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Dogs and Birds Dry Food Fumonisin FB_1 and FB_2 Contamination and Their Relation to Ingredients and Packaging Characteristics

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Abstract: Fumonisin (FB_1 and FB_2) contamination and their relation to the ingredients composition and packaging characteristics were evaluated in dry foods for dogs and birds sold in Southern Brazil. Out of 50 dogs and birds food samples analyzed, 80 and 60% had FB levels detected above, the applied method (LC/FLD) limit of quantification, thus 0.04, 0.05 and 0.09 mg kg^{-1} for FB_1, FB_2 and FB_{total}, respectively. Pet food levels ranged for FB_1 from 0.04-1.60/0.07-0.64 and FB_2: 0.04-0.27/0.12-0.45 mg kg^{-1} for dogs/birds samples, respectively. Those levels were lower than the international regulation (FDA, EU) for FB_{total} (5 mg kg^{-1}). Regarding ingredient composition, both food types (100%) had maize as the main carbohydrate energy source followed by rice (82%) and wheat (91%). The other grains toxin contamination related were in decreasing order of inclusion: Soybean, linseed and sorghum/oats mix (55/50, 37/50 and 9/50% for dogs/birds, respectively). Regarding fungi growth pet food humidity conditions while mc were high in the birds (10.0-14.0%; RSD 9.9%) food samples they were rather low in the dogs (6.8-10.4%; RSD 9%), with rather similar a_w for both food (dogs: 0.48-0.65; RSD: 6.3% and birds: 0.53-0.78; RSD 11.0%). Although, the birds samples mc and a_w were below Fusarium growth conditions, some of them could be enough for further toxigenic storage fungi growth as the pet selling food stores room temperature in Southern Brazil.

Key words: Pet food, fumonisins, moisture content, water activity, Brazil

INTRODUCTION

In recent decades, there has been a considerable increase on the pet food market worldwide which is due especially to the increase of grain production. Most of the dry pet food is made on a cereals basis as source of energy and the main raw material utilized is maize, followed by other grains such as rice, wheat, barley and oats in smaller quantities though (Diaz and Boermans, 1994; Brera *et al*., 2006). The main problem with the quality of those grains is the mycotoxins which can be produced by fungi proliferation either in the field or during storage. Apart from cereals, mycotoxins producing fungi can grow also on/in other pet food ingredients, such as pulses (soybean, peanut, peas), nuts (walnuts, cashew nuts, Brazil nuts, pistachio), dry fruits (raisins, apples) and other vegetables (tomato, carrots) (Thompson *et al*., 2011; Akande *et al*., 2006; Pacheco and Scussel, 2007). They can cause a wide variety of damages to pet's health due to their different target organs and the intensity of toxic effects-acute and chronic mycotoxicosis (Coulombe, 1993; Silva *et al*., 2009; De Souza and Scussel, 2012). Despite of toxins being present in the pet food via inclusion of contaminated raw ingredients, they can get there through final products exposed to low quality storage and selling conditions.

Field toxigenic fungi can produce mycotoxins, such as Fumonisins (FBs), Deoxinivalenol (DON) and Zearalenone (ZON) in cereals and Alternariol (AOH), Alternariol Methyl Ether (AME) and Patulin (PAT) in other vegetables such as fruits and roots (tomato, apple and carrots) all pet food ingredients. On the other hand, the storage toxigenic fungi can produce Aflatoxins (AFLs), Ochratoxin A (OTA) and Citrinin (CTR) in cereals (Pozzi *et al*., 2001; Rumbeiha, 2000; Pacheco and Scussel, 2007). Therefore, it is worth emphasizing that when a mycotoxin is found in food, one must consider that other mycotoxins may also be present, in which their interaction may worsen the clinical status of mycotoxicosis (Rumbeiha, 2000).

