Bacillaceae spores, fungi and aflatoxins determination in honey

Esporos de Bacillaceae, fungos e aflatoxinas em mel

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Summary: Consumption of honey has remarkably increased in the last years all over the world. However, the safety of these products is not regularly assessed. This paper presents the results of a study held in 80 samples of honey, randomly collected in retail markets, concerning to the contamination with Bacillaceae spores (Clostridium perfringens, Bacillus cereus), fungi and aflatoxins. The microflora was determined using conventional microbiological methods and the aflatoxins were detected by “high performance liquid chromatography” (HPLC). Spores of Clostridium perfringens were not detected in any sample. Bacillus cereus were identified in eleven samples (13.7 %); of these, five (6.2%) had levels lower than 10⁵ cfu/g, five (6.2%) samples contained levels ranging from 10⁶ to 10⁷ cfu/g and only one (1.3%) presented contamination above 10⁸ cfu/g. Yeasts and moulds were detected in 71 samples (88.8%). Three genera of moulds (Aspergillus, Penicillium and Mucor) and two genera of yeasts (Saccharomyces and Candida) were identified. The most prevalent among Aspergillus was A. flavus (57.5%), followed by A. niger (51.3%), A. fumigatus (45.0%) and A. candidus (31.3 %). Penicillium spp. and Mucor sp. were present in 38.8 and 31.3 % respectively. Saccharomyces sp. and Candida humicola had high incidence (88.8 and 75.0 % respectively). None of samples revealed to be contaminated with aflatoxins.

Key words: Bacteria, fungi, aflatoxins, honey

Resumo: Nos últimos anos, o consumo do mel tem aumentado significativamente. Contudo, a respectiva avaliação higio-sanitária não está suficientemente estudada. Neste trabalho os autores apresentam os resultados de pesquisas, em 80 amostras de mel, colhidas aleatoriamente a partir do comércio não especializado, de Clostridium perfringens, Bacillus cereus e das contaminações fúngicas obtidos por métodos microbiológicos convencionais, e de aflatoxinas detectadas por cromatografia líquida de alta resolução (HPLC). C. perfringens não foi isolado em qualquer das amostras analisadas. Bacillus cereus foi identificado em 11 amostras (13.7 %); destas, cinco apresentaram contaminações inferiores a 10⁵ cfu/g (6.2%), outras cinco amostras continham níveis compreendidos entre 10⁶ e 10⁷ cfu/g (6.2%) e apenas uma apresentava teores da ordem de 10⁸ cfu/g (1.3%). Bolores e leveduras foram isoladas em 71 amostras (88.8 %). Foram identificados três gêneros de bolores (Aspergillus, Penicillium e Mucor) e dois de leveduras (Saccharomyces e Candida). Entre os isolados de Aspergillus, o de maior prevalência foi A. flavus (57.5%), seguido de A. niger (51.3%), A. fumigatus (45.0%) e A. candidus (28.7%). Penicillium spp. e Mucor sp. foram isolados respectivamente em 38,8 e 31,3 %. Saccharomyces sp. e Candida humicola foram detectados com elevada frequência (88,8 e 75,0 %). Não se detectaram aflatoxinas em qualquer amostra.

Palavra chave: Bacteria, fungos, aflatoxinas, mel

Introduction

Honey is an interesting food that can be used as an ingredient or as a final product. New technologies and innovative uses of honey are an expanding market (Snowdon and Cliver, 1996). Honey may undergo various changes during storage, one of the most significant of which is spontaneous fermentation induced by osmophilic yeast (Jiménez et al., 1994). Honey is mainly composed by sugars, particularly the mono-saccharides fructose and glucose, though it contains a large variety of di-and trisaccharides (White, 1983). Honey is packaged for retail sale, and is presented in bulk for commercial distribution. Microbiological characteristics of honey are inherent to quality and safety. The microbes of concern in honey are primarily yeasts, moulds and spore-forming bacteria. These microorganisms may be involved in activities such as spoilage of provisions, production of enzymes, antibiotics, mycotoxins and growth factors (vitamins, amino acids), metabolic conversion of provisions, and inhibition of competing microorganisms (Goerzen, 1991). Honey has been incriminated as a source of Clostridium botulinum spores responsible for infant botulism cases (Amon et al., 1981). A different clinical form of botulism, has been recently recognised in infants under 1 year of age. In this type of botulism spores of bacteria apparently germinate and produce toxin in the intestinal tract of affected infants (Huttanen et al., 1981).

