 50°C. The separation of biogenic amines was achieved using a flow of 0.35 mL/min of a binary gradient of 0.3% heptafluorobutyric acid in water (solvent A) and 0.1% formic acid in Methanol (solvent B). Initial condition was 2% of B for 2 minutes followed by a gradient from 2 to 80% of B in 5 minutes, and a final isocratic step of 8 minutes.

The spectrometer operated in high resolution full scan mode monitoring positive ions. The quantitative data were obtained by external calibration in the range 0.05-2.5 μg/mL of a homemade mix of each biogenic amine in pure Methanol.

***Statistical analysis***

Data shown in this study, regarding the analysis of the content of biogenic amines of the raw materials used for dry pet food production, are reported as mean values of the ten analysed batches (Table 1) ± standard error of the mean (SEM). The t-Student test was used to investigate the significance of the different biogenic amine content in meat meals and fresh meats. The level of significance for the data was set at $p < 0.05$. All statistical tests were done using GraphPad Prism 6.00 for Windows (GraphPad Software, Inc., San Diego, CA).

## RESULTS AND DISCUSSIONS

The presence of biogenic amines in the raw materials was evaluated through LC/MS-QTOF. This analysis was performed following the method explained in the Materials and Methods section.

Prior to the assessment of biogenic amines by LC/MS-QTOF, the moisture level was evaluated for each raw material. The results shown in Figure 2 reveal how, as expected, fresh meats exhibit higher water content compared to meat meals.

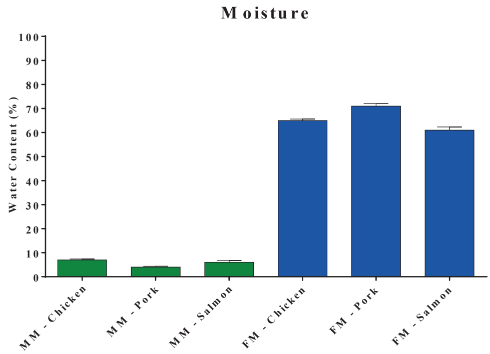


Figure 2. Water content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by stable weight reaching after oven-drying at 90°C for 6 hours, data are reported as mean ± SEM, n = 10

The humidity level in fresh meats ranges from about 60% in the case of salmon to 70% in the case of pork, whereas a water content lower than 10% is peculiar to all meat meals. This feature is the result of the high temperature treatment and dry processes used for the preparation of meat meals.

Subsequently, Mass Spectrometry analysis was performed on the same amount of dry sample for each category of raw material in order to evaluate the content of biogenic amines. The previously obtained data regarding moisture allowed to calculate the quantity of wet sample that had to be taken in order to reach the same dry quantity for each class of raw material. The content of biogenic amines was expressed in mg/kg of dry sample.

The first biogenic amines analysed was Histamine. This amine derives from the decarboxylation of the amino acid Histidine, and its intake is involved with the development of allergic phenomena (Ruiz-Capillas & Jimenez-Colmenero, 2005). The results shown in Figure 3 display how fresh meats has a significantly lower quantity of histamine compared with meat meals, and this happened for all the raw materials analysed.

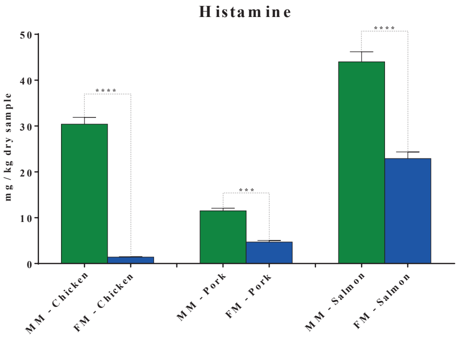


Figure 3. Histamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Histamine per kg of dry sample). Data are reported as mean ± SEM, n = 10; ***$p < 0.001$, ****$p < 0.0001$

