

CO₂ production in extruded dry foods for dogs exposed to different moisture levels with and without use of mold inhibitor

Produção de CO₂ em dietas extrusadas para cães submetidas a diferentes níveis de umidade com e sem utilização de antifúngico

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Abstract

Four extruded dog foods with different moisture contents and ammonium propionate (AP) were manufactured with the objective of evaluating CO₂ production. The diets were: low moisture (8.1%) with no inclusion of ammonium propionate (LMNP), high moisture (10.2%) with no inclusion of AP (HMNP), high moisture with inclusion of a low level of AP (0.065%) (HMLP), and high moisture with inclusion of a high level of AP (0.130%) (HMHP). The diets were stored in a chamber at 75% air relative humidity and 25-28°C temperature. CO₂ was determined on days 0, 30, 60, and 90 by titration with hydrochloric acid (HCl) 0.05N to estimate mold growth. A completely randomized split-plot experimental design was applied. The diet HMNP measured in 90 days presented the highest CO₂ concentration and mycotoxins production. Dry dog foods with moisture content higher than 10.2% without mold inhibitors may present significant mold growth and mycotoxins production after 90 days storage. Extruded dry foods for dogs with less than 8.1% moisture may not need mold inhibitors.

Key words: Dry extruded food, fungus, ammonium propionate, water activity

Resumo

Foram avaliadas quatro dietas extrusadas para cães com diferentes níveis de umidade, com adição ou não de ácido propiônico (PA), com o objetivo de avaliar a produção de CO₂ e micotoxinas. As dietas foram: baixa umidade (8,1%) sem inclusão de propionato de amônio (BUSP), alta umidade (10,2%) sem inclusão de PA (AUSP), alta umidade com inclusão de baixo nível de PA (0,065%) (AUBP), e alta umidade com inclusão de alto nível de propionato (0,130%) (AUAP). As dietas foram armazenadas em câmara com umidade relativa do ar de 75% e temperatura de 25 a 28 °C. A produção de CO₂ foi determinada nos tempos zero, 30, 60 e 90 dias por meio de titulação com ácido clorídrico (HCl) 0,05N. O experimento seguiu delineamento inteiramente casualizado em parcela subdividida no tempo. A dieta AUSP, medida aos 90 dias, apresentou maior concentração de CO₂ e produção de micotoxinas. Alimentos secos extrusados para cães com umidade superior a 10,2% sem antifúngico podem apresentar expressivo desenvolvimento de fungos com 90 dias de armazenamento. Alimentos secos extrusados com menos de 8,1% de umidade podem não necessitar de inibidores fúngicos.

Palavras-chave: Alimento seco extrusado, fungos, ácido propiônico atividade de água

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Introduction

The list of chemical compounds with mold-inhibiting action is quite extensive, but it is still very limited as compared to the number of available antibacterial drugs (NOBRE et al, 2002). This raises doubts as to the best dosage and type of antifungal agents to be added to feeds. Mold development in grains, raw materials, and feeds, in addition to compromising their quality, may cause severe problems to human and animal health.

The prevention of fungal development should start in the storage of grains by controlling their moisture content. Moreover, each level of moisture requires different adequate levels of mold inhibitor, taking into consideration period of storage, because molds may already start developing in the field, and this process may be accelerated when grains are improperly stored (KRABBE; MACIEL, 1997).

Care must be taken to prevent fungal development and to ensure the quality of the stored feeds: packages must be kept in a well-ventilated environment and not be exposed to sunlight. In addition, increased humidity should be avoided because it promotes the emergence of toxin-producing fungi.

The oldest technique for food storage, which is still used today, is decreases of moisture. Product stability, in this case, may be explained by water activity (A_w), which is determined by water availability. Water may be present as bound water and free water, resulting in total water content (moisture). This division allows the prediction of the participation of water in chemical reactions and in enzymatic or microbiological growth (LABUZA; TANNENBAUM; KAREL, 1970).

According to Azeredo (2004), the main objective of reducing water activity in foods is the reduction of microbiological changes. Labuza, Tannenbaum and Karel (1970) found that moisture content increased leads to changes in food texture and higher A_w , favouring the enzymatic reaction and non-enzymatic browning, as well as microorganism growth in feeds. Among the forms of assessment of the metabolic activity of microorganisms of food,

there is the quantification of carbon by releasing CO_2 , known as respiration (STOTZKY, 1965).

