ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 11, Issue, 09, pp. 49962-49966, September, 2021
https://doi.org/10.37118/ijdr.22843.09.2021

RESEARCH ARTICLE OPEN ACCESS

MICROBIOTA CHARACTERIZATION AND AFLATOXIN M1 DETECTION IN NEWBORNS FOOD SUPPLEMENTS FOR USE IN NEONATAL UNIT

Victor M. Farias[1],*, Beatriz C. S. F. Pereira[1], Leonardo A. Pinto[1], Robson M. Franco[1] and Luiz A. M. Keller[2]

[1]Department of Food Technology of Veterinary Medicine School, Federal Fluminense University, Avenida Almirante Ary Parreiras, 507, Niterói, RJ, Brasil.24220-000; [2]Department of Animal Science and Sustainable Agro-socio-environmental Development of Veterinary Medicine School, Federal Fluminense University, Avenida Almirante Ary Parreiras, 507, Niterói, RJ, Brasil. 24220-000.

ARTICLE INFO

Article History:
Received 20th June, 2021
Received in revised form
29th July, 2021
Accepted 03rd August, 2021
Published online 27th September, 2021

Key Words:
Food safety, Fungi,
Infant formula, Mycotoxins.

***Corresponding author:**
Victor M. Farias

ABSTRACT

Nowadays the use of dietary supplements was increasing due to changes in consumption habits. Modern society has been inducing a decrease in breastfeeding habits. These dietary changes that included supplements for newborns and infants were a demand to sustain the nutritional necessities of breastfeeding. Thus, it has become increasingly necessary to include dietary supplements in the diet of children, especially newborns. Considering the fragility of the target population, it is important to conduct studies to monitor the possible microbiological and toxicological hazards to which consumers are exposed, especially newborns in intensive care units. Microbiological analyzes were performed as described in the APHA manual and mycotoxin concentrations were detected by fluorimetry associated with immunoenzymatic methods. Analyzes revealed average values of (CFU g^{-1}): 3.54 x 10^3 for aerobic mesophiles; 3.89 x 10^1 for Staphylococcus positive coagulase; 8.14 x 10^2 and 2.34 x 10^3 for filamentous and xerophilic fungi, respectively. However, the analyzed samples presented counting values and mycotoxins levels within the recommended by the legislation, thus conforming and safe for consumption. The count values obtained in this study are a reflection of failures during product processing, choice of raw material, and handling during its reconstitution.

Citation: ***Victor M. Farias, Beatriz C. S. F. Pereira, Leonardo A. Pinto, Robson M. Franco and Luiz A. M. Keller.*** "Microbiota characterization and aflatoxin m1 detection in newborns food supplements for use in neonatal unit*", International Journal of Development Research*, 11, (09), 49962-49966.

INTRODUCTION

The international agencies encourage breastfeeding in the first months of life and do not recommend the use of dietary supplements, except for medical reasons. The importance of breastfeeding for newborns is associate with growth and development, induce passive immunity, and the development of nervous system in emotional and cognitive capacities (WHO, 2017). Oftentimes, breastfeeding is not enough to provide the nutrients a newborn needs, being necessary the use of supplements as the infant formulas., especially in the first few days. The Brazilian legislation describes infant formulas as products, either in liquid or powder form, used for newborn supplementation, constituting the main liquid element of a progressively diversified diet (ANVISA, 2011a).

The Brazilian legislation also describes the maximum limits of mesophilic bacteria, Enterobacteriaceae, *Bacillus cereus,* and *Salmonella*spp (ANVISA, 2019). The standard required by the Food and Drug Administration (FDA) for infant formula is the absence of *Salmonella* spp. and*Cronobatersakazakii* (FDA, 2018), and the European Union it is established the limits for *B. cereus, Salmonella* spp., *C. sakazakii,* and Enterobacteriaceae (EC, 2005). In these three regulations, the microbiological contaminants parameters are similar. Fungi were associated with the environmental microbiological contaminant, being a potential risk in food matrix and compromising the consumer's health. However, the limits of fungi presence in infant formulas are not present in any of the technical regulations cited. The United States Pharmacopeia (USP) has a standard for the presence of fungi in food supplements that is no more than 10^2 CFU g^{-1} or mL^{-1} (USP, 2012). For comparison purposes, this parameter was used during this study.

