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# BACTERIOLOGICAL AND MYCOLOGICAL OCCURRENCE IN FOOD SUPPLEMENTS FOR SENIORS

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## ABSTRACT

Food supplements are a beneficial source of nutrients. Designed for senior consumers, it offers a practical and easy way to supplement dietary needs even for hospitalized patients. However, due the immunocompromised nature of seniors, it is important to provide supplements that will not compromise their health further. Few studies explore the contaminants such matrix could carry and there are no microbiological standards to be taken into consideration. The aim of this study was to better characterize the sanitary quality of supplements through bacterial research and counts, determination of bacterial resistance to antibiotics, fungi counts and mycotoxicological profile of fungi´s species isolated.



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# INTRODUCTION

Supplements can be utilized in delicate and specific situations; such is the therapy of hospitalized patients. However, it can also offer risks. There is the chance of interaction with medicine and with other supplements, leading to adverse reactions. Excessive consumption of supplements for a long time can be prejudicial as well. It is recommended to notify a health care professional before introducing food supplements into a diet (FDA, 2019; OLIVEIRA, 2013). Other possible complications from food supplements are microbiological contaminations. Fungi are deeply related to the environment, being able to compromise both the food quality and the consumer's health (BENEDICT, CHILLER and MODY, 2016). Mycotoxins can also cause damage, with long term exposure to aflatoxins being associated with liver cancer and immunosuppression, AFM1 specifically being associated with liver and bile duct cancer (WHO, 2018; INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, 2019). There is also the bacteriological risk, such is the one presented by *Cronobacter sakazakii*, an opportunistic and emergent pathogen, usually associated with infant powder formulas, which can also cause infections in elderly people (FAKRUDDIN et al., 2013). However, there are no microbiological standards for dietary supplements for seniors. Such lack of standards led to the use of recommended analyses for similar products such as those described by United State Pharmacopoeia (USP) (fungi count and research for *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*) and by the Brazilian National Health Surveillance Agency (ANVISA) (counts for fecal coliforms, coagulase-positive *staphylococcus* and *Bacillus cereus*, plus research for *Salmonella* spp.) (BRAZIL, 2001; USP, 2013a; USP, 2013b).

# MATERIALS AND METHODS

Thirty-two samples of powder food supplements specific for seniors were collected for the study, all commercialized in municipal pharmacies of Rio de Janeiro and Niteroi city (RJ state) and São Paulo city (SP state). The samples belonged to four brands (X, Y, Z and W) with eight lots each. All cans were selected in perfect conditions and within valid commercial dates. Samples collected in triplicate and opened in a chamber with biological control (laminar flow chamber). All analyzes were performed immediately with the arrival of the sample in order to avoid cross contamination and later stored refrigerated (-5° C). Water activity (Wa) was measured using

the infrared method through AquaLab® cx 2 model equipment (Decagon Devices Inc. USA). Each sample was measured three, totaling ninety-six readings. Bacterial analyzes followed the Normative Instruction No. 62 of Brazilian Ministry of Agriculture, Livestock and Supply - MAPA (BRAZIL, 2003a) for standards of *Bacillus cereus* count, coagulase-positive *staphylococcus* spp. and research of *Salmonella* spp. While the total coliform and lactic acid bacteria counts followed the methodology described in *Standard Methods of the Examination of Water and Waste Water* (APHA, 1995). For research of *Cronobacter sakazakii* was used to the ISO / TS 22964 modified method (ISO, 2006). Sensitivity evaluation for antibiotics was performed following the *Clinical and Laboratory Standards Institute* (CLSI, 2012).

For the enumeration of the genus *Enterococcus* sp. the method described by the Merck Manual (2002) was used. Quantitative enumeration of fungal propagules was done using the solid mediums: dichloran rose bengal chloramphenicol agar (DRBC) (ABARCA et al., 1994) for estimation of total culturable micoflora; dichloran 18% glycerol agar (DG18) (PITT; HOCKING, 1998) for xerophilic fungi; and dichloran chloramphenicol peptone agar (DCPA) (ANDREWS AND PITT, 1986) for enumeration of *Fusarium* species. Only plates containing 10 – 100 CFU were used for counting. Representative colonies were transferred for sub culturing to tubes with malt extract agar (MEA). Nonspecific filamentous genus *Aspergillus* sp., *Pencillium* sp. and *Fusarium* sp. species were identified, respectively, according to Samson et al. (2008), Klich (2002), Pitt; Hocking (1998) and Nelson et al. (1983). Characterization of toxigenic capacity of isolated species (mycotoxin production) were evaluated by the different species being carried out the procedures according to the techniques proposed by Lin and Dianese (1976), Armando et al. (2012), Geisen (1996) and Santos et al. (2002), where selective culture in specific media allowed extraction evaluation of mycotoxin levels produced by different strains. Data analyzes were performed with transformed logarithmic function log10 (x + 1) and variance analysis (ANOVA). Duncan's test was used for comparison of enumeration data in different fungal culture media and Fisher's LSD test was chosen for the comparison of measurement data of mycotoxins. Analyses were conducted using the software PROC GLM in SAS (SAS Institute, Cary, NC).