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Regarding FBs (FB_1 and FB_2), despite the known field production, they may also be produced by Fusarium genera during storage, if grain and/or food reaches optimum growth conditions such as high mc (18-29%) and temperature (15-30°C) (Alberts *et al*., 1990; Jouany, 2007; Pitt and Hocking, 2009; Marin *et al*., 2010). Once pet food is FBs contaminated, it is difficult to be decontaminated as the food processing applied cannot guarantee these toxins elimination. As for other mycotoxins, FBs are temperature resistant (up to 260°C) which none of the processes applied such as extrusion for dry food (150-200°C) and sterilization for wet canned food (121°C) in the pet food industry can reach (Brera *et al*., 2006).

Important to emphasize that pets eat same type of food in a daily basis (Industrialized food) thus more exposed to any contaminant that may be present in it, compromising animal's health. Foods with low levels of FBs do not result in characteristic clinical signs of mycotoxicosis but increase the susceptibility of undercurrent infections caused by the animal's immune system suppression and the increase of neoplasias incidence (Osborne, 1982). FBs have been detected, mainly in maize, however they have been reported also in other different cereals in countries worldwide (Voss *et al*., 2007; Scaff and Scussel, 2004). Their levels reported vary from as low as 0.01 to as high as 41.1 mg kg^{-1} in pet food for different animal species, such as birds, dogs, cats, rabbits and fish (Hopmans and Murphy, 1993; Scudamore *et al*., 1997; Mallmann *et al*., 2010; Martins *et al*., 2003; Scussel *et al*., 2006).

As far as mycotoxicosis and small animals pet veterinarians treatment are concerned, there are still little discussed and considered among them regarding the toxicity signs and only few data on the incidence of pets poisoning (CAST, 2003) as well as their level of contamination in raw materials and final products have been reported, especially on FBs contamination.

In addition, regulation for mycotoxin in animal feed worldwide is focused on farm animals with less attention given to pets. In most of the countries were regulation include pet food, the Maximum Tolerance Level (MTL) is set in a general way, rather than pet species-specific. In Brazil, there are only official limit set for AFLs (50 µg kg^{-1}) just for farm animals. The only FBs limits recommended for pets are those set by the Food and Drug Administration (FDA, 2001) and the European Commission (EC, 2006). The FDA and EC MTL of FBs (FB_{total}: FB_1+FB_2+FB_3) for pets are 10 mg kg^{-1} for maize and 5 mg kg^{-1} for final products, respectively. Despite the lack of pet food official MTL set in Brazil, the Association of Brazilian Food Industries (Anfalpet) has developed an Integrated Program for Pet Food Quality (PIQPET). This guide established high quality standards for pet food different parameters, inclusive those for several mycotoxins which can help the industries to keep their food products quality and safety on the safe side. PIQPET recommends an MTL of FB_1+FB_2 of 5 mg kg^{-1} for finished small animal products. Therefore, considering the high inclusion of maize in pet foods, the known FBs contamination of farm animals feed and the lack of information regarding FB_1 and FB_2 in dogs and birds food, this research reports an evaluation of:

- FBs contamination of dry food for dogs and birds sold in Southern Brazil
- Their ingredients composition regarding grain as well as other sources of energy toxin related
- The packaging characteristics that can favor fungi proliferation leading to possible FBs production during commercialization

MATERIALS AND METHODS

Samples: Dry food (total: 50) for dogs (30) and birds (20) from different brands sold in polyethylene bags (bags size: 25 and 5 kg, respectively).

Chemicals: Reagents as phosphoric acid (H_2PO_4), acetic acid, potassium chloride, sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), sodium hydroxide, 2-mercaptoethanol and O-Phthalaldehyde (OPA) all analytical grade (Vetec, Rio de Janeiro, Brazil) and Solvents as Acetonitrile (ACN) and Methanol (MeOH), HPLC grade (J.T. Baker, Texas, USA) and ultrapure water (H_2O) (Millipore, Sao Paulo, Brazil); standards FB_1 and FB_2, 1 mg (Sigma Chemical, St. Louis, USA).