Mycotoxins are metabolites produced by filamentous fungi under certain conditions, during any stage of the production, storage, and processing. Mycotoxins in infant formulas are associated with the genera *Aspergillus*, *Fusarium,* and *Penicillium*. Even if they do not cause acute conditions, prolonged ingestion is related to chronic complications (PITT, 2000). Dairy products are the major ingredients in infant formulas associated with Aflatoxin M1 (AFM1) contamination. The maximum limits for AFM1 in dairy products are described in *Codex Alimentarius*, in which each country presents guidelines based on it (CODEX, 2013). Brazilian legislation contains permitted values for milk powder, including the infant formulas (5.0µg L^{-1}) and, due to the use of components of plant origin in the formulation, the values for other types of aflatoxins are also described (ANVISA, 2011b). Considering the fragility of the target population, monitoring studies are necessary to know and evaluate safety limits. This study aims to evaluate the microbiological and mycotoxicological quality of infant formulas offered in lactary units, providing the community with important data about these products.

MATERIAL AND METHODS

Obtaining samples: Samples of infant formula for early childhood were given by the milk bank of the Hospital Universitário Antonio Pedro (HUAP). A total of 36 samples were analyzed, being a total of six batches of two market-recognized brands, assessed on three forms: formula in solid form, collected at the time of opening of the primary packaging (T1); reconstituted liquid form at the time of sample reconstitution (T2) and the reconstituted liquid form after 24 hours of refrigeration maintenance (T3). The samples were evaluated in duplicates. The solid samples (T1) were collected in sterile centrifuge tubes that were opened at the time of collection and the other samples (T2 and T3) in autoclaved sterilized nipple bottles used by the lactary to offer infant formulas, as well as the other utensils used in the handling and preparation of the products. The samples were prepared following HUAP's operational handling protocols to be able to correctly evaluate the quality of the products offered to patients. The T3 sample was kept refrigerated in the lactary unit itself to evaluate the sample behavior in the unit's storage conditions. The samples were transported to the Laboratório de Controle Microbiológico de Produtos de Origem Animal at Faculdade de Veterinária of Federal FluminenseUniversiy, where the bacteriological analysis were performed. Fungi counting, toxins extraction and detection and identification of toxins were performed at the Centro Estadual de Qualidade de Alimentos (CEPQA) at PESAGRO-RJ.

Bacteriological Analyzes: In this work, it was performed the counts of total mesophilic bacteria, *Bacillus cereus*, *Staphylococcus* positive coagulase, lactic acid bacteria, total and thermotolerant coliforms, Enterobacteriaceae counts, and *Salmonella* spp. identification. The inclusion of missing microorganisms in Brazilian standardswas decided in awareness of risks that may cause to newborns. Analysis were performed according to the methodologies described in the *Compendium of Methods for the Microbiological Examination of Foods* (APHA, 2015). For the enumeration of the genus *Enterococcus*, the methodology was according to MERCK (2002) and the research of *Cronobactersakazakii* by ISO/TS 22964 method (2006).

Mycological Analyzes: For the fungi count, the serial decimal dilution in plates was performed with inoculation of 0.1mL aliquots of each dilution in two culture media: Dichloran Glycerol Agar (DG18) for xerophilic fungi and Dichloran Bengal Rose Cloranphenicol Agar (DRBC) to estimate the total fungi (PITT and HOCKING, 2009). The plates were incubated at 25°C for seven days. The analysis were performed in analytical duplicates.

AFM1 Detection and Quantification: Samples were extracted using the modified QuEChERS based extraction method following the methodology described in the Association of Official Analytical Chemists (AOAC) Official Methods Manual (AOAC, 2007).

All extractions were performed in duplicates. Sample screening was performed using commercial immunoenzymatic kits for AFM1 (Aflatest®, Vicam, Watertown, MA, USA) following the manufacturer's instructions. Quantification and analysis were performed using a VICAM® Series-4EX fluorimeter, according to the manufacturer's guidelines (Watertown, MA, USA). AFM1 standards (5mg) were purchased from Sigma (St. Louis, MO, USA).

Statistical Analysis: Data analysis were performed by analysis of variance (ANOVA). Pearson's correlation and T-test were used to compare enumeration data of different microorganisms in various dairy supplements, as well as the Pearson test in comparing data in mycotoxin contamination in different formulations and comparisons between times. Analyzes were conducted using the computer program PROC GLM in SAS (SAS Institute, Cary, NC).

