Occurrence of Filamentous Fungi in Human Milk, Infant Formula and Milk-Based Products for Young Children Nutrition

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Abstract: The safety of foods (human milk-HM; infant formula-IF; milk-based products-MBPs) aimed for children nutrition (from birth to 5 years old) through filamentous fungi & yeasts were investigated \((n = 158)\). Their analysis followed the ISO 6611: 2004 recommended for total load (isolation & enumeration) in milk and dairy products. The occurrence of filamentous fungi & yeasts was observed in 29, 51 and 60% of the HM \((n = 98)\), IF \((n = 45)\) and MBP \((n = 15)\) samples surveyed. Fungi isolated present counts above \(10^2\) CFU/g and yeasts higher (> \(10^4\) CFU/g). Aspergillus, Penicillium and Thichoderma genera were identified in all the three sample types at percentages of 100/13/9%, 78/11/22% and 32/39/25% for IF, MBP and HM samples, respectively, being Aspergillus the most isolated, especially in the IF samples. Despite deterioration that can cause, the presence of filamentous fungi in HM and other infant foods, can enable mycotoxins production as long as toxigenic species are present which are hazardous for babies.

Keywords: Fungi, Food Safety, Human Milk, Infant Formula

1. Introduction

Human milk (HM) is the best food for children from birth up to 6 months of age [1]. However, when breast feeding is not possible or desired, infant formulas (IF) are an adequate substitute [2-3]. Thus milk and milk-based products are the major nutrients for children. Quality and safety aspects of infant foods are of key importance for child health. Despite that, quite often they do not get much attention by the health care professionals whose interest tends to focus on functional benefits of early nutrition [4].

Infant foods are commonly based on cow’s milk formulations and starchy gruels [3, 5]. Food products, including powdered IF, are not sterile and may contain viable microorganisms and their spores, including pathogens such as Salmonella enterica, Salmonella typhi, Shigella dysenteriae or Cronobacter spp. (formerly Enterobacter sakazakii) which can cause serious infants infections [4, 6]. Every single IF ingredient must have an excellent quality and safety approach because (even in very small quantity in a single product) serious consequences may arise if they are not taken seriously by the manufacturer [7]. Despite that, the IF and milk powders are considered reasonably safe products for public health, however, any failure during processing may favor the occurrence of final product poor microbiological quality [8]. While most studies have focused on HM and infant foods bacteria contamination, just a few have focused on its fungal contamination, leaving a gap in the literature regarding this information.

Milk, both human and non-human, is an extremely
complex biological matrix that contains thousands of nutritional components [9]. Therefore, it is considered an ideal substrate for fungal growth [10]. Breast milk is not sterile, as the microorganisms can multiply when the milk is not handled properly. Additional exogenous contamination should be prevented. Strict hygiene and careful temperature and time control are important during the expression, collection, transport, storage, and feeding of maternal milk [11-12].

Filamentous fungi are extremely versatile; most species can assimilate any carbon source derived from food. Most of them also are indifferent regarding the nitrogen sources and can use nitrate, ammonium ions and organic nitrogen. In addition, fungi and yeasts are very resistant to adverse conditions such as low pH and water activity (aw). Most yeasts need at least aw of 0.88 and fungi 0.80 for growth [13-14]. Fungi of the genus *Aspergillus* are widely distributed in the world, however they mainly occur in subtropical and tropical regions. The *Penicillium* species are able to develop into a wider range of temperatures than *Aspergillus*; however, they are more abundant in temperate regions [15].

The major toxigenic fungi genera are *Aspergillus*, *Penicillium* and *Fusarium* and the main mycotoxins produced by them are aflatoxins - AFLs (AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$), ochratoxin A (OTA), fumonisins, zearalenone and trichothecenes (deoxynivalenol, nivalenol, T2 and HT2). AFLs can be produced mainly by four *Aspergillus* species (*A. flavus*, *A. parasiticus*, *A. nomius* and *A. pseudotamarii*). Two additional AFLs, the AFM$_1$ and AFM$_2$, are products of the AFB$_1$ hydroxylation in the liver [16]. They are excreted in the lactating animals milk and are often found in dairy products. AFB$_1$ and AFM$_1$ are human carcinogens, classified in the Group 1 [17]. OTA is produced mainly by *A. carbonarius* and *P. verrucosum* and is considered a possible human carcinogen, classified in the Group 2B by IARC [18-19].

