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Biotecnologia

Efficacy of surface microbial decontamination of beer cans and cytotoxic activity of fungal isolates[1]

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Abstract

Incorrect storage or handling of canned beverages can lead to contamination of these packages with microbiological agents that can cause various diseases and death. We investigated the surface microbial contamination of beer cans sold in supermarkets, the efficiency of different cleaning methods in the surface decontamination of beer cans, and the degree of toxicity of filamentous fungi associated with these packagings. The samples were collected in four local supermarkets, in two seasonal periods (dry and rainy season), and submitted to three sanitization methods (paper napkin, running water, and running water + neutral detergent). The cytotoxicity of fungi was determined and classified by the mortality of Artemia salina after exposure to the crude extract of the fungi. The detected contaminating fungi belong to the genera *Paecilomyces*, *Aspergillus*, or *Gliocladium*. The level of contamination was influenced by seasonality (higher in the dry season). Five isolates presented high toxicity, which means potential harm to the consumer. Hence, it is recommended to use running water + detergent in the superficial sanitization before consumption, because this method presented better results in the surface decontamination of beer cans.

Keywords: cleaning, drink, mycobiota, microbiota and packaging.

Eficácia da descontaminação microbiana superficial de latas de cerveja e atividade citotóxica dos isolados fúngicos. O manuseio ou a armazenagem incorreta das bebidas enlatadas pode favorecer a contaminação das embalagens por agentes microbianos que causam várias doenças e a morte. Foi investigada a contaminação microbiana superficial de cervejas enlatadas vendidas em supermercados, a eficiência de diferentes métodos de limpeza na descontaminação superficial e o grau de toxicidade dos fungos filamentosos associados à estas latas. As amostras foram coletadas em quatro supermercados locais, em dois períodos sazonais (estação seca e chuvosa), e

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submetidas a três métodos de limpeza (papel toalha, água corrente e água corrente + detergente neutro). A toxicidade dos fungos foi determinada e classificada pela mortalidade de *Artemia salina* após exposição ao extrato bruto deles. Os fungos isolados pertenciam aos gêneros *Paecilomyces*, *Aspergillus* ou *Gliocladium*. O índice de contaminação foi influenciado pela sazonalidade (maior na estação seca). Cinco isolados apresentaram alta toxicidade, o que significa potencial perigo ao consumidor. Para os consumidores, é recomendada a água corrente + detergente neutro na limpeza das latas de cerveja antes do consumo, porque este método apresentou maior descontaminação superficial.

Palavras-chave: limpeza, bebida, micobiota, microbiota e embalagem.

1. Introduction

The Brazilian Aluminum Can Manufacturers Association – ABRALATAS points that the Brazilian annual production of canned beverages is 26 billion cans, and over 60 % of breweries (1209 units until 2019) production is in the canned format (ABRALATAS 2016), attending a growing demand of Brazilian consumers (Caetano et al. 2015) and suggesting the relevance of this commerce for the country.

About the alcoholic beverage intake, the Brazilian index per capita – 6.2 liters – is above the world average index, highlighting the North region (Caetano et al. 2015), therefore, initiatives for ensuring the consumers welfare are vital. Consequently, the sanitization before ingestion is essential because the incorrect storage or handling may contaminate cans with microbiological agents that can cause several diseases, and death in the worst scenario (Fink et al. 2017; Gündüz et al. 2019).

Among microorganisms, the fungi can be found in foods and beverages and grows in drier and more acidic substrates than bacteria (Levinson 2016), as demonstrated by Tomičić and Raspor (2017) research on metallic surfaces. Some fungi produce mycotoxins, e.g. ochratoxin, synthesized by some *Aspergillus* and *Penicillium* species, which present nephrotoxic, hepatotoxic and carcinogenic effects (Massoud, Cruz, and Darani 2018; Paterson et al. 2018). These effects can be preliminarily estimated by bioassays using *Artemia salina* as an animal model. The tests with this species are an excellent model due to its easy execution, low cost, and easy visualization (Mesquita et al. 2015).