RESULTS

Microbiological counts: After evaluation were not found lactic acid bacteria, *B. cereus*, *C. sakazakii*, coliforms, Enterobacteriaceae, *Enterococcus* spp. e *Salmonella* spp in the samples. A linear correlation and Pearson correlation test were performed to evaluate the interrelationship of the counts in the three different treatments for each microorganism analyzed. The degree of linear correlation between the treatments was very different from the ideal, indicating that there was no trend in the count's ratio, being an indicator of different sources of contamination in the observed treatments. Pearson correlation did not present a significant correlation, for the 95% significance level among the values of the proposed treatments. The minimum and maximum count values, linear correlation (r^2), and Pearson correlation (P) for each analysis are described in Table 1 below. The average count values in CFU per gram of the evaluation of the three treatments proposed are presented in Table 2 below. The kinetic behavior of microorganisms found in the proposed treatments is described in Figure 1.

Analysis of Aflatoxin M1: From a total of 36 analyzed samples for AFM1 detection and quantification, all of the analyzes remained below the detection limit of the technique (LOD), below 0.013 µg kg^{-1}.

DISCUSSION

Bacterial Evaluation: As observed in table 1 and figure 1, the average value of total mesophilic count were below those described by Brazilian standard (5 x 10^2 CFU g^{-1}) (ANVISA, 2019). Despite initial contamination, the sample is safe and fit for consumption right after preparation, although these microorganisms can be potential pathogens. Beuchat et al. (2013) highlighted the possibility of microbial growth after rehydration, especially when the product is not subjected to heating after preparation and stays a long period without any refrigeration. According to literature, the addition of heated water at least 70ºC for resuspension of infant formula is capable of reducing the microbial load through cell damage and inactivation of viable cells as proven by Kim and Park (2007). This statement is evidenced in T2 where a decrease was observed before an exponential growth in T3. Despite the use of water at 70ºC, microorganism growth can be attributed to the use of improperly sanitized utensils to prepare infant formula, as evidenced by Rossi and his collaborators (2010), who observed an average of 5.24 x 10^6 CFU g^{-1} in the mesophiles count, showing the significant microbial load present in the utensils. The handler contamination during preparation should also be considered since poorly hygiene can introduce microorganisms into the products and utensils used. In this work, the statement can be attested when observing the values and the kinetic curve for *Staphylococcus* positive coagulase in table 1 and figure 1, which was analyzed due to its clinical importance, although it is no longer present in the current legislation (ANVISA, 2019). At the first point analyzed (T1), the *Staphylococcus* positive coagulase count found was above the Brazilian standard, indicating a possible risk to the consumer.

Table 1. Minimum and maximum count values and linear correlation and Pearson correlation for each microorganism analyzed

Microorganism	Minimun (CFU g^{-1})	Maximun (CFU g^{-1})	Linear correlation (r^2)	Pearson correlation (P)
PCA	$\leq 1.0 \times 10^1$	1.84×10^4	0.35	0.21
Staphylococcus positive coagulase	$\leq 1.0 \times 10^1$	2.17×10^2	0.30	0.25
DRBC	4.0×10^1	2.55×10^3	0,16	0,42
DG18	1.17×10^2	6.61×10^3	0,67	0.68

*LOD: $\leq 1.0 \times 10^1$ CFU g^{-1}; ** Pearson correlation for a 95% significance level

Table 2: Average of microbial count for mesophilic aerobic (PCA), *Staphylococcus* positive coagulase, filamentous fungi (DRBC), and xerophilic fungi (DG18) in infant formulas

Sample	PCA (CFU g^{-1})	*Staphylococcus* positive coagulase (CFU g^{-1})	DRBC (CFU g^{-1})	DG18 (CFU g^{-1})
T1	$2.52 \times 10^2 \pm 2.52 \times 10^2$ a	$1.08 \times 10^2 \pm 1.08 \times 10^2$ a	$1.39 \times 10^3 \pm 1.16 \times 10^3$ a	$3.38 \times 10^3 \pm 1.69 \times 10^3$ a
T2	$4.46 \times 10^2 \pm 4.38 \times 10^2$ b	$\leq 1.0 \times 10^1$ b	$5.17 \times 10^2 \pm 3.50 \times 10^2$ b	$2.33 \times 10^2 \pm 1.17 \times 10^2$ b
T3	$9.93 \times 10^3 \pm 8.48 \times 10^3$ c	$\leq 1.0 \times 10^1$ c	$5.33 \times 10^2 \pm 4.93 \times 10^2$ c	$3.43 \times 10^3 \pm 3.19 \times 10^3$ c

*LOD: $\leq 1.0 \times 10^1$ CFU g^{-1};**a, b e c: Averages with the same letter in columns are equivalent, according to Tukeytest ($P \leq 0.05$).