The amount of Histamine in the chicken fresh meats, for all the batches analysed, is about twenty times less than in meat meals; as for pork and salmon fresh meats, the quantity of Histidine is about halved with respect to the corresponding meat meals, with the highest concentrations being recorded for the salmon. These results highlight how pet food products made with meat meals may contain more Histamine and therefore be more harmful to pets, which may thus undergo toxic and allergic reactions (Bjeldanes et al., 1978; Blonz & Olcott, 1978; del Rio et al., 2017; Kovacova-Hanuskova et al., 2015; Lewis, 1998; Linares et al., 2016; Maintz & Novak, 2007; Privitera et al., 1969; Taylor & Eitenmiller, 1986; White, 1990). Subsequently the concentration of Cadaverine, a biogenic amine deriving from the decarboxylation of the amino acid Lysine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was evaluated. The results show that the concentration of Cadaverine in chicken fresh meats is more than three times smaller than in meat meals; while in pork fresh meats it is less than half as compared to its content in meat meals. In salmon fresh meat was found a concentration of Cadaverine four times lower than in the corresponding meat meal, which also showed the highest concentration among those analysed. (Figure 4). In general, all the fresh meats analysed show a statistically significant lower content of Cadaverine compared to meat meals.

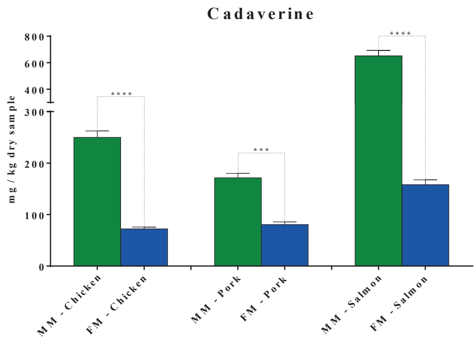


Figure 4. Cadaverine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Cadaverine per kg of dry sample). Data are reported as mean ± SEM, n = 10; ***$p < 0.001$, ****$p < 0.0001$

These values therefore show how meat meals have probably gone through more intense microbial processes, particularly lysine decarboxylation processes, than fresh meats, with the consequent formation of Cadaverine, a potentially toxic amine for organism (del Rio et al., 2019; Lewis, 1998; Til et al., 1997).

The concentration of Tyramine, a biogenic amine deriving from the decarboxylation of the amino acid Tyrosine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was then evaluated. In Figure 5 it is shown how the concentration of Tyramine in chicken fresh meats is more than five times smaller than in meat meals; while it becomes half in the case of pork and more than three times lower in the case of salmon samples. The highest concentrations of Tyramine are peculiar to chicken and salmon meat meals, both being more than double when compared to pork meat meals. Again, all the fresh meats analysed show a statistically significant lower content of Tyramine compared to meat meals.

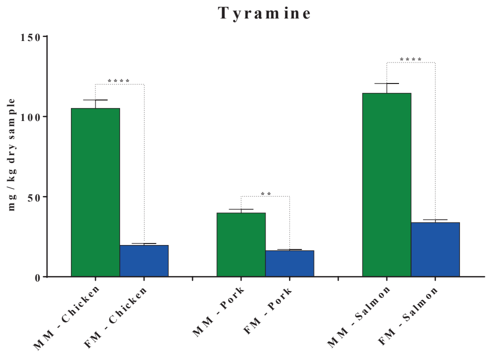


Figure 5. Tyramine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Tyramine per kg of dry sample). Data are reported as mean ± SEM, n = 10; $^{**}p < 0.01$, $^{****}p < 0.0001$