Antifungal agents present different modes of action, routes of administration, actions on superficial or systemic mycoses, and can be classified according to their target site and chemical structure. These products act mainly on the cell membrane (LACAZ; PORTO; MARTINS, 1991).

Other factors may also affect fungal growth, such as humidity, temperature and amount of antifungal added. Organic acids lower feed pH, which in **turn** acts as flavour enhancer and delays enzymatic degradation. They also act as chelating agents that bind to metals forming the metal chelates, which prevent or reduce oxidation caused by the catalysis of metal ions. Organic acids are strong inhibitors of microbial growth, and may be used for the conservation of grains and feeds and sanitization of the meat as growth promoter additives in diets (BELLAYER; SCHEUERMANN, 2004).

There are few studies in literature on the use of pet foods with different moisture levels and the addition or not of antifungal agents. This study aimed to evaluate the effects of AP as a mold inhibitor on extruded dry foods varying moisture by CO_2 and mycotoxins production.

Material and Methods

The experiment was carried out in the Animal Nutrition Laboratory of the Universidade Federal do Paraná. A completely randomized experimental design in split plot in time was applied. Four dry extruded dog diets (treatments) were used: For treatments were used two moisture levels (low 8.1%, high-10.2%) with or without the addition of ammonium propionate, and the four treatments were LMNP (low moisture, no addition of ammonium propionate); HMNP (high moisture without addition of ammonium propionate) HMLP (High moisture with addition of 0.065% ammonium propionate) and HMHP (high moisture e with the addition of 0.130% ammonium propionate. The

nutritional composition of the basal diet is shown in the Table 1.

A reference diet contained 8.1% moisture and 0.524 (26.5°C) Aw. Temperature was maintained between 25 to 28°C and relative humidity around 75% during manufacturing. After that, the dryer was set to increase moisture to 10.2% and water activity to 0.622 (26.7°C) in order to produce the high-moisture feed. The water activity readings were determined using a water activity indicator (Rotronic, HigrPalm, model AW1 Set, Huntington, NY, USA). After drying, all diets were coated in mixer with 25 kg capacity. Firstly, AP (Myco Curb Liq, Kemin, AP, containing 65% ammonium propionate acid) was applied, by compressed air gun, in the treatment HMLP (0.065%) and HMHP (.13%), then was 3.0% poultry fat added and finally 3.0% poultry hydrolyzate in all treatments.

Treatment diets were stored for 15 days before analyses to allow stabilization. Diet samples (700g) were stored in plastic cups in duplicate (identified as A and B for each treatment) in a chamber at 75% relative humidity and 25-28°C temperature for CO₂ determination at times zero, 30, 60, and 90 days of storage by titration with hydrochloric acid (HCl) 0.05 N. Mold development was evaluated by determining CO₂ production, using the method adapted from Stotzky (1965). Approximately 25g of feed were weighed and placed in dark vials with wide mouth and seal (cap lined with a rubber washer that prevents gas exchange with the external environment). A plastic cup with 20mL NaOH was placed next the sample in the vials, which were then sealed for 24 hours. All analyses were performed in triplicate, using A and B duplicate cups from each treatment, yielding six replicates per treatment per storage time.

Table 1. Ingredient and chemical composition of the experimental diet.

Ingredient (%)	
Corn	44.0
Brewers rice	4.0
Soybean meal	15.0
Meat and bone meal	15.0
Fish meal	1.0
Poultry viscera meal	14.0
Poultry fat	3.0
Poultry hydrolysate	3.0
Vitamin and mineral premix ¹	0.5
Sodium chloride	0.5
Chemical composition (%)	
DM (%) ²	91.93
OM	90.04
CP	30.59
AHF	8.51
CF	2.34
NFE ³	48.60
ME ⁴ (Mcal/kg)	3.12

¹D3 = 2340 IU; Vit. E = 104 ppm; Vit. K = 1,3 ppm; Vit. B1 = 3,9 ppm; Vit. B2 = 6,5 ppm; Pantothenic acid = 19,5 ppm; Niacin = 32,5 ppm; Choline = 1150,75 ppm; Zinc = 156 ppm; Iron = 104 ppm; Copper = 13 ppm; Iodine = 2,6 ppm; Manganese = 45,5 ppm; Selenium = 0,26 ppm; Antioxidant = 240 mg.