# RESULTS AND DISCUSSION

Water activity reading provided an overall average of 0.47 ± 0.15, with maximum and minimum value of 0.84 and 0.20 respectively. Beuchat et al. (2013) highlight the possibility of microbial growth after rehydration, a fact that should be considered in the consumption of dietary supplements considering the lack of heating after preparation and the fact the product will not be consumed in a single time, allowing the remaining product to absorb moisture from the air. Fungal counts showed high coefficient of variation (CV ≥0.33), which suggests there are suitable conditions for maintenance of fungal conidia. There is also a potential risk for contamination by fungi sprouting process in this substrate, due to the hygroscopic potential of the matrix.

Astoreca et al. (2012) used modeling to describe the development variation of *Aspergillus* spp. based on Wa and temperature. Values inferior to 0.77 e 27° C, respectively are considered the ideal conditions for *Aspergillus* spp. However, Wa below 0.84, such as found in some samples, can allow fungi to grow in a 24-48 hours period, which is far superior to the time the consumers take to finish one package of supplements. In Brazil, during the realization of this study, variations in temperature between 27.5° to 37.5° C were observed, as well as humidity levels above 60% (BRAZIL, 2016b). Those variations provide a conducive environment for fungal growth, which is also benefited from the heterogeneity of the substrate and the ability to absorb moisture from the environment, exposing consumers to both fungi intake and mycotoxins. Long term consumption could increase the risk to the senior population.

**Table 1. Fungi count (log10 CFU / g) in the Dichloran Rose Bengal Chloramphenicol agar (DRBC), Yeast Peptone Dextrose agar (YPD), Glycerin Dichloran agar (DG18) and Nash-Snyder agar (NSA) in food supplements samples for elderly**

| Marcas | DRBC | YPD | DG 18 | NSA |
|---|---|---|---|---|
| X | 4.06[a] | 4.15[a] | 3.82[a] | 2.75[a] |
| Y | 3.41[b] | 3.65[b] | 3.58[a] | 3.03[a] |
| Z | 3.30[b] | 3.45[b] | 3.58[a] | 3.12[a] |
| W | 3.29[b] | 3.53[b] | 3.54[a] | 3.41[a] |
| Média | 3.65 | 3.79 | 3.65 | 3.14 |

* Means with the same letter in column are equivalent in accordance with Duncan test ($P \leq 0.005$).
** Technical Limit of Detection: ≤ 1.0 log10 CFU /g.

**Table 2. Frequency of fungal strains of the genera found in samples of supplements for seniors**

| Fungi Genus | Quantitative strains CFU.$^{g-1}$ | Frequency (%) |
|---|---|---|
| Quantitative Fungal Evaluated in the Samples | | |
| *Aspergillus* sp. | 63 | 50.00 |
| *Eurotium* sp. | 13 | 10.36 |
| *Penicillium* sp. | 23 | 18.26 |
| *Cladosporium* sp. | 8 | 6.34 |
| *Mucor* sp. | 2 | 15.87 |
| *Fusarium* sp. | 12 | 9.81 |
| *Curvularia* sp. | 2 | 1.57 |
| *Alternaria* sp. | 3 | 2.38 |
| Total | 126 | 100.0 |

As for bacterial analyses, *B. cereus*, *C. sakazakii*, *E. coli*, *Salmonella spp*. and *S. aureus* were not found in any of the samples. Total coliforms were isolated in only two of the 32 samples analyzed, from brand Y and W. Using the table for Most Probable Number (Número Mais Próvável or NMP in Portuguese) the results were 3.0 coliforms/g and 3.6 coliforms/g, respectively. There are no standards for these bacteria in supplements in any of the references used in this study. However, there is a standard for fecal coliforms by RDC nº 12 of ANVISA (10 coliforms/g or ml), which would enable both samples for consumption (BRAZIL, 2001). These results are not consistent with the exposed by Cortez et al. (2013), who found coliforms in all evaluated whey samples, with the presence of *E. coli* in 30% of them. It is possible that the difference in counts is due the difference of materials analyzed since Cortez et al. (2013) chose the serum of fresh milk for their study. The drying process, which subjected the whey, for the production of protein powder may influence the final bacterial load. It was observed growth of lactic acid bacteria in just two samples, from brands X and W, both outcomes below 3.0 CFU/g. Considering probiotics were not listed as ingredients in any brand, there is the possibility of the lactic acid bacteria found have not been added in order to benefit the production of supplements but be a result of contamination. The absence of a standard for the count of lactic acid bacteria in food supplements for the elderly prevents the final classification of the brands analyzed. However, because the counts found were very low and define only 6.25% of the samples, it is thought that all would be considered suitable for human consumption. There are no standards for *Enterococcus* spp. counts in any of the consulted bibliography, therefore there is no way to define if the samples tested would be considered adequate for human consumption or not. However, it is possible to draw some conclusions based on the data obtained.