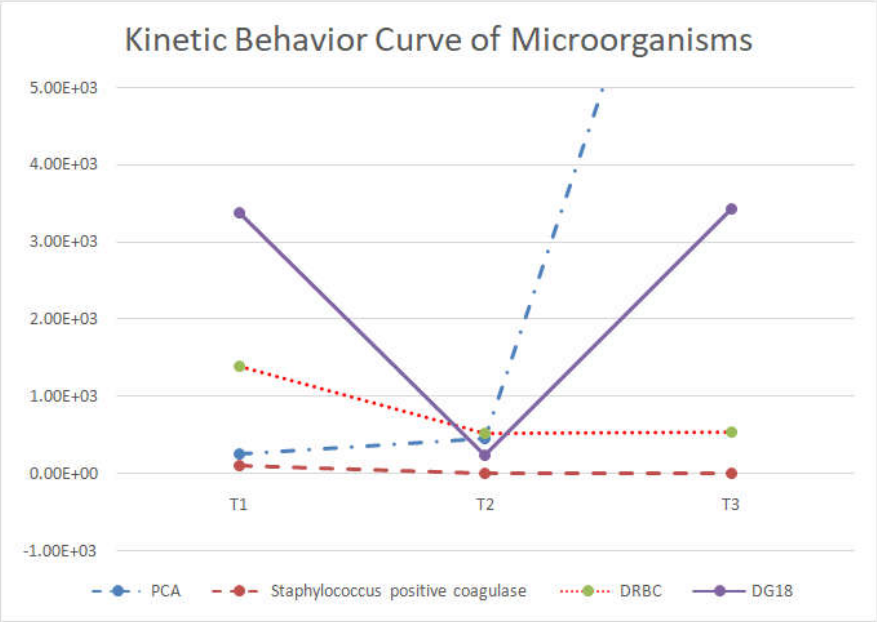

Figure 1. Kinetic behavior curve of microorganisms over the three evaluated treatments

For powdered dairy products, the Brazilian standard does not allow values above 10^1 CFU g^{-1} (ANVISA, 2019). The contamination observed at T1 occurred due to handling failures during the opening of the package as described by Nienov et al. (2009). Buchanan and Oni (2012) also pointed out that microbiota occurs only sporadically and at very low levels in infant formulas, especially because they colonize the handlers' skin, mouth, and nose. When comparing the Kinect curve for mesophilic bacteria and *Staphylococcus* positive coagulase, an opposite behavior can be observed (Figure 1) where there was a reduction in count values over time until no detection in T3 after resuspension with heated water. An important factor to be considered about these microorganisms is the presence of extreme heat-resistant strains. The presence of these strains in this type of food can be harmful, requiring greater care during handling to prevent this kind of contamination. However, they can survive heat treatment during manufacturing, the use of heated water in the resuspension is capable of inactivating injured microorganisms as described by Nema et al (2007).

Fungi evaluation: Since the packages were opened at the time of collection, the initial contamination with filamentous fungi (DRBC) at T1 may come from the raw material and handling during manufacturing (Table 1 and Figure 1). The high counts are justified by the diversity of ingredients present in food supplements, each of these ingredients is subject to contamination during production, storage, and transport (Santos et al. 2014). The addition of hot water to reconstitute the samples was enough to cause cell inactivation at T2, reducing the fungal count until the sample has adequate conditions for growth of the less affected individuals (SALOMÃO et al., 2009; GROOT et al, 2019). After 24 hours of storage, T3 presented an increase concerning T2. This increase is directly related to environmental contamination during handling, which introduced new viable cells that did not suffer cellular damage by the addition of heated water, being capable to grow during the 24 hour period of storage.

Sample handling locations are critical points to be analyzed for fungal contamination, especially in hospitals. Due to the ease of dispersal of these microorganisms and ineffective cleaning protocols, fungi can be found in closed ventilation systems being biological indicators with a maximum limit of 7.50×10^2 CFU m^{-3} (ANVISA, 2003). High values of fungal contamination were observed in hospital environments, considering the air conditioning system tray as the main source of microbial proliferation and the genera *Aspergillus* spp. and *Penicillium* spp. as the most frequent fungal contaminant, confirming the contamination found due to ineffective control of the sanitization of the air conditioning system (MOBIN and SALMITO, 2006).

