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MICOBIOTA AND AFM1 LEVELS IN FOOD SUPPLEMENTS FOR INFANTS AND SENIORS

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Abstract – This study aimed to show the presence of fungi in powder supplements for infants and for seniors, comparing counts as well as aflatoxin M1 levels. The overall average of fungal count and variety was high. As for aflatoxin M1 levels, approximately 11.6% samples were above the European Union limits. One sample was above United States limits. Data show the persistence and viability of fungi even after the manufacturing process. It highlights the importance of good manufacturing practices and storage conditions, by both the industry and by the consumers. It also can be considered a potential health risk due the inherent fragility of the target consumers. More studies are suggested to better understand fungi growth and mycotoxin production in such matrix.

INTRODUCTION

Aflatoxins are metabolites produced by the fungi genus *Aspergillus*. It is commonly associated with crops, compromising livestock and food production worldwide. Animal products can also be compromised, such is the case of Aflatoxin M1, found in milk (Marchese, 2018; WHO, 2018).

Humans are gravely affected by aflatoxins, with a 2004 outbreak resulting in a death rate of 39% (CDC, 2004). Long term exposure can be associated with liver cancer and immunosuppression, with the potential for birth defects and stunted growth in children (WHO, 2018). Such exposure can be intrinsically related to a person's diet, which is influenced by cost, lifestyle, environment and culture. Such factors are not stable and can make a person's diet change over time (WHO, 2003).

The consumption of powdered supplement formulas can be a risk considering the amount of ingredients, both plant and animal based. The long shelf life can also allow for fungi growth and for aflatoxins to be produced. According to Neves *et al.*, (2020) there seems to be an increase in consumption of infant formulas, starting with the rich and eventually reaching low-income families. This increase reflects a higher number of infants exposed.

No data is available for the consumption of supplements for seniors. However, reconstituted powdered feeds can be used in hospitalized patients, even the ones in critical care (ICSI, 2012).

Microbiological standards vary for infant formulas. There is a focus on bacteria such as *Salmonella* spp., *C. sakazakii* (Food and Drug Administration, 2019), *Bacillus cereus* and enterobacteriaceas (European Union, 2005) but no standards for fungi counts. Supplements for seniors have no standards whatsoever.

Such discrepancies, as well as the fragility of the targeted market reinforce the need to assert the risk the consumer is exposed, unify the regulations available as well as establish new ones.

METHODOLOGY

Sampling

A total of 86 samples were used, all presented as powder. Of those 18 were infant formulas (sample code IN) and 64 were supplements for seniors

(sample code SE). Brands and batch distribution are described on Table 1.

Table 1. Infant and senior brands and batches used in the study

Type of supplement	Brand	Number of batches
Infant	IN 1	6
	IN 2	7
	IN 3	4
	IN 4	2
	IN 5	1
	IN 6	1
	IN 7	1
Senior	SE1	16
	SE2	16
	SE3	16
	SE4	16

Whey protein is the main source of protein in most brands. Other common ingredients include vitamins (A, C, D, E and B complex), calcium, iron, manganese and plant-based ingredients, like soy lecitin and oils. Caloric intake varied from 67 to 164kcal per portion. Most infant formulas required the use of heated water for preparation. Sugar intake was only listed by one brand (SE2). Cans selected were in proper conditions, with all samples collected in triplicate. Analyses were performed immediately at the arrival of the sample with subsequent storage (-5° C).

Label information varied or was absent. Information about storage was dissonant among infant formulas, with 42.8% not specifying dry storage. Time for consumption, once the package was opened, had an average of 4 weeks, with hot water suggested for most preparations. Supplements for seniors presented the opposite problem, with all brands recommending dry storage but with a time for consumption varying from 4 to 7 weeks, an average of 5.5 weeks. The use of hot water was never suggested.

Most supplements (approximately 72.7%) did not inform an estimated number of portions per package and none left clear the amount of sugar per portion.