Researches that points to this contamination in cans and its biological activity are useful for vendors and consumers of canned beverages, reducing hazards and risks associated with its handling. Therefore, this research aims to determine the surface microbial contamination of beer cans commercialized in supermarkets, the influence of different sanitization methods on its decontamination and the toxicity grade of isolated filamentous fungi.

2. Material and Methods

2.1 Sampling

The aluminum beer cans without top protection seal were sampled in four different supermarkets (S1, S2, S3, and S4), in October 2016 (dry season) and February 2017 (rainy season), in Santarém city, state of Pará, Brazil.

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In each sampling, 12 beer cans were acquired per supermarket, counting 48 cans per season. The behavior during the sampling (including the transport pack) simulated the normal consumer, with only one specificity: the cans were removed from the shelves by the bottom side.

2.2 Sanitization assays

Three sanitization methods – paper napkin (PN), running water (RW) and water + neutral detergent (WD) – all described as reported below, were applied per season, where three units per supermarket per sanitization method were analyzed. For the PN method, a napkin was rubbed during four seconds around all top and neck (top border of the can body, slightly in funnel form) parts. For the RW method, each beer can was washed under a tap in high flow mode, for four seconds, with artesian well water, not chlorinated (common in local pubs), reaching all top parts. For the WD method, every top part of each can was rubbed with the non-abrasive side of a new synthetic sponge, embedded in a liquid neutral detergent solution, turning 360 degrees twice, followed by washing under a tap (in high flow mode) until the detergent was eliminated. No sanitization method was applied for the control group (C).

2.3 Fungi detection and enumeration

In aseptic conditions, the surface smear technique was performed using sterile swabs during four seconds, rubbing only the semi-circle part of the top panel, which leads the access to the internal liquid, and the neck, where consumers put their mouth. The inoculation was made on the surface of the Potato Dextrose Agar medium supplemented with chloramphenicol 0.01% (PDAC). Then, the plates were incubated at 25±2 °C/48 hours. The microbial contamination was determined by the Forming Colony Unit (FCU) technique, and the results were expressed by FCU per can and FCU per cm^2. Only the filamentous colonies were isolated in PDAC and, posteriorly they were submitted to separations (by monosporic technique) to obtain pure cultures, which were identified by macro and microscopical characteristics according to specialized literature (Kern and Blevins 1999; Pitt and Hocking 2009) and conserved in sterile water according to Castellani (1968).

2.4 Toxicity of isolated fungi against *Artemia salina*

The crude extracts of each identified fungi genus were obtained after static culture in Potato Dextrose Broth (PDB) according to Vargas-Isla and Ishikawa (2008) and Teixeira et al. (2011). Three discs of 5 mm diameter were cut off from the medium zone of pure culture, inoculated in 100 mL of PDB, and incubated at 25± 2 °C/10 days, in absence of light. To obtain the crude extract, the fungal cells were separated from the liquid medium by filtration under vacuum using a 0.22 µm filter. The microbial toxicity was determined and classified by the mortality of *A. salina* after exposure to each crude extract according to Teixeira et al. (2011), with adaptations (changing the agitated to static culture and multiwell plates to 1.5 mL centrifuge tubes).

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2.5 Statistical analyses

Due to the non-normality of the data, the transformation (n + 1) was used to verify the existence of significant differences and their interactions regarding contamination between seasonality and supermarkets, using the ANOVA two-way. For the sanitization methods, the Kruskal-Wallis non-parametric test was used, and, in the end, the averages were compared by the Mann-Whitney test with Bonferroni correction. All the tests used 5.0 % of significance, using the program PAST version 3.19 (Hammer, Harper, and Rian 2001).

3. Results

3.1 Fungi contamination of beer cans

In both seasons [dry, pluviometric index (PL) = 25-50 mm; rainy, PL = 150-200 mm according to CPTEC/INPE (2017)] we found 100% of contamination below 5 x 10 FCU/cm^2, which is indicated as safe by Mendes, Santos, and Carvalho (2016). In the rainy season, the contamination per can did not vary as much as in the dry season. The dry season presented the higher contamination. Regarding contamination in each supermarket, cans from S4 were the most contaminated, while those in S2 were the ones with the lowest indexes. According to the variation, the contamination in S2 and S3 presented the lowest indexes, especially the last supermarket cited (Table 1).