Equipment: Mill (Romer, Miami, USA), vacuum pump (Tecnal, Sao Paulo, Brazil), blender (Metvisa, Santa Catarina, Brazil), SPE monifold (Phenomenex, California, USA), heating block (Tecnal, Sao Paulo, Brazil), oven (Fanem, Sao Paulo, Brazil), analytic scale (Shimadzu, Kioto, Japan), a_w reader (Aqualab, Sao Paulo, Brazil), solvent filtration system (Millipore, Sao Paulo, Brazil). High performance Liquid Chromatography (LC) with fluorescence detector-FLD (Gilson, Vivier le Bel, France), injector of 20 µL loop (Rheodyne, California, USA) and reverse phase column C_{18} with length, inner diameter, particle size of 250, 4.6, 5 mm, respectively (Phenomenex, California, USA).

Quaternary amino Solid Phase Extraction (SPE) cartridges 500 mg packaging size and 6 mL volume (Applied Separations, United States); nitrogen gas, analytical grade (White Martins, Rio de Janeiro, Brazil), filter paper Whatman No. 4 (Whatman, Maidstone, England), desiccators (Ø 200 mm), micro syringe (50 µL) with lock needle (Hamilton, Nevada, USA) and membrane filter 0.45 μm and 0.45 mm for porosity and diameter, respectively (Millipore, Sao Paulo, Brazil).

Sample collection and preparation: Dogs and birds food were purchased randomly from six pet stores in Santa Catarina State, Southern Brazil. Each sample was grounded in a mill, homogenized and divided into portions for further analysis. Packaging was kept for labels ingredients and characteristics data gathering.

Packaging and sample characteristics: Data on dogs and birds food packaging regarding vegetable ingredients, either cereals/seeds/nuts (energy mycotoxin related) and others were obtained as described on the label composition list, in decreasing order of inclusion and presentation format of whole or ground (meal/grits), apart from dry fruits (raisins, apple, carrots, tomatoes) and presence of dye; bags light protection and material type (opaque or translucent), inner atmosphere (vacuum), packaging selling integrity and sample characteristics which were obtained from samples visual analysis and labels displays.

FBs LC determination

Standard solutions: Individual FB_1 and FB_2 stock solutions (100 μg mL^{-1}) were prepared in 10 mL of ACN: H_2O (1:1) according to Visconti *et al*. (1994). A series of working FB_1 and FB_2 solutions at increasing concentration and a mix of toxins were prepared for calibration curves. All were stored in sealed amber vials at -18°C.

LC determination: The method applied was of AOAC, art. 995.15. Briefly, portions of ground samples (50 g) were FBs extracted with MeOH:H_2O (3:1) followed by filtration. After pH adjustment to 5.8-6.5 (with NaOH) extract was cleaned-up through SPE (C_{18}) cartridge (conditioning-MeOH, washing-MeOH:H_2O (3:1), extract addition/washing-MeOH:H_2O (3:1) and MeOH then FBs elution-MeOH:acetic acid (9.9:0.1)). The FBs elution extracts were concentrated in a heating block (40°C, under nitrogen flow) and quantified after OPA derivatization (25 μL extract, 225 μL OPA, 2 min) by LC-FLD (ex. 335 nm, em. 440 nm) with mobile phase MeOH:NaH_2PO_4 (77:23) adjusted to pH 3.3 (with H_2PO_4) at 0.8 mL min^{-1} flow rate. Method validation procedure was carried out through calibration curve (Linearity, LOD and LOQ), recovery and evaluation of repeatability/reproducibility. The method LOQ for FB_1/FB_2/FB_{total} was 0.04/0.05/0.09 mg kg^{-1}, respectively and recovery was 87±11.5%.

Mc and a_w: Mc was determined by gravimetric method art. 930.15 of AOAC (2005) (average of 3 data). The a_w was obtained by measuring samples an a_w meter at 25°C.

Statistical analysis: Pearson Coefficient of Correlation Test (PCCt) was applied to evaluate the correlation between data of the nonparametric variables of FBs with mc and a_w and the t-test student, for comparison of FBs detection in dogs and birds food data obtained with significance level of 1%.