Analysis

Fungi counts were made using dichloran rose bengal chloramphenicol agar (DRBC) (Abarca *et al.*, 1994) for estimation of total culturable micoflora and dichloran 18% glycerol agar (DG18) (Pitt and Hocking, 1997) for xerophilic fungi. Nonspecific filamentous genus, *Aspergillus* sp., *Pencillium* sp. and *Fusarium* sp. species were identified, respectively, according to Samson *et al.* (2004), Klich(2002), Pitt and Hocking (1997) and Nelson *et al.* (1983).

Aflatoxin extraction method was based on modified QuEChERS, following methodology described in by the Association of Official Analytical Chemists International (2007). Screening was performed using AFM1 commercial kits (Aflatest®, Vicam, Watertown, MA, USA), with quantification and analysis done with VICAM® Series-4EX fluorimeter (Watertown, MA, USA) and HPLC quantification. The limits of detection (LOD) and quantification (LOQ) were found by the variation of concentrations of the standard solution and subjected to extraction and quantification until the lowest detectable concentration (LOD) and the lowest quantifiable concentration (LOQ) under suitable conditions of repeatability (n = 5, RSD <15%). The detection and quantification limits were 0.013 μg kg^{-1} and 0.055 μg kg^{-1}, respectively.

Statistics

Data analysis was performed by ANOVA. Pearson's correlation and T-test were used to compare the enumeration data of the different microorganisms in the various dairy supplements. Pearson test also compared contamination data by mycotoxins, in the different formulations and comparisons between the times. The analyses were conducted using PROC GLM software in SAS (SAS Institute, Cary, NC).

RESULTS

The different CV obtained in fungi counts show heterogeneity between groups of data obtained. Filamentous fungi counts, made in DRBC agar, varied from 1.00×10^2 to 9.00×10^3 CFU/g in SE samples and from 1.00×10 to 5.00×10^3 CFU/g in IN samples, with a CV of 1.43 and 1.36, respectively. Both can be considered homogeneous due to the low CV. Filamentous fungi are plant pathogens, thus associated with plant base ingredients. They can also be associated to the production of mycotoxins.

The DG18 agar, used for xerophilic fungi counts, had a higher coefficient of variation in supplement for seniors (2.12), indicating a high dispersion of data. There was also a big difference between minimum and maximum scores (1.00×10^2 to 2.00×10^4 CFU/g respectively). Such high levels were not mirrored by infant formulas, which varied from 1.00

x 10^2 to 7.20 x 10^4 CFU/g, with a CV of 0.53.

Fungi counts are described on more detail on Table 2.

Table 2. Fungi counts in DRBC and DG18

Brand	DRBC (CFU/gram)	DG 18 (CFU/gram)
IN1	1.28 x 10^{3a}	5.37 x 10^{3a}
IN2	1.05 x 10^{2a}	1.80 x 10^{4a}
IN3	2.85 x 10^{2a}	1.01 x 10^{3a}
IN4	7.50 x 10^{2a}	1.10 x 10^{3a}
IN5	4.10 x 10^{2a}	7.20 x 10^{4a}
IN6	4.20 x 10^{2a}	1.00 x 10^{3a}
IN7	4.20 x 10^{2a}	1.00 x 10^{2a}
Average	5.24 x 10^2	1.4 x 10^4
SE1	2.99 x 10^{3a}	1.25 x 10^{3a}
SE2	1.40 x 10^{3b}	3.30 x10^{3a}
SE3	1.30 x 10^{3b}	3.26 x 10^{3a}
SE4	2.99 x 10^{2b}	4.35x 10^{2a}
Average	1.50 x 10^3	2.06 x 10^3

[a,b] Means with the same letter in column are equivalent in accordance with ANOVA associated with Tukey and Pearson correlation (P ≤ 0.005).
[c] Technical Limit of Detection: ≤ 1.0 X 10^2 CFU/gram.