These findings therefore confirm what was previously seen for the other biogenic amines, corroborating the hypothesis that meat meals have probably gone through intense microbial processes, resulting in more efficient Tyrosine decarboxylation reactions. This leads to the formation of Tyramine, a toxic compound for the body (Lewis, 1998; Til et al., 1997), which has been shown by recent studies to be particularly impactful to intestinal cells (del Rio et al., 2017; Linares et al., 2016).
Afterwards the concentration of Tryptamine, deriving from the decarboxylation of the amino acid Tryptophan (Ruiz-Capillas & Jimenez-Colmenero, 2005), was evaluated. Again, the concentration of this biogenic amine in fresh meats is significantly lower than in meat meals for all the raw materials analysed. In particular, for chicken fresh meats the concentration is about twenty times lower; while in the other samples the concentration is less than half as compared to that found in meat meals (Figure 6).
As is the case with the other biogenic amines previously analysed, the highest concentrations are found in chicken and salmon meat meals. These findings again underline how fresh meats contain less biogenic amines in comparison with meat meals, being therefore confidently better in terms of quality and thus less harmful for pets.
The exposure to Tryptamine, which is formed following unwanted microbial degradation processes, is indeed responsible for toxicity phenomena such as serotonergic neurotoxicity and hallucinations, mediated by agonism at the 5HT1A and 5HT2A receptors (Tittarelli et al., 2015).

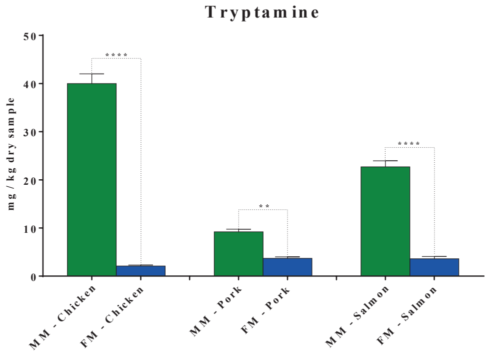


Figure 6. Tryptamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Tryptamine per kg of dry sample). Data are reported as mean ± SEM, n = 10; $^{**}p < 0.01$, $^{****}p < 0.0001$

The concentration of 2-Phenethylamine, a biogenic amine deriving from the decarboxylation of the amino acid Phenylalanine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was also tested. Even in this case, evidence emerges of how fresh meats are qualitatively better, as they show a statistically significantly lower content of this biogenic amine compared to meat meals (Figure 7).

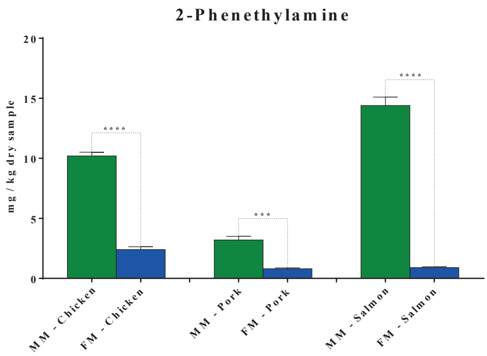


Figure 7. 2-Phenethylamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of 2-Phenethylamine per kg of dry sample). Data are reported as mean ± SEM, n = 10, $^{***}p < 0.001$, $^{****}p < 0.0001$

2-Phenethylamine formed as a result of unwanted microbial degradation processes was found to be four times less concentrated in chicken and pork fresh meats as compared to the corresponding meat meals, but the largest difference was found for salmon samples, whose fresh meats contain 2-Phenethylamine in a concentration around fourteen times lower. Similarly to what seen before, the highest concentrations were observed for chicken and salmon meat meals.

The intake of this biogenic amine is toxic to the body, in that studies have shown that 2-Phenethylamine itself or its derivatives can accumulate in the kidneys and induce nephrotoxicity phenomena (Mossoba et al., 2016).