RESULTS AND DISCUSSION

From the dogs and birds dry food data obtained, it was possible to observe that apart from maize, rice followed by wheat were the main carbohydrates energy sources which could be FBs related; FB_1 and FB_2 were present in different levels depending upon the animal food type and composition; the packaging material and inner atmosphere were light protective and food inner atmosphere were air except for one vacuum bird food sample. The ingredients characteristics mycotoxin related (cereal/pulses/nuts/seeds), FBs (FB_1 and FB_2) contamination data; humidity conditions (mc/a_w) and inner packaging environment are shown in Fig. 1 and Table 1-3.

Packaging and samples characteristics: The packaging material utilized for the dogs and birds samples varied regarding light incidence: in the dog's food bags the materials utilized were opaque which can lead to food ingredients light protection. In contrary, the birds were made with transparent material which allows light transfer into the food (Table 1).

Apart from food lipid oxidation, light can stimulate FBs production due to temperature enhancement on the substrate (Fanelli *et al*., 2011). Regarding the packaging inner atmosphere, all pet food except for one bird sample (vacuum treated) had air inside. Despite the nature of substrate/a_w/temperature, the bag gas composition

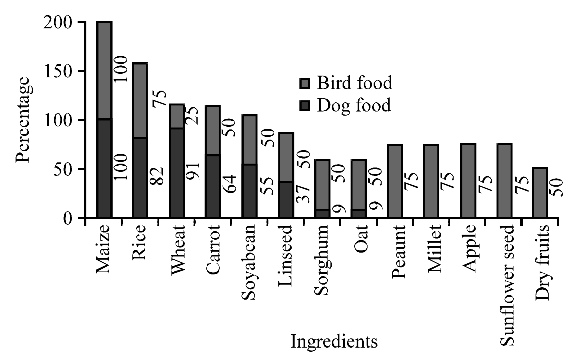

Fig. 1: Percentage of some ingredients mycotoxin related (grains, cereals, seeds, nuts and dry fruits) added to dogs and birds complete dry foods

Table 1: Dry foods for dogs and birds ingredient composition, packaging and samples characteristics data collected from their label/inner content and their relation to fungi and FBs/other toxins contamination

	Pet food brands														
	Dogs											Birds			
Pet food	A	B	C	D	E	F	G	H	I	J	K	A	B	C	D
Composition related to fungi and mycotoxins contamination															
Cereals/nuts/seeds															
Maize (ground)	1**	4	1	1	2	2	2	2	1	2	1	NA	NA	1	1
Maize (gluten meal)	3	1	NA	2	6	4	NA	3	3	1	3	5	3	NA	13
Rice (coarse)	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	1	4	2
Rice (broken)	2	NA	4	NA	1	1	NA	1	2	4	2	NA	NA	NA	NA
Wheat (whole grain)	NA	NA	NA	NA	5	NA	NA	NA	NA	NA	4	NA	NA	NA	NA
Wheat (meal)	4	5	2	4	4	3	6	4	NA	3	NA	NA	NA	NA	8
Oats (pressed)	NA	NA	NA	NA	NA	9	NA	NA	NA	NA	NA	15	NA	NA	3
Millet	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	2	14
Sorghum	NA	6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6	NA	10
Soybean (meal)	5	NA	3	3	3	NA	NA	6	NA	5	NA	6	NA	3	NA
Beans (meal)	NA	NA	NA	NA	NA	NA	NA	5	NA	NA	NA	NA	NA	NA	NA
Pea	NA	NA	NA	5	NA	NA	NA	NA	NA	NA	NA	NA	7	NA	NA
Peanuts	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	9	2	NA	6
Peanuts skin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	10	NA	NA	NA
Birdseed (whole)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA
Linseed (whole)	6	7	NA	NA	7	NA	NA	NA	NA	6	NA	14	NA	NA	7
Pumpkin seed	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	11	NA	NA	NA
Sunflower seed	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	8	4	NA	5
Dry fruits/roots/other vegetables/by-products															
Dry fruits	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	7	NA	NA	11
Raisins	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12
Carrot	7	3	NA	6	9	7	3	NA	NA	NA	6	12	NA	7	NA
Tomato	NA	NA	NA	NA	NA	NA	4	NA	NA	NA	NA	NA	NA	NA	NA
Apple	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4	NA	6	9
Vegetable oil	8	8	NA	NA	NA	5	NA	NA	6	NA	5	1	5	5	4
Package and sample characteristics															
Pack size (kg)	20	25	25	20	20-25	15-20	20	25	25	25	25	5	5	5	5
Packaging															
Material type	Pe[a]	Pe	Pee	Pe	Pe	Pe	Pe	Pe	Pe	Pe	Pe	Pe	Pe	Bo	Pe
Protection to light	Op[d]	Op	Op	Op	Op	Op	Op	Op	Op	Op	Op	Tr[e]	Tr	Tr	Tr
Inner atmosphere	air	air	air	air	air	air	air	air	air	air	air	vacuum	air	air	air
Particles:															
Format	R,S &B	R,S &H	R&S	R&B	R&T	R&T	R,H &B	R,S &B	R	R&S	R,S,H, B&T	R&S	S	R&S	S
⌀ (mm)	10	10-20	5-12	10-15	7-12	6-10	12	15-20	8	8	10	5-10	8	3-10	2-10
Dye added*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Age recommended	A[b]	A	A	A	P[c]	A	A	A	P	A	A	Aa	Aa	Aa	Aa
Sample															
Number per brand	2	5	2	2	5	3	2	4	1	2	2	7	2	3	8
Total general	30											20			