There was a significant difference between seasonal periods and between supermarkets (Table 2 and Figure 1).

Table 1 - Surface microbial contamination on beer cans commercialized in supermarkets, Santarém, Pará, Brazil.

Analytical category (group)	Category level	Contamination (in FCU*)			The incidence between levels (in %)
		FCU ± SD per can (average)	FCU per cm^2	Variation (min.-max., in FCU)	
Season	Dry	$1.59 \times 10^2 \pm 1.88 \times 10^2$	1.45×10^1	12 - 418	73.45
	Rainy	$5.75 \times 10^1 \pm 2.67 \times 10^1$	5.24×10^0	29 - 102	26.55
Supermarket	S1	$9.98 \times 10^1 \pm 1.52 \times 10^2$	9.09×10^0	29 - 409	22.82
	S2	$4.50 \times 10^1 \pm 3.49 \times 10^1$	4.10×10^0	12 - 102	10.29
	S3	$5.63 \times 10^1 \pm 3.07 \times 10^1$	5.14×10^0	19 - 95	12.91
	S4	$2.36 \times 10^2 \pm 2.04 \times 10^2$	2.15×10^1	29 - 432	53.98

Abbreviations: *FCU = Former colony unit, SD = Standard deviation.

3.3 Identification and toxicity of isolated fungi

Six fungi were isolated from beer cans, which were identified as *Aspergillus* spp. (n = 3), *Gliocladium* sp. (n = 1) and *Paecilomyces* spp. (n = 2), and most of them were highly toxic against *A. salina* (Table 3).

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Table 2 – ANOVA *two-way* results by category about surface microbial contamination of beer cans commercialized in supermarkets, Santarém, Pará, Brazil.

Contamination by analytical category (groups)	Analytical level	Statistical index				
		SS	df	MS	F	p-value*
Season	Dry x Rainy	62,322.0	1	62,322.0	10.470	0.005178
Supermarket	S1 x S2 x S3 x S4	133,476.0	3	44,492.2	7.473	0.002396
Interaction	Season x Supermarket	169,832.0	3	56,610.7	9.508	0.000766
Within groups		95,259.3	16	5,953.7		
Total		460,890.0	23			

Abbreviations: SQ = sum of squares, df = degrees of freedom, MS = medium square.
*p-value ≤ 0.05 means significant statistical difference at 5% level.

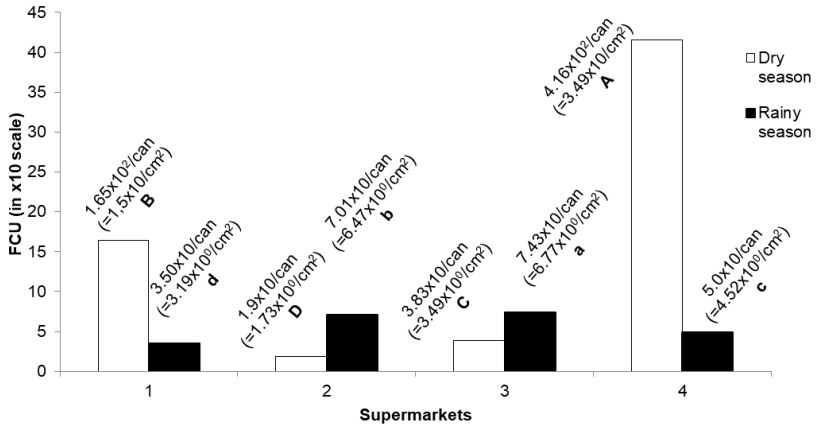

Figure 1 – Surface contamination (average) of beer cans by fungi in supermarkets of Santarém city, state of Pará – Brazil, in dry (white filled columns) and rainy (black filled columns) seasons. Letters represent the statistical comparison between supermarkets in dry (capital letters) and rainy (small letters) seasons. Averages followed by different letters mean a significant statistical difference between supermarkets by the Tukey test at 5 % level of probability.