Pollen may be the original source of microbes for honey (Gilliam et al., 1983). The honey bees appear to be seeded microbiologically by pollen consumption and by other bees in the colony through food exchange (Snowdon and Cliver, 1996). In Portugal, in a preliminary study on honey, Martins et al., (2001) showed...
that there was a low percentage of contamination with *Bacillus cereus*, and with fungi: yeasts, *Mucor* sp, *Penicillium* spp and several species of genus *Aspergillus*, particularly *Asp. flavus*, *Asp. candidus*, *Asp. fumigatus* and *Asp. niger*. These potentially pathogenic species can be harmful to predisposed patients. The infestation of natural food by these toxigenic fungi, and the resultant toxin contamination (mycotoxins) have been deemed as unavoidable contamination by the US Food and Drug Administration (FDA). Among mycotoxins, aflatoxins could pose a significant threat to human health because they are toxigenic, carcinogenic, mutagenic and teratogenic (Hsieh, 1986), besides the economic loss due to food contamination. A great deal has been written about the acute toxicity and carcinogenicity of the aflatoxins as well as the pathways leading to their biosynthesis by some strains of *Asp. flavus*, *Asp. nomius* and *Asp. parasiticus* (Betina, 1989). Other safety problems related with pesticide residues are constantly monitored (Garica et al., 1995; Fernandez et al. 1997).

The objective of the present study is to give a preliminary assessment of microbial and aflatoxin contamination of multifloral honey from Portugal.

**Material and methods**

**Sampling procedure**

Eighty multifloral honey samples were randomly collected at the retail public markets, in the city of Lisbon, Portugal. All packaged samples (200 g) were transported and stored at room temperatures (20 °C).

**Pre-treatment of samples**

Ten grams of each sample were homogenized for 3 min in 90 ml (10⁻¹ suspension) peptone water (Oxoid, code CM 9, Basingstoke, England) in a Colworth 400 Stomacher (Seward Medical, London, UK). Ten-fold dilutions were prepared till 10⁻⁴.

**Detection of *Bacillus cereus* spores**

For *Bacillus cereus* spores enumeration, 1 ml of the each dilution was spread on five Petri plates of Mannitol-bromothymol blue agar supplement with polymyxin (Oxoid code number SR 99 Basingstoke, England) and egg yolk Emulsion (Oxoid code number SR 47 Basingstoke, England) (0.25 ml/plate), after thermal inactivation of each inoculum at 80 °C for 10 min in a water bath. The plates were incubated in inverted position at 37 °C for 48 h. After enumeration of morphologically typical colonies, peacock blue colonies with blue halos, were picked for biochemical identification according to ICMSF (1996), ISO 7932, (1987) and Bergey’s Manual of systematic bacteriology (Sneath et al., 1986).

**Detection of *C. perfringens* spores**

For the enumeration of *Clostridium perfringens*, 1 ml of each decimal dilution was incorporated onto Tryptose sulfae cycloserine Agar (TSC) (Oxoid code number CM 0587D; SR88 Basingstoke, England) after thermal treatment at 80 °C for 10 minutes (ISO, 1995). The plates were incubated in inverted position, under anaerobic conditions, for 48 h at 45 °C.

**Mycological examination**

For enumeration and identification at genus level of moulds and yeast, 1 ml of honey of each dilution was spread into each of five plates (0.2 ml/plate) of Glucose Yeast Extract Sucrose Agar (GYES) (King et al., 1984) and incubated at 28 °C for 3-5 days. Each isolated mould colony was observed microscopically for morphological characterization and identification (Domsh et al., 1980; Samson and Pitt, 1989). Selected yeast strains from each group were identified using API ID 32 C galleries system (Bio Merieux - 32200, France). Complementary biochemical tests were performed for *Saccharomycses* and *Candida* species (Barnett et al., 2000).

**Determination of aflatoxins B1, B2, G1 and G2 by HPLC**

The samples were analysed for the quantification of AFs using immunoaffinity columns supplied from Rhône - diagnostics Technologies Ltd (Spain), and quantified by high performance liquid chromatography (HPLC) according to the method described by Stroka et al. (2000), with modifications an in the initial extraction phase. The solvent mixture was water + methanol (8+2) instead of methanol+water (8+2,v/v). The detection limit was 1 µg/kg. The sample extract was filtered, diluted and applied in an immunoaffinity column containing antibodies specific to aflatoxins B1, B2, G1 and G2. Standard AFs B1, B2, G1 and G2 were purchased from Sigma-Aldrich (Ref. A-6636, A-9887, A-0138 and A-0263 respectively) (Quimica S.A. Spain). The stock solution, working standards and the calibration curve were prepared and determined as described by Stroka et al., (2000). The recoveries were done in duplicate, in blank samples of honey (1kg), spiked with levels of 4.0 mg/kg of AFB1 and AFG1, and 2.0 mg/kg of AFB2 and AFG2. The average recoveries were 89.5 % for AFB1, 87.0% for AFB2, 88.3% for AFG1 and 86.2 % for AFG2. Samples with AFs levels below 1 mg/kg were considered negative (inferior to the quantification limit).