[a]Polyethylene; [b]Adult; [c] Puppy; [d]Opaque packaging; [e] Transparent packaging; NA: Not Added; R: Round; S: Square; H: Heart; T: Triangle; B: Bone; BO: Bottle; AA: All Ages; *Food with artificial dyes added: Green, red, yellow and orange; **Means the decreasing order of quantities included on the package ingredients list; ⌀The higher the number, the lower the quantity added/the lower the number the higher the quantity added into the pet food final product

(air/CO_2/N_2/vacuum) is the most important parameter that can affect fungal growth and mycotoxin production post-harvest (Magan *et al*., 2003; Magan and Aldred, 2007). Pet food packaging with air inside can allow lipid ingredients (fatty acid) oxidation and if enough mc and a_w, fungi growth. Packaging is an important part of pet food safety and the choice of material, the pack inner atmosphere and the sealing process can add to it. As far as sample characteristics, both pets are concerned as expected birds had higher size variation due to whole grains and seeds included than dogs which had all ingredients ground, extrusion cooked and shaped into similar size pellets.

All dogs food had dyed pellets, i.e., different colorants (artificial dyes) added which although, make pet food more attractive for animal owners, no effect causes to the animal preference except for activating some skin, throat and lungs allergies/intolerance (example: tartrazine-orange/yellow color).

Table 2: Dry food for dogs and birds levels of fumonisins, moisture content and water activity in samples of different brands sold in Southern Brazil

