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Nucleotide inclusion in pet food: effect of heat treatment and storage

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Summary. Nucleotide supplementation in pet foods aims to reinforce the immune system and promote intestinal function. Data on alteration of nucleotides during pet food processing are lacking, however. With this study we compared the recovery percentage of nucleotides in dry and canned food after processing and controlled environment storage. Selected dry and canned feed were supplemented with 0.4g/100 g dry matter basis of Prosol petMOD™ (free nucleotide concentrate) before exposure to high temperature. In detail, the highest temperature applied to dry pet food was 110° C and around 125° C to canned food. The recovery percentage for dry food was 75% at the end of processing and 74% after storage for 12 months versus 43% and 41%, respectively, for canned food.. These results indicate that dietary supplementation with nucleotides to pet food may benefit animal health; however, a high loss of these semi-essential nutritional components was observed, particularly in the canned pet food.

Key words: dry pet food, canned pet food, recovery, nucleotides, thermic treatment, storage

Introduction

Supplementation with nucleotides to baby food and parenteral preparations, in particular, has increased following research demonstrating that including nucleotides in these products can reinforce the immune system and improve gut function (1). Nucleotides are now recognized as "semi-essential" nutrients: endogenous production satisfies requirements in normal health conditions, while supplementation is beneficial for the growth and development of young animals and for tissue repair (1). Nucleotides are phosphoric nucleoside esters formed of three components: a weakly basic nitrogenous compound, a pentose sugar, and one or more phosphate groups. They constitute the basic units of the nucleic acids DNA and RNA (2-3). The most important are adenosine, guanosine, inosine, cytidine, and uridine monophosphates. Generally, there are three sources of nucleotides: de novo synthesis, salvage pathways, and dietary nucleotides (4- 5).

De novo synthesis of nucleotides is a metabolically costly process requiring substantial amounts of energy in the form of ATP (2). Another mechanism for maintaining the cellular nucleotide pools is the salvage pathway. Since this pathway recycles 90% or more of the purine bases, it is thought to be dependent on the availability of free purine and pyrimidine bases (4). The salvage pathway requires less energy than the reactions needed for the de novo synthesis of nucleotides; it is characterized by linkage of a ribose phosphate moiety to free bases formed by the hydrolytic degradation of nucleic acid and nucleotides (6). Finally, since some tissues have limited capacity for the de novo synthesis of nucleotides, they require exogenously supplied bases that can be utilized by the salvage pathway (7).

For example, since the intestinal mucosa, hematopoietic cells of the bone marrow, leucocytes, erythrocytes, and lymphocytes are incapable of de novo nucleotide production (8), they all utilize the salvage pathway. This suggests that dietary supplementation

with nucleotides may be important for these rapid turnover cells (4).

Nucleotide supplementation has been variously studied in farm animals but much less in companion animals. Rutherfurd-Markvick et al. (9) demonstrated an increased proliferative response of post-vaccination lymphocytes in 43 cats fed with an integrated diet supplemented with nucleotides. Improvement in humoral and cellular immunity (IgG, IgA, IgM) was observed in puppies fed with supplemented food (10-11). The dietary effects of nucleotides were correlated with increased CD4+/CD8+ ratio, improved protein electrophoretic pattern and acute phase response in dogs suffering from leishmaniasis (12).

Dietary inclusion of nucleotides into commercial pet food has raised questions about whether and how nucleotides undergo alterations during pet food processing and storage. Commercial pet food is manufactured by extrusion/expansion (dry food) and retort sterilization (canned food). Conventional dry pet food is obtained by extruding a finely ground mixture of ingredients of animal and vegetable origin in variable percentages, depending on the species (dog or cat) and the age or size of the animal for which the food product is intended. Extrusion enhances starch digestibility, which is essential for cats and dogs since they lack a complete enzyme pattern for digesting starches (13). The mixture is treated with water and steam, followed by a short but intense heat and mechanical treatment. To guarantee storage at room temperature, dry pet food is dehydrated in ovens for 20-50 minutes. During this industrial process, the food components are exposed to high temperatures, which can degrade any heat-sensitive and easily oxidizable nutrients (14).

From a food technology perspective, canned pet food is simply a preserved food with a pH close to neutral that ensures its microbiological, chemical, and physical stability and that prolongs product's shelf life. Since it is subjected to intense heat treatment, overdosage of thermolable ingredients is necessary to guarantee adequate final recovery net of losses due to heating (15).