Xerophilic fungi are often found in foods with a low water activity (Aw), such as powdered foods, especially foods rich in protein and carbohydrates (PITT and HOCKING, 2009). The ease of fungi to grow in foods with low Aw, the production of conidia and the ability of powdered supplements to absorb humidity from the environment are also factors to consider, especially when considering the group in which the formulas are intended (CODEX, 2008). Due to the product's characteristics, the initially isolated mycobiota came from the sample itself. So the quality of the raw materials used in its formulation should be controlled to avoid this contaminant load. The kinetic curve behavior for xerophilic fungi was the same as the filamentous fungi in T2, with a decrease of cell counts due to resuspension using water heated to 70ºC, being similar to the data described in the literature (SALOMÃO et al., 2009; GROOT et al, 2019). For the third treatment, the kinetic curve presented a more accentuated growth in comparison to the general count of filamentous fungi. Although the samples have a high fungal load on T1, most of the initial cells were inactivated at T2. Some individuals did not suffer cell damage severe enough to interrupt growth during the 24 hours of storage, which, associated with contamination during handling, led to an increase in cell count in T3. These microorganisms are extremely dangerous for hospitalized patients due to their low immunity, and more efficient cleaning protocolsare highly recommended to avoid the spread of the entire hospital.

Evaluation of mycotoxins: After the fluorimetric analysis, none of the samples presented detectable levels of AFM1. According to the European Community, the maximum limit for AFM1 in infant formulas is 0.05µg kg^{-1} (CODEX, 2013). The Brazilian standard for AFM1 5.0µg kg^{-1} is still far from the international standard (ANIVSA, 2011b). Despite that, the absence of mycotoxins in the samples suggests a gradual increase in mycotoxin prevention and control worldwide as described by Tonon et al (2018).

CONCLUSION

The products analyzed are in accordance with Brazilian legislation and current international regulations, being considered safe for consumption, but the need for immediate consumption after resuspension and adequate storage after opening the package is recommended to avoid microbiological development. Any hour after that period confers risk for consumers. Although the products analyzed are in compliance, the identified contaminations are indicative of the need for greater efforts in the control of the manufacturing process, whether the raw material quality or the equipment's cleaning processes. The constant review of professional training and hygiene protocols used in lactary units are also important to minimize the risks to consumers.

ACKNOWLEDGMENTS

The authors would like to thank the course of HigieneVeterinária e Tecnologia de Produtos de Origem Animal of the University Federal Fluminense and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their scholarship. To the Nutrition sector and the lactary unit of Hospital UniversitárioAntônio Pedro (HUAP). Tothelaboratoryofthe Centro Estadual de Pesquisa em Qualidade de Alimentos (CEPQA) ofthe Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO-RJ).

REFERENCES

ANVISA - Agência Nacional de Vigilância Sanitária, Brasil, 2011a. Resolução da diretoria colegiada - RDC nº 44, de 19 de setembro de 2011. Regulamento técnico para fórmulas infantis de seguimento para lactentes e crianças de primeira infância. *Diário Oficial da União*, Brasília, DF, n. 182, p. 92, 21 set. 2011a. Seção 1.

ANVISA - Agência Nacional de Vigilância Sanitária, 2011b. Resolução da diretoria colegiada - RDC nº 7, de 18 de fevereiro de 2011. Dispõe sobre limites máximos tolerados (LMT) para micotoxinas em alimentos. *Diário Oficial da União*, Brasília, DF, n. 7, p. 72, 22 fev. 2011b. Seção 1.

ANVISA - Agência Nacional de Vigilância Sanitária, Brasil, 2019. Resolução da diretoria colegiada - RDC nº 60, de 23 de dezembro de 2019. Estabelece as listas de padrões microbiológicos para alimentos. *Diário Oficial da União*, Brasília, DF, n. 249, p. 133, 26 dez. 2019. Seção 1.

ANVISA - Agência Nacional de Vigilância Sanitária, Brasil. 2003. Resolução – RE no 9, de 16 de janeiro de 2003. Padrões Referenciais de Qualidade do Ar Interior em Ambientes Climatizados Artificialmente de Uso Público e Coletivo. *Diário Oficial da União*, Brasília, DF, n. 114, p. 35, 20 jan. 2003. Seção 1.

AOAC - Association of Official Analytical Chemists International, 2007. Official Methods of Analysis of The Association of Official Analytical Chemists, 19th ed. Author. Gaithersburg, Maryland.

APHA - American Public Health Association, 2015. Compendium of Methods for the Microbiological Examination of Foods, third ed. Author, Washington, DC.