	FB[a] ($mg\ kg^{-1}$)														
Dry pet food	FB_1			FB_2			ΣFBs			mc[b] (%)			a_w[c]		
Animal/Brand	Average	Min/max	±SD	Average	Min/max	±SD	Average	Min/max	±SD	Average	Min/max	±SD	Average	Min/max	±SD
Dogs															
A	ND[d]	NA[e]	NA	ND	NA	NA	ND	NA	NA	8.5	8.1/9.0	0.63	0.58	0.57/0.59	0.14
B	0.18	0.04/0.61	0.24	0.132	0.04/0.29	0.10	0.31	0.06/0.91	0.34	9.0	8.5/9.9	0.55	0.58	0.55/0.61	0.02
C	0.53	0.43/0.64	0.14	0.315	0.04/0.59	0.38	0.83	0.64/1.02	0.56	8.9	8.5/9.3	0.56	0.63	0.60/0.65	0.03
D	0.10	0.08/0.12	0.02	ND	NA	NA	0.10	0.08/0.12	0.02	9.4	9.3/9.6	0.21	0.61	0.61/0.61	NA
E	0.29	0.04/0.86	0.34	0.168	0.04/0.64	0.26	0.29	0.08/0.86	0.37	9.5	8.4/10.3	0.75	0.59	0.56/0.64	0.03
F	1.60	0.34/0.83	0.63	ND	NA	NA	1.60	0.64/1.6	0.51	8.7	8.6/8.9	0.15	0.62	0.59/0.65	0.03
G	0.14	0.05/0.23	0.12	0.095	0.04/0.15	0.07	0.22	0.05/0.39	0.24	9.0	6.2	1.97	0.65	0.48/0.65	0.12
H	0.75	0.12/1.60	0.66	0.16	0.04/0.15	0.16	0.89	0.12/2.00	0.84	8.4	8.4/8.5	0.05	0.60	0.55/0.65	0.04
I	ND	NA	NA	0.22	NA	NA	0.22	NA	NA	9.9	NA	NA	0.58	NA	NA
J	0.28	0.03/0.54	0.35	ND	NA	NA	0.29	0.04/0.54	0.31	7.4	7.4/7.4	NA	0.55	0.55/0.56	0.01
K	0.70	0.03/1.36	0.93	ND	NA	NA	0.70	0.04/1.36	0.89	9.9	9.4/10.4	0.71	0.61	0.60/0.62	0.01
Total samples (30)															
Positive (%)	23 (76.7)			13 (43.3)			24 (80)			NA (NA)			NA(NA)		
>MTL[f]	0			0			0			NA			NA		
Average (min/max)	0.50 (0.04/1.60)			0.29 (0.04/0.64)			0.64 (0.05/2.00)			8.96 (6.2/10.4)			0.59 (0.48/0.65)		
Sd[g] (RSD %[h])	0.49 (98)			0.20 (69)			0.55 (86)			0.86 (9.9)			0.03 (6.3)		
Birds															
A	0.13	0.03/0.14	0.03	1.35	0.04/0.42	0.13	0.14	0.04/0.42	0.13	11.6	10.0/12.9	0.98	0.69	0.56/0.77	0.06
B	0.03	0.03/0.03	NA	0.04	0.04/0.04	NA	0.08	0.08/0.08	NA	10.9	10.2/11.7	1.06	0.65	0.59/0.71	0.08
C	0.03	0.03/0.05	0.01	0.17	0.04/0.45	0.23	0.22	0.08/0.5	0.23	11.1	10.2/13.3	1.66	0.60	0.53/0.71	0.56
D	0.07	0.03/0.27	0.08	0.14	0.04/0.44	0.15	0.19	0.08/0.44	0.14	11.9	10.7/14.0	0.94	0.70	0.56/0.78	0.06
Total samples (20)															
Positive (%)	9(45)			6 (30)			12 (60)			NA (NA)			NA (NA)		
>MTL[f]	0			0			0			NA			NA		
Average (min/max)	0.09 (0.04/0.27)			0.32 (0.12/0.45)			0.22 (0.04/0.50)			11.7 (10./14.0)			0.67 (0.53/0.78)		
Sd[g] (RSD %[h])	0.07 (78)			0.13 (40)			0.16 (73)			1.05 (9)			0.07 (11.0)		

[a]Fumonisins (FB_1, FB_2 and FB_{total} LOQ: 0.04, 0.05 and 0.09 $mg\ kg^{-1}$); [b]Moisture content; [c]Water activity; [d]Not detect; [e]Not applicable; [f]Maximum tolerable level (USA max. recommended level for maize pets: 10 $mg\ kg^{-1}$ (FDA, 2001) and EU max. recommended level for pet foods 5 $mg\ kg^{-1}$); [g]Standard deviation; [h]Relative standard deviation

Table 3: Coefficient of correlation between fumonisins, moisture content and water activity in dog and bird food commercialized in Santa Catarina state, Southern Brazil