**Results**

From the 80 samples, only nine do not revealed any microbial contamination (11.3%) in one gram. Eleven
of the samples (13.8%) were contaminated with spores of *B. cereus*, five (6.2%) of which had levels lower than $10^1$, five samples (6.2%) with levels greater than $10^3$ cfu/g; one sample (1.3%) revealed to be contaminated with $10^3$ spores/g. Spores of *C. perfringens* were not detected in any sample (1 g) (Table 1).

Of the 80 samples analysed, 71 (88.8%) were contaminated with fungi; of these samples, 46 were contaminated with moulds and yeasts, and 25 samples presented only yeasts. The moulds identified were: *Asp. candidus* (28.7%), *Asp. flavus* (57.5%), *Asp. fumigatus* (45.0%), *Asp. niger* (51.3%), *Mucor* sp. (31.3%) and *Penicillium* spp. (38.8%), with levels ranging from $10^1$ to $10^2$ cfu/g (Table 1).

Two yeast species were identified: *Candida humicola* (75.0%) and *Saccharomyces* sp. (88.8%); Its level of contamination ranged between $10^1$ and $10^2$ cfu/g.

None of samples revealed to be contaminated with aflatoxins.

### Discussion

Huttanem *et al.* (1981), in 80 honey samples collected from apiaries in Pennsylvania, Illinois and New Jersey, did not found *Clostridia* spores. In another study, Kautter *et al.* (1982), in 100 samples of honey found only two samples (2.0 %) contaminated with *Clostridium botulinum* spores. The present study does not revealed contamination superior to 1 spore/g (limit of quantification).

Hauschild *et al.* (1988), state that honey generally contains few botulinic spores. According to Bonvehi and Jordá (1993) the presence of *Bacillus* spores are at low incidence in multifloral honey. The results of the present study revealed contamination by *B. cereus* in 11 samples; one of the samples had a heavy contamination ($>10^3$ spores/g). Potential toxigenic effects are achieved on levels above $10^3$ spores/g.

Jiménez *et al.*, (1994), studying raw honey referred that the dominant mycoflora included *Aspergillus flavus*, *Asp. niger*, *Asp. candidus*, *Asp. terreus*, *Penicillium* spp., *Saccharomyces* sp. and *Zygosaccharomyces*. This study partially confirms this findings.

The yeast species identified (*Candida humicola* and *Saccharomyces* sp.) were detected in a very high frequency and at high levels of contamination. This osmophylic yeast are probably good indicators for microbiological quality of honey.

There are few informations concerning mycological contamination and simultaneous co-occurrence of *Asp. flavus* or *Asp. parasiticus* and aflatoxins detection in honey. Hilldrup *et al.* (1977), studied fungal growth and aflatoxin production on apiarian substrates (unprocessed honey, pollen, brod comb, whole larvae and whole bees), and verified that fungi grew, sporulated and produced aflatoxins in low levels in all substrates except the unprocessed honey. Other similar study, carried out by Wellford *et al.* (1978), inoculated unprocessed honey with toxigenic strains of *Asp. flavus* NRRL 5862 and *Asp. parasiticus* NRRL 2999, the fungal growth was observed, but none of the cultures produced detectable levels of aflatoxins. The results presented by these researchers agree with those obtained in the present study and allow us to conclude that microbial contamination levels in honey is generally low. It is not surprising to find that none of the stains of *Asp. flavus* have produced aflatoxins, because the production depends not only of the genetic competence of the strains, but is also influenced by a quite wide range of factors (substrate composition, very low aW, and acidity of honey) and ecological conditions.

### References


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### Table 1 - Frequency of microflora identified in honey (Total samples - 80 )

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Number of positives samples / levels of contamination ( %)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>&lt; 1 cfu/g</td>
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<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>69 (86.3)</td>
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<tr>
<td><em>Clostridium perfringens</em></td>
<td>80 (100.0)</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus candidus</em></td>
<td>57 (71.3)</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>34 (47.5)</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>44 (59.0)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>39 (48.8)</td>
</tr>
<tr>
<td><em>Mucor</em> sp.</td>
<td>55 (68.8)</td>
</tr>
<tr>
<td><em>Penicillium</em> spp.</td>
<td>49 (61.3)</td>
</tr>
<tr>
<td><em>Candida humicola</em></td>
<td>20 (25.0)</td>
</tr>
<tr>
<td><em>Saccharomyces</em> sp.</td>
<td>9 (11.3)</td>
</tr>
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