²DM = Dry Matter; OM = Organic matter; CP = Crude Protein; AEE = Acid-hydrolysed fat; CF = Crude fiber; NFE = Nitrogen-free extract; ME = Metabolizable energy.

³NFE = DM – (Ashes + CP + AHF + CF).

⁴ME = (14.65 x CP + 35.58 x AEE + 14.65 x NFE).

Source: Elaboration of the authors.

Vials were kept at room temperature in the absence of light. After 24 hours, the plastic cup was removed from the sample vials and barium chloride (1 mL) and phenolphthalein (2 drops) were added as indicator. The cup was placed in shaker (Imam) and titrated with standardized HCl 0.05 N. Five blank tests were performed, using pieces of styrofoam to equate the volume of feed.

CO₂ was titrated according to the volume of acid used to change the sample colour from pink to no colour. Increasing acid volumes indicated that more O₂ was consumed and more CO₂ was released by microorganisms.

CO₂ content was obtained using the following formula:

$$\% \text{ CO}_2 = \frac{[(B-A) \times N \times 22 \times CF] \times 100}{\text{sample weight}}$$

B = Volume of acid used for blank titration

A = Volume of acid used for sample titration

N = Normality of the acid

CF = acid correction factor

22 = grams of CO₂ equivalent

The mycotoxins Aflatoxin (B1, B2, G1 and G2), Fumonisin (B1 and B2), Ochratoxin A, Trichothecenes, Vomitoxin (DON), and Zearalenone were determined in all treatments at the beginning and end of the experiment by high performance liquid chromatography (HPLC).

The results were tested for normality and variance homogeneity. CO₂ data were log transformed, and submitted to analysis of variance. Means were compared by Tukey's test (P<0.05).

Results and Discussion

There was a significant interaction among diets and storage time (P<0.01), demonstrating longer times are required for mold growth (Table 2).

Table 2. Concentration of CO₂ (%) produced by 25 g per 24 hours in extruded diets with different moisture levels, with or without ammonium propionate, stored for 90 days.

Time (days)	Treatments				SEM ^b	P
	LMNP	HMNP	HMLP	HMHP		
0	0.25 ^c	1.09 ^{bB}	3.32 ^a	4.74 ^{aA}	0.385	<0.001
30	0.86 ^b	2.49 ^{aB}	2.64 ^a	2.82 ^{bAB}	0.209	<0.001
60	1.44 ^b	3.58 ^{abB}	4.01 ^a	3.68 ^{abB}	0.365	0.042
90	1.09 ^b	145.76 ^{aA}	4.70 ^b	2.19 ^{bbB}	17.087	0.001
SEM	0.233	17.099	0.415	0.256	4.443	
P	0.337	0.001	0.343	<0.01		<0.001

^aLMNP = Low moisture (8.1%) without ammonium propionate; HMNP = High moisture (10.2%) without ammonium propionate; HMLP = High moisture with low inclusion of ammonium propionate (0.065%); HMHP = High moisture with high inclusion of ammonium propionate (0.130%).

^b SEM = Standard error of means.

^{ab} Means followed by different small letters in the row and capital letters in the column are different by the Tukey's test (P<0.05).

Source: Elaboration of the authors.

The highest CO₂ levels observed in treatments containing ammonium propionate on time 0 may be explained by the high volatility of propionic acid, which reacts with sodium hydroxide, simulating fungus respiration. Acid gas volatilization

commonly happens and it is more intense during the first hours after its application (COELHO, 1996). Therefore, periods longer than 15 days are required for ammonium propionate stabilization. The stabilization of ammonium propionate is

demonstrated by inverse relationship with CO₂ values measured at 30, 60 and 90 days. So the treatment was LMNP who presented the lowest values of CO₂ concentration indicating that the ammonium propionate was unstable and there was less mold growth. At 90 days, the highest CO₂ concentration was observed in the HMNP treatment ($P < 0.01$), indicating higher fungal development. The other treatments were not different at this time period ($P > 0.05$) (Table 2), showing the efficiency in diets with added ammonium propionate or low moisture content (8.1%) also inhibits mold growth up to 90 days of food storage.