		Pet food					
		Dogs			Birds		
Parameters	PCCt[a]	ΣFBs[b]	Mc[c]	a_w[d]	ΣFBs[b]	mc	a_w
ΣFBs	CC[e]	1	−0.027	0.290	1	0.013	0.495
	p value[f]	-	0.888	0.120	-	0.957	0.026
	N[g]	30	30	30	20	20	20
mc	CC	−0.027	1	0.620	0.013	1	0.254
	p value	0.888	-	0.000	0.957	-	0.281
	N	30	30	30	20	20	20
a_w	CC	0.290	0.620	1	0.495	0.254	1
	p value	0.120	0.000	-	0.026	0.281	-
	N	30	30	30	20	20	20

[a]Pearson correlation coefficient test; [b]ΣFBs = FB_1+FB_2; [c]Moisture content; [d]Water activity; [e]Correlation coefficient; [f]$p < 0.05$; [g]Number of samples

Regarding carbohydrate energy ingredients, cereals were the most prevalent with 100% of maize inclusion in dogs/birds food followed by 82/75% of rice and 91/25 of wheat distributed in the different brands and samples evaluated. The other grains were in decreasing order of inclusion: 55/50, 37/50 and 9/50% of soybean, linseed-sorghum and oats for dogs/birds, respectively. Figure 1 shows the percentage of grain-based ingredients apart from other vegetables added to dogs and birds dry food. Whole nuts, seeds and dry fruits were present only in the birds food samples which can be mainly storage mycotoxin related (AFLs, OTA and CIT).

FB_1 and FB_2 contamination versus pet food regulation: Regarding the pet food samples FB_1 and FB_2 contamination, 80% (24) for dogs and more than a half of the food for birds, 60% (12) had levels detected above the method LOQ (0.04/0.05/0.09 $mg\ kg^{-1}$ for FB_1/FB_2/FB_{total}, respectively). Levels varied for FB_1 and FB_2 being the first higher than the second, ranging for FB_1: from 0.04-1.60/0.07-0.64 and for FB_2: from 0.04-0.27/0.12-0.45 mg kg^{-1} in dogs/birds food, respectively. In the dogs, dry food the percentage of FB_1 positive samples was higher (76.7%) than FB_2 (43.3%). On the other hand in bird's food, FB_1 was present in less than a half (45%) of the samples, the same for FB_2 (30 %) (Table 2).

In a study carried out by Martins *et al.* (2003) evaluating mycotoxins in 60 dogs dry foods, FB_1 was detected in 5% with levels ranging from 0.012-0.024 mg

kg^{-1}. On the other hand, Cruz evaluating FBs in 24 samples of maize (Grain) for the animal feed manufacturing company, these mycotoxins were detected in 83.3% of the samples with an average concentration of 3.27 and 1.30 mg kg^{-1} for FB_1 and FB_2, respectively, levels rather high compared to the previous researchers and the current study in dogs and birds food. Indeed, the addition of carbohydrate energy source ingredients shows that maize and rice were the main source followed by wheat. Except for rice, those grains have been reported being field mycotoxin contaminated (Rumbeiha, 2000). Among other sources of digestible carbohydrates added to dogs and birds food in the current study, there were also other cereals (sorghum, oats) and pulses (soy) meals. Also fiber sources ingredients were added such as bran (wheat/rice) and hulls (soybean). It is important to emphasize that bran and hull are the first part of the grain that fungi spores can get in contact and the most contaminated part of the grain, apart from germen. Despite this, the possibility of the FBs presence and the contamination levels are dependent on the food type (whether or not grains, brans and hulls are included), the proportion of an added grain and its safety.

Regulation: Comparing the FBs results obtained in the current study to the MTL levels recommended by FDA (2001), EC (2006) and the PIQPET for pet food, no sample had levels higher than those recommended, although 80 and 60% where detected higher than the method LOQ. One of the reasons may be the increase of rice inclusion as the carbohydrate energy source to the pet food produced in Brazil which apart from being cheaper than maize and easier to get in the country due to its high production for human consumption as staple food, it has been reported being less FBs contaminated (Brera *et al*., 2006). Indeed, rice is not a good substrate for Fusarium and those toxins have not been reported in detectable levels, different of maize worldwide.