Table 3 – Toxicity classification of fungi isolated from the surface of beer cans commercialized in Santarém city, state of Pará - Brazil.

Fungi	Mortality (%)	Classification	Incidence of classification (in %)
Aspergillus sp. 1	100	Highly toxic	83.33
Aspergillus sp. 2	100	Highly toxic	
Aspergillus sp. 3	100	Highly toxic	
Gliocladium sp.	100	Highly toxic	
Paecilomyces sp. 1	100	Highly toxic	
Paecilomyces sp. 2	82	Toxic	16.67

4. Discussion

This research confirmed the influence of weather conditions on microbial contamination. A similar research by Mendes, Santos, and Carvalho (2016) during the autumn [PL = 25-50 mm (CPTEC/INPE (Centro de Previsão do Tempo e Estudos Climáticos/Instituto Nacional de Pesquisas Espaciais) n.d.), similar to dry season on this research] verified 91.67 % of the beer cans sold in

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Itabuna – BA with low contamination index. For the rainy season, no other research was found in that same period or with an approximate PL for comparison.

The greatest contamination in the dry season may be related to the high humidity (up to 80 %) simultaneously with the heat (up to 40 °C) in the Amazon, which promotes the natural growth of microorganisms (Oliveira Martins Junior et al. 2011). Even in this period, there are many particles in suspension, being an environment where fungi survive better than other microorganisms (Franco and Landgraf 2008).

Maybe, the cleaning conditions (not measured or qualified in this research) are the motivators of the surface contamination of cans. According to Rodrigues et al. (2015), incorrect handling and neglect of hygienic standards favor contamination by pathogenic microorganisms, which can multiply in sufficient numbers to cause illnesses to consumers.

In this study, all supermarkets have large fluctuations in the order of contamination within the same seasonal period and no supermarket presented the highest or the lowest contamination rate in both seasonal periods (Figure 1). There was a significant difference in microbial contamination in each supermarket, regardless of the seasonal period. Therefore, despite the seasonal period being an important factor, it is assumed that the hygienic-sanitary quality adopted by the commercial establishment at any time of the year is the determining factor for the contamination of beer cans.

About sanitizations methods, the findings of this research (in FCU/cm^2) differ from those obtained by Pascoal et al. (2007) about surface microbial contamination after sanitization methods on metallic beverage cans, that detected the following increasing order of efficiency: water and soap (1.82×10^2), only water (8.99×10) and paper napkin (4.45×10).

For PN, the inefficiency is due to the removal of less than 50 % of microorganisms, possibly because it contributes to the spread of fungal propagules. Decontamination by RW was also not efficient, probably due to the lack of friction in the beer can and because only the tap flow was not able to properly remove the attached microorganisms.

According to Price (1938), to disinfect bacteria from hands and arms, the amount of vigor during brushing is more important than the sort of soap, sterility and temperature of water washed in. So, this concept may be extended to friction for cans hygienization. Additionally, soap and detergent have little value as an antiseptic but, in some situations, they contribute to dissolve the lipid membrane of microbes and release its cellular content, and to disperse microorganisms and dirt, facilitating their mechanical removal during rinse (Niessen 2018; Jing et al. 2020).

It was not possible to make a careful comparison with the similar work by Pascoal et al. (2007) due to the lack of details on its protocol. Possibly, these authors obtained greater efficiency using napkins due to the use of more time, force in friction or material composition of napkin, different from the method used in this research. Still, it is possible that the friction used in WD method was greater than that adopted by Pascoal et al. (2007).