The aim of the present study was to compare dry and canned pet food supplemented with nucleotides, the recovery percentage at the beginning and after production and after storage for 12 months, and to determine whether or not heating and prolonged storage cause significant degradation in these components.

Material and Method

Preparation of dry food

A commercial dog kibble formulation was prepared with a percentage of inclusion of 0.14 g/100 g on dry matter (DM) of Prosol petMOD™, a *Kluyveromices fragilis* yeast cell derivative containing a high concentration of free nucleotides 5'-mono phosphate (> 40%) and total nucleic acids (> 80%) (PROSOL S.p.A, Madone/Bergamo, Italy). The supplemented nucleotides were adenosine (AMP), cytidine (CMP), uridine (UMP), and guanosine (GMP) (Table 1). The control was the same commercial dog kibble formulation without nucleotides.

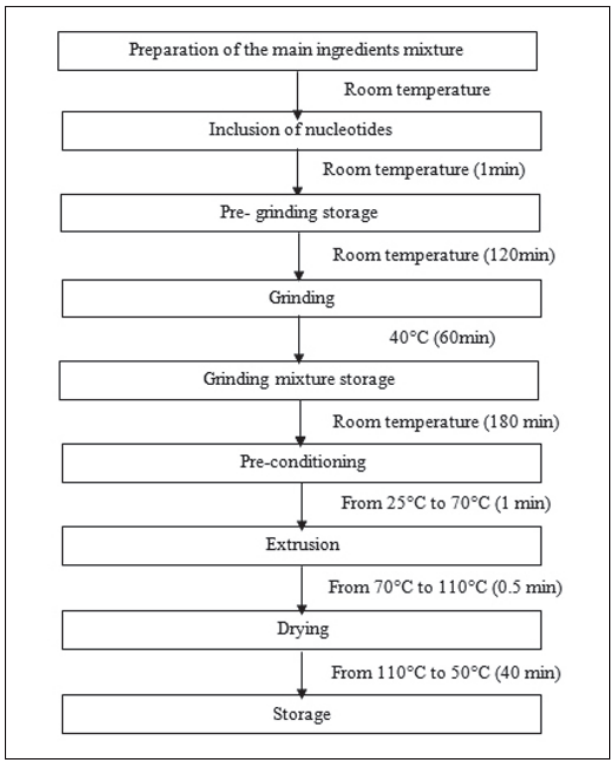
The tested dry pet food (with Prosol petMOD™) and the control food (without Prosol petMOD™) contained the same ingredients combined in a mixture of cereals, mixed poultry meal, oils and fat derivatives of vegetable origin, and minerals. Chemical composition was determined in triplicate according to methods approved by the European Pet Food Industry Federation (16). Table 2 presents the results (expressed on dry matter bases (DMB)). Moisture of finished product was 7.9% and activity water (aW) was 0.497 at 21° C.

The production flow for dry food preparation is illustrated in Flow chart 1. In detail, nucleotides were added during the mixing phase. The percentage of inclusion was calculated at the beginning and at the end of processing and after 12-month storage of the finished product: three vacuum packed lots of the dog kibble formulation. The products were stored at room temperature to simulate domestic environmental conditions.

Table 1. Free 5'-mono-phosphate nucleotide pool in Prosol petMOD ™.

Nucleotide	Concentration (g/100g)
GMP	10
AMP	20
CMP	40
UMP	30

GMP, guanosine; AMP, adenosine; CMP, cytidine; UMP, uridine.



Flow chart 1. Dry food processing, temperatures, and duration.