Brand X showed no contamination by *Enterococcus* spp., which characterizes it as the best from a hygienic-sanitary point of view regarding this genus. Considering the target population of the supplements studied, this brand would be the only one without risk to consumer's health. Brand Y showed lower contamination, where only two out of the eight samples had contamination by *Enterococcus* spp. (4.6 and 9.2 CFU/g). The heterogeneity of the samples can be observed by the following: Standard Deviation (SD) of 3.42 and Coefficient of Variation (CV) of 1.98. Despite the lack of microbiological standards, when compared with the provided by

ANVISA as the maximum allowed for fecal coliforms ($1.0 \times 10^1$ CFU/g), and the maximum allowed for coagulase-positive staphylococci and *Bacillus cereus* (both with limit of $5.0 \times 10^2$ CFU/g) (BRAZIL, 2001), it is arguable that the samples of brand Y would also be classified as fit for consumption. Brand W, despite having an average higher than brands X and Y also have the majority of their samples approved for human consumption, since five out of the eight samples tested negative for *Enterococcus* spp. and two of which were within the limits considered in the analysis of brand Y (1.5 and 4.6 CFU/g.). Only one sample could be regarded as unfit for use, since the count *Enterococcus* spp. was 120 CFU/g. It was also observed in brand W a heterogeneity of data due to high DP (42.14) and CV (2.67). Alas, the greatest risk to the population was observed in brand Z, where all eight samples presented high levels of *Enterococcus* spp., with the lowest score found 240 CFU/g and the highest 655 CFU/g.

This risk confirmed by the low coefficient of variation (CV ≤0.41), which demonstrates the homogeneous distribution of the counts. Of the strains obtained in all samples, most were identified as *E. faecalis* (96%), with the rest identified as *E. faecium* (4%). The presence of *Enterococcus* spp. can be explained by the presence in the human gastrointestinal tract, indicating contamination during handling of food supplements (KAARME et al., 2014). These data were confirmed by Soares-Santos Barreto and Semedo-Lemsaddek (2015), who reported the presence of *Enterococcus* spp. widespread both in production environments and marketing of food, *E. faecalis* being the most common (about 67%) but also reporting the presence of *E faecium* (approximately 9.8%). The authors also pointed out the need to distinguish the technological use of *Enterococcus* spp. from pathogenic variants. The high counts of *Enterococcus* spp. found, as well as the identification of the pathogenic species *E. faecalis* and *E. faecium*, are a hazard to the consumer and should not be present in foods and food supplements for the elderly. The fact that probiotic cultures were not listed as ingredients in any of the brands analyzed only reinforces the idea that such bacteria are of contaminant source, not being deployed as a benefit for the product but a hygienic and sanitary failure. Regardless of the absence of established standards, *Enterococcus* spp. counts. found should not be permitted in the final product.

As for antibiotic resistance, the follow substances were used: ampicillin, penicillin, vancomycin, teicoplanin and chloramphenicol. Only the strains of *Enterococcus* spp. were tested. Chloramphenicol was included due to the prohibition of its use in veterinary products and other products used in animal feed in Brazil since 2003 (BRAZIL, 2003b). Only two strains (approximately 9%) of *E. faecalis* were considered to have an intermediate degree of resistance to vancomycin but all strains were considered sensitive to the other antibiotics. The *E. faecium* strain was sensitive to all antibiotics used. These results are consistent with those reported by Kaarme et al. (2014) who described the absence of *Enterococcus* spp. vancomycin resistant. Soares-Santos, Barreto and Semedo-Lemsaddek (2015) also reported that low resistance to vancomycin (1%), and sensitivity of all isolates from the study to ampicillin and teicoplanin. However, Soares-Santos Barreto and Semedo-Lemsaddek (2015) also reported resistance from some isolated to penicillin G (6%) and chloramphenicol (4%), data that were not compatible with those obtained in the present study. As for fungi counts, it was observed the following minimum and maximum variations in the counts for the proposed incubation schemes: DRBC (2.00 to 4.86 CFU/g); DG18 (1.88 to 4.40 CFU/g); YPD (2.00 to 4.90 CFU/g); DCPA (2.0 to 3.90 CFU/g).