Finally, the microbial degradation pathways of Arginine and Glutamine amino acids were analysed. Although starting from different compounds, these pathways then converge to a common route. Arginine can initially be decarboxylated by Arginine decarboxylase, obtaining a biogenic amine called Agmatine (Galgano et al., 2012; Ruiz-Capillas & Jimenez-Colmenero, 2005), which is not directly responsible for toxicity problems, but can be further degraded to form another biogenic amine called Putrescine, responsible for toxicity phenomena (De Vera et al., 1992; del Rio et al., 2019; Lewis, 1998; Til et al., 1997). This amine, following the transfer of the propylamine group from S-adenosylmethioninamine, can subsequently be converted initially into Spermidine and then into Spermine (Ruiz-Capillas & Jimenez-Colmenero, 2005), secondary biogenic amines which can be toxic to the nervous system and give rise to disorders such as emaciation, aggressiveness, convulsions and paralysis phenomena (Til et al., 1997).

Another route for Arginine degradation is attributed to the action of other microbial enzymes, Arginase, which initially catalyse a hydrolysis reaction with the formation of Ornithine (Ruiz-Capillas & Jimenez-Colmenero, 2005), a non-proteinogenic amino acid. The latter is subsequently decarboxylated by Ornithine decarboxylase with the formation of Putrescine, which can then undergo the pathway described above.

The formation of the intermediate amino acid Ornithine can also be obtained starting from another amino acid, *i.e.* Glutamine, which is first converted into Glutamate and then in Pyrroline-5-carboxylate before the formation of Ornithine (Jones, 1985).

The results shown in Figure 8 indicate that Agmatine has a concentration about sixteen times lower in chicken fresh meats and six times lower in pork fresh meats compared to their meat meals. The greatest difference is recorded for salmon samples, whose fresh meats exhibit almost two hundred times smaller quantity of Agmatine with respect to salmon meat meals. The latter were also found to have the highest concentrations among the different meat meals analysed. In general, the highest Agmatine content in all the meat meals analysed is always statistically significant ($p < 0.0001$) when compared with the respective fresh meats.

As for Ornithine, which is produced from both Arginine and Glutamine, the same trend is always recorded, characterized by a statistically significant higher biogenic amine content in meat meals compared to fresh meats. All fresh meats tested have a quantity of Ornithine corresponding to about half of what is measured in meat meals, with chicken meat meals showing the highest concentration.

Putrescine, another meeting point between the two pathways of amino acid degradation, is significantly less concentrated in all the fresh meats analysed compared to the meat meals, in particular for chicken and salmon fresh meats, where the greatest differences were found: salmon fresh meats indeed possess a quantity of Putrescine about twenty times lower than meat meals. Again the highest concentrations found were those in chicken and salmon meat meals.