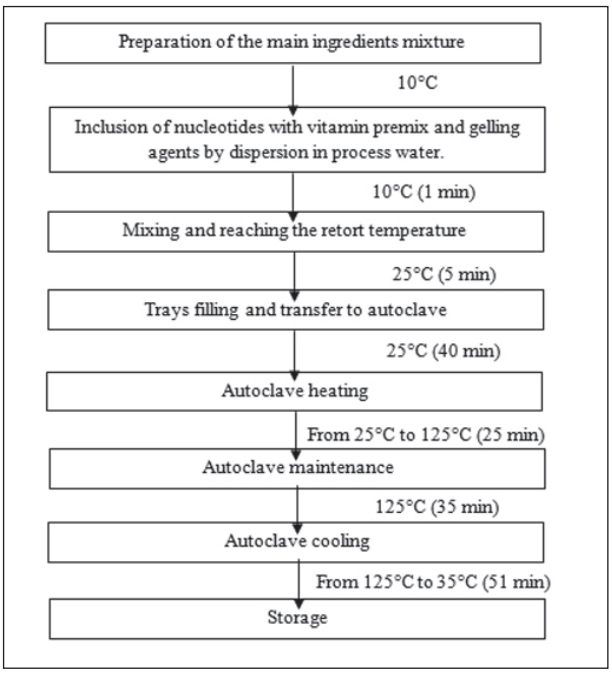
Preparation of canned food for cats

A commercial pâté formulation (final moisture 80%) was prepared containing a Prosol petMOD™ percentage of 0.4g/100g raw materials. The pâté was composed of uncooked chicken and rabbit meat, minerals with (tested food) or without (control food) the addition of nucleotides. Table 2 presents the chemical composition expressed on DMB. Flow chart 2 illustrates the production of the canned food. The product was packed in a 100 g aluminum tray, which was chosen because this form is the one usually subjected to the intense sterilization. The nucleotides were added immediately before packaging to minimize their permanence in the unsterilized batch and to prevent decay

Table 2. Chemical composition of dry and canned foodproducts (g/100 g DMB).

Chemical composition	Dry food	Canned food
Crude Protein	27	47.5
Ether Extract	12.4	30
Ash	7	9
ME (kcal)[a]	423	504

[a]Metabolisable energy (NRC, 1985).



Flow chart 2. Processing of canned food during phase 1, temperature, and duration.

due to fermentation of the nucleotides by the bacterial flora present before sterilization. As suggested by Sevenich et al., sterilization was performed based on the unwanted food processing contaminants (FPCs) value (17). The percentage of nucleotide inclusion was determined at the same time points and under the same atmospheric conditions as for the dry food. Measurements were performed in triplicate on three different lots of the same canned food for cats in the tested and the control formulations.

Method for food processing contaminants (FPCs)

The indicator we used to identify the intensity of heat treatment in pet food is the so-called sterilization value (FPCs). By convention, the FPCs value expresses the number of minutes necessary to obtain a lethal effect against a guide germ (i.e., a pathogen of reference) at a temperature of 121.1° C (i.e., classical sterilization). The FPCs value for a process is the number of minutes needed to kill a known population of microorganisms in a given food under specified conditions.

The FPCs value was calculated using the following equation, where Dr is the decimal reduction time

of a bacterium at 121.1° C (250° F) and Dn is the number of decimals that will be obtained with the heat treatment. The equation is:

$$FPCs = Dr_{121.1} \times Dn$$

Given that the $D_{121.1}$ value for *Clostridium botulinum* spores is 0.21 minutes (min) and that, by convention, a decimal reduction of 12 is considered acceptable, we will have:

$$FPCs = 0.21 \times 12 = 2.52 \text{ min (3 min)}$$

The F0, or lethality value designation, is the number of minutes required to destroy a specific number of *Clostridium botulinum* spores at 250° F. The F0 value was defined as the equivalent number of minutes at 250° F (121.1° C) when no time is required to heat to 250° F or cool to sublethal temperatures when the thermal death time curve slope (Z) is equal to 18° F. The minimum time to obtain commercial sterility is 2.78 minutes at 250° F or its equivalent, or in other words, an F0 of 2.78. With these parameters, assuming a very high initial contamination (1000 spores / container), the probability of survival is reduced to 1 spore per 100 mln of containers. Fc represents the calculation of this parameter at the core of the product. According to EU legislation, wet pet food must undergo a core treatment of at least 121.1° C for at least 3 minutes.

Method for nucleotide determination

Nucleotide determination was modified from Gill et al. (18). Briefly, 50 mg of each standard sample (AMP free acid, CMP free acid, GMP Na2, IMP Na2, and UMP Na) were weighed and diluted in high-performance liquid chromatography (HPLC)-grade water to reach a final volume of 100 ml. After solubilization, 10 ml of this solution were diluted in 50 ml of the solvent. A volume of 10 µl of the final solution was injected into the HPLC system.