The average and SD of the samples are shown in Table 1, where it is observed statistically significant differences between the brands and lots, which amounted to total sample ($P \leq 0.005$). The overall average of fungal count was high, compared to the average of some bacterial counts. Coefficient of variation obtained were different, indicating variation in homogeneity between the groups of data obtained. Counts made in DCPA (CV ≤0.04) considered homogeneous due to the low coefficient of variation, which is confirmed by the low number of *Fusarium* spp. isolates the intended medium. It is important to note these genera are plant pathogens of excellence, thus the addition of vegetal compounds is the possible way of contamination by this genre. The culture medium used for filamentous fungi total count (DRBC) and the one used for yeast count (YPD) present variation coefficients slightly higher (CV ≤0.07 and ≤0.08 respectively), indicating a higher scattering of data in relation to the DCPA and DG18. This dispersion explained by the wide variation in counts obtained, where minimum values are inferior to even half of the maximum values. Finally, DG18 agar for xerophilic fungi showed the highest coefficient of variation (CV ≥0.13), indicating dispersion of counts. Again, there was a big difference between minimum and maximum counts (1.88 and 4.40 CFU/g respectively), confirming the wide dispersion of data. The USP determines the maximum fungi count allowed for food supplements in general as 10 CFU/g. As a benchmark, the MAPA in their legislation to control contaminants in products intended for animal nutrition, refuses products with fungi counts equal or greater than $1.0 \times 10^4$ CFU/g. Therefore, some samples would not be considered acceptable even for animal consumption in Brazil according to order no. 709/11 1988 revoked for update by Normative Instruction No. 30 of August 5, 2009 (BRAZIL, 2009). In addition to the high counts it was also found a wide variety of fungi. Were isolated the genus *Alternaria*, *Aspergillus*, *Cladosporum*, *Eurotium*, *Fusarium* and *Penicillium*. Among the *Aspergillus* genus were found the species *A. flavus*, *A. fumigatus*, *A. ocracius*, *A. oryzae*, *A. parasiticus* and *A. niger*, among the *Penicillium* genus, species *P. citrinum*, *P. citronigrum* and *P. clavatus*, and the *Fusarium* species *F. verticillioides* and *F. chlmydosporum*.

High CFU counts as well as the variability of genres and species can be justified by the variety of ingredients present in food supplements for seniors, each vulnerable to contamination during production, storage and transportation. The easiness in which fungi grow on foods with low aw, spore production and the ability of the powder supplements to reabsorb moisture from the environment are also very important factors, especially when considering the population group, the food supplements, analyzed are destined to and the absence of heat treatment after rehydration of the product. The major yeasts isolated during this study were identified as *Saccharomyces cerevisiae*, however it is not possible to relate their presence to the addition of probiotic cultures in supplements since they are not listed as ingredients. Therefore, the presence of yeasts considered a result contamination of the product and not something beneficial for the processing. It is noteworthy that the fungal contamination of all samples was above recommendations and both national and international regulations (USP, 2013th, GMP, 2008), which may cause harm to consumers. Toxigenic capacity of strains was observed in *A. flavus*, *A. fumigatus*, *A. parasiticus* and *P. citrinum*. It was observed the following percentage frequency of isolates, respectively, for each fungal genus: 30%, 12%, 8% and 50%. Considering these data, a significant toxigenic potential from the strains, which characterizes potential risk since, with in the right conditions, they may grow and produce toxin at 24 hours as proposed Astoreca et al. (2009). All strains isolated during this period were tested for the ability of aflatoxin production (at levels from 1 to 20 µg/kg), gliotoxin (at levels from 0.1 to 0.5 µg/g) and citrinin (1 levels in 2 µg/g). In *F. verticilloides* strains not identified significant levels of fumonisin in the capacity tests. The effects of ingestion of mycotoxins can cause chronic infections and generally has no specific clinical symptoms for most of these dosages. Mycotoxicosis rarely manifest as acute illness due the fact intoxications are closely linked to the dose and time consumption of each mycotoxin (DILKIN, 2010).

## CONCLUSION

In relation to bacteriological analysis, all supplement brands analyzed showed results consistent with the standards established by Brazilian legislation and the stipulated by USP. It wasn't observed the presence of resistant or multi resistant bacteria in the samples. Fungal counts were at odds with the proposed laws (APHA, 1995; USP, 2013a & 2013b), and can compromise the consumers' health. Further studies

are recommended to better understand the food matrix and its contaminants.

**ABBREVIATIONS**

IARC - International Agency For Research On Cancer
ANVISA - Brazilian National Health Surveillance Agency
USP - United State Pharmacopoeia
MAPA - Brazilian Ministry of Agriculture, Livestock and Supply
DRBC - Dichloran Rose Bengal Chloramphenicol Agar
DG18 - Dichloran 18% Glycerol Agar
DCPA - Dichloran Chloramphenicol Peptone Agar
MEA - Malt Extract Agar

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