In addition to the high counts, a wide variety of fungi genus was isolated as shown on table 3. Predominant species included *A. flavus*, *A. fumigatus*, *A. ochracius*, *A. oryzae*, *A. parasiticus* and *A. niger*, *P. citrinum*, *P. citronigrum* and *F. verticillioides, F.solani* and *F. chlamydosporum*.

For AFM1 detection the Limit of Detection (LOD) used was ≤ 0.013 μg/kg and the Limit for Quantification (LOQ) was 0.055 μg/kg, with most AFM1 levels below one or both limits. There were also few samples above the maximum levels of AFM1 in milk and milk products determined by the European Union (EU) (0.05 μg/kg), United States (US) and Brazil (0.5 μg/kg both) (Food and Agriculture Organization, 2004; Brazil, 2011).

Table 3 Fungi frequency of identified genera

Fungi Genus	Frequency (%)	
	IF[a]	SE[b]
Aspergillus sp.	31.09	50.92
Eurotium sp.	23.52	7.97
Penicillium sp.	18.48	16.56
Cladosporium sp.	7.56	6.13
Mucor sp.	6.72	1.84
Fusarium sp.	10.08	13.49
Curvularia sp.	-	1.22
Alternaria sp.	2.52	1.84
Total	100.0	100.0

[a] Total of isolates = 119
[b] Total of isolates = 163

For infant formulas, one sample was above EU limits (0.39 μg/kg) and another above all limits considered in this study (0.59 μg/kg). Supplements for seniors had nine samples considered above the EU limits but no other limits were surpassed.

DISCUSSION

Beuchat *et al.* (2013)considers rehydration a key factor for bacterial growth in low water activity foods. Fungi could also benefit from the increase in water absorption, with Astoreca *et al.* (2012) considering water activity (aw) below 0.84 enough to allow fungi growth in 24-48 hours. Bearing in mind the time for optimal consumption of the supplement packages (averages of 4 and 5.5 weeks for infant formulas and supplements for seniors, respectively), it is possible to assume both rehydration and fungi growth could occur, especially under inadequate storage conditions. The increase of fungi growth also increases the chance of exposure to mycotoxins, which are detrimental to the consumer's health.

The use of heated water should also be highlighted. While recommended in infant formula labels, with studies showing cell damage and inactivation (Kim and Park, 2017; Chen *et al.,* 2009; Osaili *et al.,* 2009). The same is not proposed for senior supplements despite the similarities of both products. If such step was used, similar results would most likely be observed.

There are no official limits for fungi counts in infant formulas or senior supplements, therefore it was used the limit proposed by USP (2015) of 10 CFU/g for food supplements in general. By that standard most samples were above the limit and should not be considered acceptable for consumption. Santos *et al.* (2015) attributed the contamination of this type of product to the inferior quality of the raw materials that make up the final product, as well as failures in the processing during its production and filling in the factory. The high counts, as well as the variety of fungi found, are justified by the diversity of ingredients present in food supplements, each subject to contamination during production, storage, and transportation processes.

The capacity to absorb water from the environment during both production and consumption is also a facilitating factor, especially in

powdered foods, especially foods that are rich in protein and carbohydrates(dos Santos *et al.*, 2015; Moura *et al.*, 2014).

Perhaps more worrying than fungi counts is the toxigenic potential of some strains. Sibaja *et al.* (2019) found AFM1 in all milk powder samples of their study, half above EU established limits, proving not only the presence of AFM1 but the possibility of it to remain even after manufacture. While this study had fewer samples above the same limit (around 13.4%), it doesn't diminish the risk to which infants and seniors are exposed. Long term exposure to aflatoxins can be associated with liver cancer and immunosuppression, with AFM1 specifically being associated with liver and bile duct cancer (WHO, 2018), International Agency for Research on Cancer 2019), conditions that could be devastating to the already fragile target population.

There are ingrained risks for both infants and seniors. The presence of fungi growth and AFM1 production bring to light concerns for storage conditions, be it industrial or domestic. More studies are suggested to better understand and quantify the risks, preserving the health of infants and seniors.

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