The microbiological quality of foods intended for children consumption, especially for infants hospitalized in Neonatal Intensive Care Units (NICUs) is a subject of interest to public health, since these children have low resistance to neonatal infections [20]. In addition, those toxigenic fungi species can produce toxins that produce a wide variety of toxic effects [16, 21-24].

This study aimed to evaluate the presence of filamentous fungi and yeasts in foods (HM, IF and milk-based products-MBP) intended for children (from birth to 5 years old) consumption in order to contribute with information about their contamination occurrence.

2. Materials and Methods

2.1. Material

2.1.1. Samples

Foods for young children nutrition (n = 158) being (a) HM–bottles with 20-60 mL content (n = 98) from the Human Milk Bank of Blumenau (HMBB-SC), Santa Catarina State (SC), Southern Brazil and (b) IF and MBP- packs of 400 g (n = 45 & 15, respectively) from manufacturers and chemist stores, commercialized in Florianopolis city, SC (Table 1).

2.1.2. Reagents and Culture Media

Peptone, glucose, yeast extract, malt extract, agar and chloramphenicol, all from Vetec (Duque de Caxias, RJ, Brazil). They were used to prepare the yeast extract glucose chloramphenicol agar (YEGC) and the malt extract agar (MEA) culture media.

2.1.3. Equipment

Autoclave, Phoenix (Araraquara, SP, Brazil), laminar flow, Vecho (Campinas, SP, Brazil), semi-analytical balance, Mettler (Barueri, SC, Brazil), stomacher, Marconi (Saint Nom, France), incubator, Fanen (Sao Paulo, SP, Brazil) colony counter, Phoenix (Sao Paulo, SP, Brazil) and optical microscope, Olympus (Tokyo, Japan) were used to the analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Product</th>
<th>Package</th>
<th>Total number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human milk</td>
<td>in natura</td>
<td>in natura</td>
<td>NA 20 98</td>
</tr>
<tr>
<td>Infant formula</td>
<td>powder</td>
<td>cans</td>
<td>A, B, C 400 45</td>
</tr>
<tr>
<td>Milk-based</td>
<td>powder</td>
<td>cans</td>
<td>D 400 15</td>
</tr>
</tbody>
</table>

*bovine protein-based products for infants from birth to a year old; * bovine milk-based product with addition of vegetable oils for children from 1 to 5 years old

NA: not applicable

Note: this study was approved by the Ethics Committee of the University of Blumenau. All lactating donors who provided the HM samples were informed about the content of this study and when agreed to participate, an informed written consent was signed by both parties before inclusion in the study.

2.2. Methods

2.2.1. Sample Collection

(a) HM - were collected (20-60 mL) after milking expression, in glass bottles, then frozeed at the HMBB and stored frozen until the time of analysis at the LABMICO. (b) IF and MBP- were purchased randomly from manufacturers and pharmacies/chemists in Florianopolis city, SC and stored in their own sealed packs/bags at room temperature (+ / -
20°C) until analysis (Table 1).

2.2.2. Total Fungi Load
(a) isolation - the analysis of fungi and yeasts followed the ISO 6611: 2004 recommendations, directed to their enumeration in liquid milk, milk powder and other dairy products [25] as follows (b) HM, aliquots of HM (0.1 ml) and two decimal dilutions in peptone water 0.1% (10⁻¹ and 10⁻²) were plated in duplicate by the spread plating technique in YEGC and incubated at 25 ± 1°C for 5 days and (b.1.2) IF & MBP- portions of each sample (25g) were diluted in sterile peptone water (0.1%) and homogenized in stomacher for 1 min. From these solutions two decimal dilutions in peptone water 0.1% (10⁻¹ and 10⁻²) were prepared. Aliquots from each dilution (0.1 ml) were plated and incubated at 25 ± 1°C for 5 days. (c) Enumeration of yeasts & fungi: the total yeasts and fungi counts were prepared as per the IN62 regulation [26], ISO 7218: 2007 [27] and Silva et al. [13].

2.2.3. Identification of Filamentous Fungi Genera
The fungi grown on YEGC plates were isolated in MEA for the slide cultures preparation, according to Riddell [28] and Weber and Pitt [29]. The fungigenera identification was carried out microscopically according to Pitt and Hocking [30].

3. Results and Discussion
From the total food samples (HM, IF and MBP) aimed for children nutrition (in their early ages), it was possible to register a variation on the filamentous fungi & yeasts presence and so among the products type. Tables 2 to 4 show data including differences on fungi genera isolated per product surveyed and their frequency distribution.