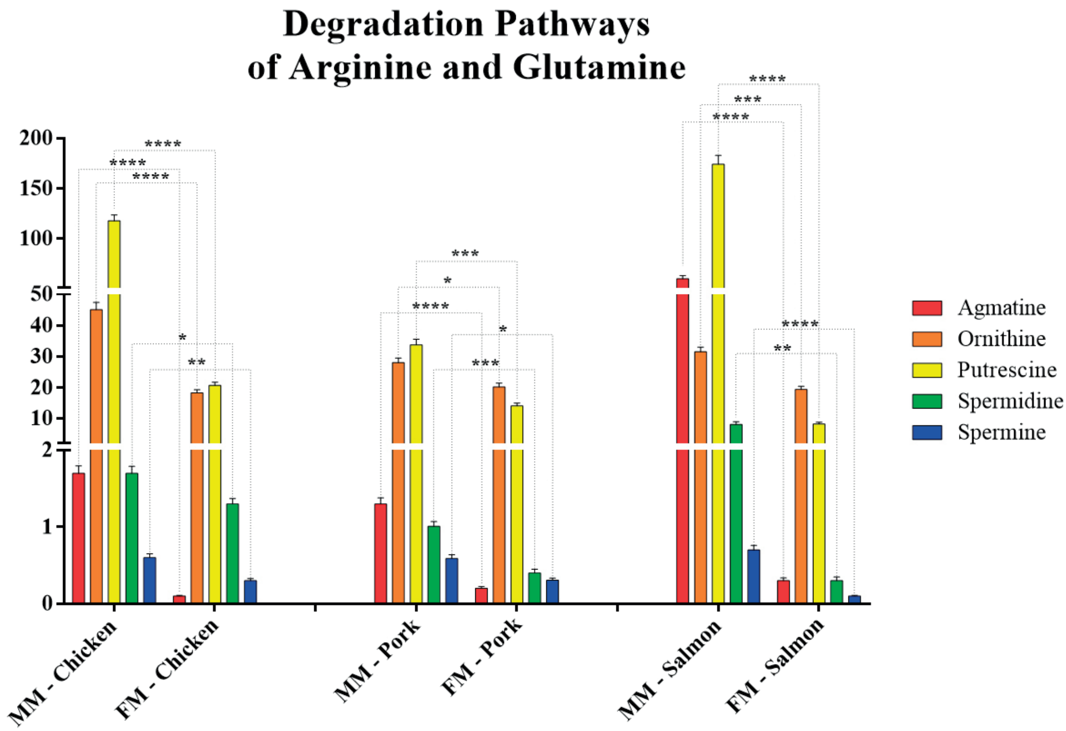


Figure 8. Agmatine, Ornithine, Putrescine, Spermidine and Spermine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of biogenic amine per kg of dry sample). Data are reported as mean ± SEM, n = 10; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$

The fact that the biogenic amines belonging to the Arginine and Glutamine degradation pathways are more represented in the meat meals instead of in fresh meats implies that almost certainly meat meals are qualitatively lower from this point of view, potentially more harmful to pets and probably inadequately stored.

All of the findings listed above could be justified by the fact that meat meals are produced through intensive industrial processing that may cause the partial degradation of raw materials (Camire et al., 1990; Lankhorst et al., 2007; Piergiovanni & Limbo, 2010; Rokey, 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006). The degraded protein material could be the substrate of microorganisms, which, following decarboxylation reactions, can lead to the formation of biogenic amines, which are toxic to the organism, especially for puppies (Bjeldanes et al., 1978; Blonz & Olcott, 1978; Learey et al., 2018; Privitera et al., 1969; Radosevich, 2006).

In this study, it has been shown how there are significant differences in the content of biogenic amines of different raw materials used for dry pet food production. Fresh meats appear to contain lower quantity of biogenic amines, probably thanks to the fact that, since they are generated as meat by-products intended for human consumption, they are not produced with intense industrial processes such as thermals or mechanicals. By doing so, fresh meats result less degraded, better preserved and more protected from the action of microorganisms.

In fact, as they are less degraded, the substrates for the microbial decarboxylating enzymes are only available to a lesser extent, and the decarboxylases cannot thus efficiently convert the amino acids into biogenic amines. As mentioned before, this is supposedly due to the fact that fresh meats are probably qualitatively better and more adequately stored than meat meals, reducing the range of action for microbial decarboxylases.

All of these aspects can heavily influence the quality of the final products and could help the manufacturer companies to understand which raw materials are the best choice for making healthier dry pet food.

## CONCLUSIONS

This study revealed the different concentration levels of biogenic amines between fresh meats and meat meals.

Meat meals, for all the categories of raw materials analysed, *i.e.* chicken, pork and salmon, showed a higher concentration of biogenic amines compared to fresh meats, and this is the case for all the decarboxylation products analysed.

Therefore, these results suggest that meat meals, regardless of the raw material considered, go intensely through microbial degradation processes, probably as a result of both the aggressive industrial processes they undergo and the incorrect handling and storage conditions.

In conclusion, this study has disclosed that fresh meats, for all the categories taken into account, are qualitatively better from a toxicological point of view, being less subject to microbial degradation processes, and therefore probably better preserved than meat meals.

These findings allow us to confidently state that fresh meats, for all the categories analysed, can be considered the best choice as raw materials for dry pet food production.

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