Beuchat, L.R., Komitopoulou, E., Beckers, H., Betts, R. P., Bourdichon, F., Fanning, S., Joosten, H. M., Kuile, B. H. T., 2013. Low–Water Activity Foods: Increased Concern as Vehicles of Foodborne Pathogens. J. Food. Prot. 76, pp. 150-72.

Buchanan, R. L., Oni, R., 2012. Use of microbiological indicators for assessing hygiene controls for the manufacture of powdered infant formula. J. Food. Prot, 75, pp. 989-997.

Codex Alimentarius Commission - FAO, 2008. Code of hygienic practice for powdered formulae for infants and young children. *Codex Alimentarius*. FAO/WHO, Rome. CAC/RCP 66-2008.

Codex, S., 2013. General standard for contaminants and toxins in food and feed. FAO/WHO, Rome, (CODEX STAN 193-1995).

European Commission, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 50, pp. 1-26.

FDA – Food and Drug Administration, 2018. Infant Formula Requirements Pertaining to Current Good Manufacturing Practice, Quality Control Procedures, Quality Factors, Records and Reports and Notifications, 21 C.F.R. § 106.

Groot, M. N., Abee, T., van Bokhorst-van de Veen, H., 2019. Inactivation of conidia from three *Penicillium* spp. isolated from fruit juices by conventional and alternative mild preservation technologies and disinfection treatments. Food Microbiol. 81, pp. 108-114.

International Organization for Standardization ISO/TS 22964:2006 (IDF/RM 210: 2006), 2006. Milk and Milk products – Detection of *Enterobacter sakazakii*.

Kim, S. H., Park, J. H., 2007. Thermal resistance and inactivation of *Enterobacter sakazakii* isolates during rehydration of powdered infant formula. World J. Microbiol. Biotechnol. 17, pp. 364-368.

MERCK, 2002. Microbiology Manual. Berlin, Germany.

Mobin, M., Salmito, M. D. A., 2006. Microbiota fúngica dos condicionadores de ar nas unidades de terapia intensiva de Teresina, PI. Rev. Soc. Bras. Med. Trop. 39, p. 3.

Nema, V., Agrawal, R., Kamboj, D. V., Goel, A. K., Singh, L., 2007. Isolation and characterization of heat resistant enterotoxigenic*Staphylococcus aureus* from a food poisoning outbreak in Indian subcontinent. Int. J. Food Microbiol. 117, pp. 29-35.

Nienov, A. T., Macedo, M. B., Félix, C., Ramos, D., Moreira, A. N., Silva, P. E. A., 2009. Hygienic-sanitary quality of infant formulas given to neonates. Nutrire Rev. Soc. Bras. Aliment. Nutr. 34, pp. 127-138.

Pitt, J. I., Basilico, J. C., Abarca, M. L., Lopez, C., 2000. Mycotoxins and toxigenic fungi. Med. Mycol. J. 38, pp. 41-46.

Pitt, J.L.; Hocking, A. D., 2009. Fungi and Food Spoilage**.** Springer US, ed. 3., Black Academia & Professional Chapmam& Hall, New York. p. 520.

Rossi, P., Kabuki, D. Y., Kuaye, A. Y., 2010. Avaliação microbiológica do preparo de fórmula láctea infantil em lactário hospitalar. Rev. Inst. Adolfo Lutz (Impr.). 69, pp. 503-509.

Salomão, B. C., Churey, J. J., Aragão, G. M., Worobo, R. W., 2009. Modeling *Penicillium*expansum resistance to thermal and chlorine treatments. J. Food. Prot. 72, pp. 2618-2622.

Santos, J. S., Okano, W., Arrais, B. C. D., Costabeber, I. H. Santana, E. H. W., 2014. Aflatoxin M1 in Dairy Products and Acid Lactic Bacteria as Biocontrol Agent in Milk. Uniciências, 18, pp. 51-56.

Tonon, K. M., Savi, G. D., Scussel, V. M., 2018. Application of a LC–MS/MS method for multi-mycotoxin analysis in infant formula and milk-based products for young children commercialized in Southern Brazil. J Environ Sci Health. C. 53, pp. 685-691.

USP - United State Pharmacopeia, 2012. Microbiological attributes of nonsterile nutritional and dietary supplements – Nutritional and Dietary Supplements <2022>

WHO - World Health Organization, 2017. Guideline: protecting, promoting and supporting breastfeeding in facilities providing maternity and newborn services (No. 9789241550086). World Health Organization.

****