3.1. Total Filamentous Fungi Load Versus Different Foods for Children Nutrition
As far as the young children nutrition food samples (HM, IF and MBP) filamentous fungi total load are concerned, it was observed that their occurrence was most detected in the MBP samples, followed by IF and HM at 60% (n = 9), 51% (n = 23) and 28% (n = 28), respectively (Tables 2 and 3).

HM: data also showed that, apart from the fungi load in the HM samples, it was also possible to observe & separate filamentous fungi counts from the yeasts, i.e., only filamentous fungi total (> 10² ≤ 10⁵ CFU/mL n = 5+1). The others samples were just yeasts contaminated or not contaminated at all (NG = 30%) (Table 2). On the other hand, a higher percentage (42%) of samples was registered containing only yeasts reaching rather high counts above 10⁵ CFU/mL (n = 3+7). It should be emphasized that high occurrence of yeasts in the HM samples may come from the mother’s skin and often produce maternal infections such as mammary candidiasis by Candida incluindo C. albicans and C. parapsilosis yeasts [31-32].

IF & MBPs - although IF&MBP percentage of positive samples was higher than HM, the occurrence of filamentous fungi isolated from them (dry/powder products) reached similar counts at the range: > 10² ≤ 10⁵ CFU/mL with n = 4 and n = 1 samples, respectively and no yeasts were detected in those dry samples as expected (Table 3).

Table 2. Enumeration of filamentous fungi and yeasts in HUMAN MILK samples for young children nutrition from HMBB**.

<table>
<thead>
<tr>
<th>Plate content</th>
<th>HM contamination</th>
<th>Range (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive n (%)</td>
<td>&lt; 1 CFU/mL (est)</td>
</tr>
<tr>
<td>Filamentous fungi + yeasts (%)</td>
<td>22 (22)</td>
<td>NA</td>
</tr>
<tr>
<td>(a) yeasts</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>(b) filamentous fungi</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Only filamentous fungi (%)</td>
<td>6 (6)</td>
<td>5</td>
</tr>
<tr>
<td>Only yeasts (%)</td>
<td>41 (42)</td>
<td>16</td>
</tr>
<tr>
<td>No fungal growth</td>
<td>29 (30)</td>
<td>29</td>
</tr>
</tbody>
</table>

CFU: colony forming units; * percentages from the total samples (n = 98); ^ yeasts and filamentous fungi were enumerated separately; ^ estimated enumeration due to the lower number of colonies than the accuracy and repeatability range of the method (15-150 colonies); NA: not applicable; NG: no growth*HMBB Human Milk Bank of Blumenau

Table 3. Occurrence of filamentous fungi in INFANT FORMULA AND MILK-BASED PRODUCTS for young children nutrition from HMBB**.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Children nutrition products contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>IF</td>
<td>Total</td>
</tr>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>MBP</td>
<td>D</td>
</tr>
</tbody>
</table>

MPs: milk products CFU: colony forming units; IF: infant formula; MBP: milk-based products; * total samples (n = 45 & 15); NG: no growth *HMBB Human Milk Bank of Blumenau.
3.2. Filamentous Fungi Genera Isolated Versus Children Food Types

Regarding the main filamentous fungi isolated and identified in the different samples types (HM, IF and MBP), they were those belonging to the Aspergillus, Penicillium, Mucor, Paecilomyces, Trichoderma and Geotrichum genera. Some samples 6% (n = 6) showed growth of more than one fungus genus. Table 4 presents the frequency of occurrence of each genus isolated from the young children food positive samples. Important to emphasize that, as HM has a quite high humidity (aw & moisture content-mc) when compared to those processed IF & MBP food (low humidity), other filamentous fungi were also isolated and identified. They were from the Alternaria, Botrytis and Cladosporium genera which need high humidity to grow and are called field fungi, as they can grow under high moist in open environments.