All fungi identified in this research (Table 3) are implicated in human health disorders, directly or indirectly. The fungal group *Aspergillus* is

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notorious for contaminating food products, with some species capable of producing mycotoxins, which are potentially toxic substances to humans and animals (Niessen 2018). Some species, especially *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*, are the causes of pulmonary aspergillosis, an infectious and non-contagious disease. Although the pulmonary form is the most common, in extra-pulmonary forms there may be cerebral, ocular, cutaneous, bone, and cardiovascular involvement (Campos Cavalcante et al. 2018). The species *A. fumigatus* was the etiologic agent of primary laryngeal aspergillosis in an immunocompetent or moderately immunosuppressed individual (Dutta et al. 2017). Therefore, the detection of *Aspergillus* spp. in the cans analyzed in this research implies that consumers are subject to contamination if they mainly inhale their spores during the swallowing of the drink.

Gliocladium spp. are fungi commonly used as biocontrol agents in association with *Trichoderma* spp. to combat some phytopathogenic fungi (Ilyas et al. 2015). Recently, the first case of eye infection involving *Gliocladium* sp. in a diabetic person was described (Venkatesh et al. 2017). Thus, the contact of canned beer consumers with this fungus can also pose a risk to their health.

Paecilomyces species are implicated in several negative effects on human health. *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) had its presence detected in different types of substrates, sometimes as a parasite of other fungi, nematode eggs, human skin and cornea of immunocompromised or immunocompetent individuals (Abbas et al. 2016; Chen and Chen 2016; Toker et al. 2016). *Isaria farinosa* (formerly *Paecilomyces farinosus*) has already been reported as an infectious agent of the human cornea (Lakhundi, Siddiqui, and Khan 2017) and *Paecilomyces formosus* is an agent of skin infection in premature children (Kuboi et al. 2016); *Paecilomyces variotii*, of urinary tract infection and rhinosinusitis in immunocompetent people (Çolakoglu et al. 2016; Swami et al. 2016) and also pneumonia in immunocompromised people (Feldman et al. 2016). Our finding of *Paecilomyces* sp. in beer cans shows a risk to consumers health, especially those who are immunocompromised and to those who neglect to clean hands and cans before consumption, especially if they drink beer directly from the can.

The toxicity of all genera fungi isolated in this research could be a consequence of the biological activity detected by other researchers. The fungi *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Aspergillus* sp. 3 revealed toxicity percentages equal to the 100 % obtained by Qureshi et al. (2011) when studying *Aspergillus niger* and higher than 75.7 % by Zainudin and Perumal (2015) for *Aspergillus flavus*. According to the high percentage of toxicity presented by the *Aspergillus* spp. in this research, we can assume its ability to produce toxins.

Regarding *Gliocladium*, high toxicity was detected. Maybe, this biological activity is a result of gliotoxin production, a secondary metabolite synthesized by *Gliocladium fimbriatum* and other fungi species. This toxin has a histopathological effect and may alter the immune response in humans and animals (Hussain et al. 2020).

Paecilomyces sp. 1 and *Paecilomyces* sp. 2 expressed toxicity percentages higher than the 70 % observed by (Qureshi et al. 2011) evaluating the species *Paecilomyces*

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lilacinus against *A. salina*. Another study pointed out *Paecilomyces variotii* as a mycotoxin producer (Fasoyiro, Gourama, and Cutter 2017), which corroborates the high toxicity verified for the studied strain of this genus.

Due to the high toxic activity presented by isolated fungi, the consumer of canned drinks may be susceptible to health problems due to contamination by its metabolites or by the pathogenicity of these contaminants. This condition found in Santarém-PA can be extended to other tropical places of sale of this product.

5. Conclusion

The superficial microbiota indexes of beer cans indicate low contamination, despite including filamentous fungi with high potential for toxicity. The hygienic conditions of each establishment were also considered a determining factor for this contamination, although it is influenced by seasonality. To guarantee the consumer welfare and to indicate the ideal sanitary conditions for the trade of canned drinks, the national legislation needs to establish adequate microbiological standards, which currently do not exist. Important hygiene measures are required and must be taken by all agents involved in the stages of transportation, storage, and sale of the product to minimize risks to the consumer. Such measures should cover from the first contact with the beer can on the shelf until the consumption of the product. The use of water + detergent for surface cleaning before consumption should be recommended to consumers, as this method has shown better results in the superficial decontamination of beer cans.

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