For the dry food samples, 50 g of kibbles were weighed and ground with pestle and mortar. A precise quantity of 1 g of ground kibbles was weighed and dissolved in 100 ml of HPLC-grade water, stirred for 15 min, and filtered through 30-micron filter paper. A filtrated volume of 10 µl was injected into the HPLC system. For the canned food samples, 50 g of pâté were weighed, crushed with a few drops of water, and dissolved in 100 ml of HPLC-grade water. The emulsion was settled for 30 min, and 2 ml of supernatant were collected for HPLC injection. The retention time of the standards and the sample were compared. The coefficient of variation (CV) was calculated and the data with a CV ≤ 5% were validated.

The fraction of nucleotides from the raw materials included in the dry and the canned food of the same analytic composition was measured during previous experiments (data not shown). These quantities were then deducted from the values obtained at each time point.

Statistics

Statistical analysis was performed using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). Nucleotide inclusion data were processed using one-way ANOVA on ranks (rank-based nonparametric test) for the time points (T0 vs. T1 vs. T12). Statistical significance was set at $p<0.05$. The results are reported as mean percentage of nucleotides in dry and canned pet foods ±standard deviation (SD).

Results and Discussion

Nowadays, a growing trend in nucleotides supplementation is occurring in pets with the final goal of enhancing immune function and gut health, especially in young and debilitated animals. In these subjects, indeed, the cellular turnover is aximized and this aspect leads to an increased demand in dietary nucleotides as well. Segarra et al. (12) demonstrated that nucleotides improve biological markers of immune response in dogs and helped to a better- health status. This is particularly true for puppies since all tissues are in rapid growth and moreover the immune system is still incomplete (10-11). The best way to achieve a dietary integration of nucleotides is to add them directly to a commercial food, even if the main source of these elements for healthy subjects is usually represented by meat and its by-product, which represent one of the main components of their maintenance diet. Despite the beneficial function of nucleotides administration

is well recognized, little is still known about the capability of these elements to resist to technological processes. The present study was conducted to improve the knowledge in this field.

The recovery percentage of nucleotides in the dry food after processing was 75% and 74% after 12 months of storage, respectively, and 43% and 41%, respectively, in the canned food (Fig. 1). The difference in recovery percentages between the dry and the canned food is due to the fact that the much higher temperatures for sterilization of the canned food mixture led to greater deterioration of all the ingredients (16). Furthermore, the recovery percentage of nucleotides is related to FPCs: the higher the FPC value the longer the duration of sterilization. The FPC values we estimated for sterilization of the moist food was 69, which is a standard value for canned pet food. It should be said, however, that the intensity of heat treatment varies according to the volume of the container and the resistance of its contents to the penetration of heat. Pâté was chosen precisely because, unlike chunks in sauce, it is more resistant to heat penetration and so requires more thermal energy to achieved the same sterilizing effect. Nonetheless, despite the high percentage of degraded nucleotides, their concentration remained stable after 12 months of storage.

N denotes nucleotides, T0 before processing, T1 after processing, T12 after 12-month storage.

Processing of the dry food did not significantly degrade the raw materials, leading to a higher recovery percentage as compared with the canned food. The concentration remained stable during the 12 months of storage. A plausible explanation for this finding is that the temperatures reached at the product core during processing were lower than those in the canned food.

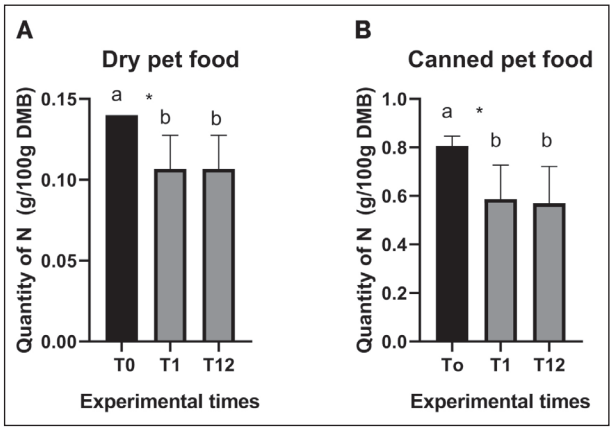


Figure 1. Nucleotides in dry (A) and canned (B) pet food before and after processing and after 12 months of storage. Data are expressed as mean ±SD. ($p < 0.05$)

Conclusions

Nucleotides for dietary supplementation in pet food differ in their resistance to processing for dry or canned food. Pet food manufacturers should be aware of this difference and ensure that sufficient levels of nucleotides are present in the finished product so that it exerts the expected beneficial effects on the immune system and the gastrointestinal tract.

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