Krabbe and Maciel (1997) also observed that the use of 1.2 kg propionic acid/t diet in broiler diets containing 11.6% of moisture effectively controlled CO₂ production during seven days of storage. Moreover, Bueno, Silva and Oliver (2001) relates that moisture contents lower than 11.5% are sufficient to prevent fungal development in dog and cat foods, as fungi require available water in foods to grow. However, in the present study, 10.2% of moisture without mold inhibitor did not prevent fungi growth in the feed. Thus, the water activity above the recommended values, considering his role in the growth of microorganisms and extend the shelf life of foods, should be viewed cautiously.

The highest A_w increased CO₂ production in HMNP, which contained high moisture level and without ammonium propionate. In addition, it was also the only treatment where fumonisin B1 (393.3 mg kg⁻¹) was detected at the end of the experiment. The other analyzed mycotoxins were not detected in the other treatments, neither in the beginning nor after 90 days of challenge. Water is produced by fungi metabolism as a result of nutrient consumption. Although HMNP, HMLP, and HMHP were produced in the same batch, and therefore started with the same A_w , as HMNP had higher microbiological activity, water must have been generated during the experimental period, contributing to the increase in A_w , as detected on day 90.

High moisture levels promote fungi growth both in grains – still in the field or stored – and in already processed and ready-to-eat foods. Poor humidity control during storage leads to fungi development, which may damage the entire food due to the contact of non-contaminated with contaminated particles (KRABBE; MACIEL, 1997).

During drying in the feed mill, some extruded foods may present higher moisture levels than the expected, which may trigger fungal development when raw materials are contaminated. Fungi metabolism produces water, and high levels of water available in the food may promote further fungi development and mycotoxin production.

Evaluating mold inhibitors, Krabbe (1996) observed that copper sulphate added to ground corn during storage was not effective against molds. Moreover, the authors cited above observed that propionic acid used in correct dosage was effective to control fungi growth, reducing nutritional losses in corn. Similar results were obtained by Gomez, Rodriguez and Rincón (2008), who evaluated fungal growth in contaminated maize stored for seven weeks in laboratory, using three products based on propionic acid, and reported that all of them were effective in reducing fungi over time. Thus, organic acids may be used to preserve grain cereals containing high moisture levels and to inhibit fungi growth in foods.

Assessing the effect of moisture level and storage time on fungal development in oat grains, Rupollo et al. (2006) found that the greatest impact of the genera *Aspergillus* and *Penicillium* occurred at 18.0% of moisture, followed by 21.0%, and 15.0%. The highest incidence of the genus *Fusarium* was found in grains stored at 21.0% of moisture. As to the storage time, *Aspergillus* fungi showed higher incidence after three months and six months of storage. The present experiment showed that 75% relative humidity (this moisture level is frequently found in the field and in storage facilities) and 90-d storage time is sufficient to allow fungal growth when no propionic acid is added. Ramos, Brasil and

Geraldine (2008) studied maize grain contamination and detected the presence of *Aspergillus* spp. in 100% of samples from areas where it rained during harvesting, and grains were stored with 13.0% of moisture, confirming the fact that grains can be contaminated in the field and also during storage, remaining on the grains during processing.

Once established in grains and feeds, fungi utilize the nutrients from these sources to develop, particularly fat and carbohydrates, which are energy sources essential for growth. This was shown by Rupollo et al. (2006) in *Aspergillus*, *Penicillium*, and *Fusarium* spp.: when grains contained 12% moisture, unsaturated fatty acid content was markedly reduced, especially of linoleic and linolenic acid.

Conclusion

Extruded dry foods for dogs with moisture level above 10.2 % and A_w 0.801 without inclusion of AP promoted intensive CO_2 production after three months of storage. On the other hand, dog foods with less than 8.1% moisture and less than A_w 0.719 the addition of 0.065% ammonium propionate present adequate control of CO_2 production in the conditions and times studied.

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