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3.3. HM Fungi Contamination, Hygiene and Temperature Treatment

The HM data of filamentous fungi enumerated (load), isolated and identified in the current work showed that some samples were exposed to inadequate conditions either, of handling, storage or feeding environment leading to certain contamination. That could be either, the HM temperature treatment, contains hygiene and/or substrate exposure conditions. Regarding HM data reported in the literature on HM hygiene and/or treatments, Almeida [33], registered filamentous fungus prevalence in 69.4% of the samples surveyed (with counts reaching 10^3 CFU/mL). After employing the mammary gland hygiene with soap and water, authors reported that contamination declined to 16.7% (counts up to 3.0x10^2 CFU/mL). On the other hand, Novak et al. [10] observed the occurrence of fungi & yeasts in 5.2% (43) of the samples surveyed with counts reaching 10^3 CFU/mL. From those positive samples, authors identified mainly Penicillium (60.4%), followed by Syncephalastrum (14.5%), Paecilomyces (12.6%), Aspergillus (4.2%) and Rhizopus (2.0%) generagroup. Authors were able also to isolate and identify the ochratoxigenic species of Aspergillus niger (6.3%). Regarding temperature treatment, Serafini et al. [20] reported rather similar contamination being in 22% (43) of crude HM samples, and in 25.7% (37) of pasteurized HM - a worrying result indicating possible environment contamination after pasteurization or the processing ineffectiveness. The presence of pathogenic fungi in pasteurized HM suggests that this could be a source of infection to newborns during lactation and also to early exposure to mycotoxins [21-23].

![Figure 1. Fungi colony and reproductive structures of: (a) Aspergillus - (a.1) macroscopic & (a.2) microscopic [a; a2] and (b) Penicillium- (b.1) microscopic [b; b2] genera isolated from the human milk samples, infant formula and milk-based products for young children nutrition.](image)

<table>
<thead>
<tr>
<th>Fungi genera isolated</th>
<th>Children nutrition products positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------</td>
</tr>
<tr>
<td>Aspergillus sp</td>
<td>9</td>
</tr>
<tr>
<td>Penicillium sp</td>
<td>11</td>
</tr>
<tr>
<td>Trichoderma sp</td>
<td>7</td>
</tr>
<tr>
<td>Mucor sp</td>
<td>2</td>
</tr>
<tr>
<td>Alternaria sp</td>
<td>1</td>
</tr>
<tr>
<td>Botrytis sp</td>
<td>1</td>
</tr>
<tr>
<td>Paecilomyces sp</td>
<td>1</td>
</tr>
<tr>
<td>Cladosporium sp</td>
<td>1</td>
</tr>
<tr>
<td>Other*</td>
<td>2</td>
</tr>
</tbody>
</table>

*positive samples; a human milk (n = 28) * infant formula (n = 23) * milk-based products (n = 9) * not isolated/identified * Geotrichum.

3.4. HM Versus IF and MBP Contamination

Despite of filamentous fungi HM contamination detected in the current work at reasonable lower counts [≤ 10^2 (n = 17]
the occurrence of filamentous fungi in the low aw samples such as IF and MBP (aw 0.2), can also be derived from environmental contamination [30].

3.6. Regulation for Filamentous Fungi

One of the difficulties of the current work was to find other data on their occurrence in IF and MBP for infant feeding and also the lack of regulated microbiological limits to fungi and yeasts in these products, and so for HM. Some microorganisms presence is indicative that the food has been exposed to conditions that pose an increased risk of pathogens contamination or having been held under conditions that would allow their proliferation.

The Brazilian Health Ministry (RDC 12), established standards for IF and MBP just for coliforms, coagulase positive *Staphylococci, Bacillus cereus* and *Salmonella* sp. Breast milk from HM banks, has standards established for Mesophilic Aerobic Bacteria, coliforms, coagulase positive *Staphylococci* and *Salmonella* sp [26, 38]. Also international regulations such as from Australia, China, EU, Japan, USA established similar standards with some differentiations though [39].

4. Conclusions

Data obtained on HM showed filamentous fungi contamination. It is likely that the fungi spores present in the HM (handled by milk donors) were the source of fungi contamination detected, as their characteristics were quite similar to those of food deterioration. When HM is exposed to microbiologic contaminants, usually it is related to (a) mothers skin or hands / breast pump components / milk containers and/or (b) the environment where the milk is expressed and exposed.

It is assumed that HM pasteurization (62.5°C / 30 min) at the HM Banks inactivates filamentous fungi. However when it comes to the product (HM) transfer to hospitalized premature babies in NICUs, fungi spores can get into and proliferate. Therefore, it is essential to comply with proper conditions (during collection / storage / transportation) to avoid the presence and multiplication of such contaminants.

Regarding the IF and MBP filamentous fungi presence, the results showed that did not represent a serious public health problem. The low occurrence of fungi in these products is explained by their low aw (± 0.2) and mc, which hinders the development of fungi. Care should be taken after their dissolution in water prior children feeding.

This is the first study reporting IF and MBP filamentous fungi and yeasts enumeration and identification.

References