Pet exposure and toxic effects: As far as pet's health and low levels of FBs are concerned, it is important to emphasize that the pets intake of industrialized food is continuous therefore the presence of FBs, even in small amounts in the food, can lead the animal to a continuous exposure to that contaminant. Long-term exposure is known to produce cumulative damages over the years. Exposure to 1.75 mg of FB_1/kg/day is lethal for rabbits, resulting in liver and kidney toxicity. For equine FBs can cause leukoencephalomalacia and cerebral hemorrhage with a minimum dose of 5 mg kg^{-1} of FB_1 and may lead to development of neural tubes defects in rats (Riley *et al*., 1994; Haschek *et al*., 2002; Voss *et al*., 2007).

Mc/a_w and the risk of FBs/other toxins contamination in dogs and birds dry food: Mc ranged from as low as 7 to as high as 14% and 0.5-0.8 for a_w in both pet foods. Birds food samples had higher mc (10-14%) than dogs (7-10.5%) which may be explained by the different packaging material utilized (birds: transparent) and O_2 permeability; dogs: 2 layers of polyethylene and flexible aluminum foil. Also, their samples characteristics; for birds, there were whole seeds and grains, also nuts without extrusion, on the other hand for dogs the dry food had ground grains and ingredients that were extruded-high temperature processing (150-200°C) and pressure (34-37 atm) which also reduces moisture. Values <12% for mc are considerable safe to control biological contaminants development, such as insects, mites, bacteria and fungi (Crane *et al*., 2000). Therefore, regarding mc in the present study out of the total of dogs food evaluated, all of them (100%) had mc <12% (RSD: 9.9%). In contrary, the birds food had about half (51%) of them ≥12% (RSD: 9%), reaching a maximum of 14% which should be of concern as toxigenic storage fungi could grow producing AFLs, OTA and CTR in substrates, such as cereal, pulses, nuts and dry fruits (all ingredients present in birds food). Regarding a_w, the values ranged from 0.48-0.65 and 0.53-0.78 for dog and bird food, respectively. This measure is the main factor responsible for final products deterioration and favors microbial growth. Fungi are more tolerant to low a_w than bacteria and yeast. In general, fungal growth occurs in a_w varying from 0.65-1.0 and for mycotoxins production, from 0.79-0.90 (Jouany, 2007; Pitt and Hocking, 2009). In the current study, 75% of the bird samples had a_w>0.65 which allow fungi growth. Under these birds food humidity conditions (mc and a_w), it is possible to find microbial growth and mycotoxins.

Considering the characteristics of pet food composition, some ingredients are quite prone to absorb humidity (grain/dry fruits) in rainy or high relative humidity days. That condition allows fungi growth if together with high temperature. The PCC test performed to investigate any correlation between the FB_1, FB_2, mc and a_w variables obtained in the dogs and birds food are in Table 3.

There was a strong positive correlation between FBs and mc in foods for dogs (p = 0.888) and birds (p = 0.957). This correlation is important as FBs can be produced in pet food stored at inadequate moisture conditions (high mc) allowing development of *Fusarium* sp. and possible mycotoxin production (Rumbeiha, 2000; Orsi *et al*., 2000).

CONCLUSION

The FBs levels in the dogs and birds food positive samples were lower than the MTL reported in literature. One of the reasons of low FBs may be the increase of rice inclusion as the carbohydrate source which is cheaper than maize and easy to get in Brazil. Indeed for Fusarium growth, rice is not a good substrate and those toxins have not been reported in detectable levels in that grain.

In addition, the mc and a_w detected in the samples, were not high enough for Fusarium growth. The extrusion process which is applied in the pet food production highly reduces the moisture increasing feed stability for fungi to take place.

FBs even in small quantities, continuous exposure of pets to these toxins, due to their monotype diet can lead to development of chronic diseases including neoplasias. Pet food monitoring to keep bellow levels the MTL is important to ensure the safety to minimize fumonisin-related diseases and problems with pet's health.

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