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# Microbiological quality control of marketed pollen

**Hani Belhadj[1], Daoud Harzallah*[1], Saliha Dahamna[2] and Seddik Khennouf[2]**

*[1]Laboratory of Applied Microbiology, Faculty of Natural and life Sciences, University Ferhat Abbas Sétif1, Sétif, Algeria*
*[2]Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Natural and life Sciences, University Ferhat Abbas Sétif1, Sétif, Algeria*

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**ABSTRACT**

*Microbiological quality control of 13 pollen samples purchased from local markets and 2 other samples provided by apiarists was investigated. TAMC ranged from 3.00 to 5.48 Log CFU/g. TMYC was between 2.3 and 6.99 Log CFU/g. Staphylococcus aureus was recovered with high density in 14 samples (up to 8.32 Log CFU/g) and Enterobactericeae count ranges from 4.18 to 8.018 Log CFU/g. Moreover, Salmonella spp. and Listeria spp. were detected in seven and ten pollen samples respectively. Potent toxinogenic molds isolated from pollen such as Aspergillus flavus, A. niger, A. alliaceus, Penicillium sp., Alternaria alternata, Alternaria sp., Monila sitophilia, Rhisomucor pusillus and Mucor hiemalis were characterized by conventional methods. In addition, analyzed pollen samples contain pathogenic members of Enterobacteriaceae (Salmonella sp. Shigella sp., Proteus mirabilis, Citrobacter diversus, Klebsiella sp., Escherichia coli, Providencia sp. and Enterobacter cloacae) as revealed by biochemical identification tests. Interestingly, this type of marketed pollen has poor microbiological aspect, unacceptable, and a hazardous food material.*

**Keywords**: Pollen, quality control, mycotoxigenic fungi, enterobacteria, *Salmonella*, *Listeria*.

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## INTRODUCTION

For centuries, plant products have been used for a variety of functions. Trading of plants and plant products used for medicinal purposes were expanded in various societies **[1, 2]**. This situation probably stimulated by the fact that people believe that the consumption of natural products is healthier and safe than conventional medicine. As a plant product, pollen collected either by man or by honey bees, is used for nutritional and medicinal purposes. Because of its richness by proteins, amino-acids, carbohydrates, minerals, as well as vitamins, it was used as a dietary and a fortifying food. During the last few years, numerous pollen formulas are developed and marketed worldwide. Furthermore, researches that revealed the promising biological activities (therapeutic effects) of pollen extracts in recent years enhanced greatly the importance of pollen consumption in the society. In Algeria, as well as in other countries, pollen is used by people for various reasons, principally, as a weight raising food, general health wellbeing, as a fortifying agent and prostate hypertrophy treatment. In practice, apiarists collect pollen using pollen traps. These later are non sterile and may contain various and different microbial populations originated during either manufacturing, transport, storage or handling. In addition, from flowers to beehives, pollen is exposed to microbial contamination by dust, air, insects, animals, and man. Most bee combs in Algeria, are not far away from industrial zones and metropolitan activities. Therefore, during their foraging, bees collect other man manufactured products, such as patisseries, sweet cakes; which may be contaminated by human or animal wastes. From another aspect, apiarists give, usually, for their bees, home made syrup basically prepared from sugar, milk and water. These latter ingredients are sources for microbial contamination and suitable growth medium for bacteria and fungi. Bee-Pollen, after collection, will be dried, purified, packed, stored and marketed to the consumers without an effective

hygienic and sanitary control. During each step of this important manufacturing chain, pollen continues to load and kept the original microbial flora as the first step, drying (40 °C max), is insufficient to removing and/or reducing microbial populations. Pollen is a suitable carrier for Gram positive and Gram negative bacteria, fungi and yeasts. Fungi and some bacterial species are spore formers. Also, growth and aflatoxin production, carcinogenic secondary mycotoxins, have been reported by Pitta and Markaki **[3]**. In addirion, our preliminary study **[4, 5]** on pollen microbial flora revealed the existence of elevated numbers of molds, coliforms, *Staphylococcus aureus* as well as aerobic and anaerobic microorganisms **[6]**. Some of these bacteria like *Bacillus cereus* and *Clostridium perfringens* are recognized as potential pathogenic organisms and have been incriminated in food poisoning **[7, 8]**. Pollen formation (flowering) and harvesting occur during warmth and humid seasons. Such environmental conditions favour microbial contamination and proliferation. Pollen can be classed as important vehicle for various micro-organisms implicating possible health problems for consumers and shelf life problems. Foodborne diseases are perhaps the most wide-spread health problem in the contemporary world and an important cause of reduced economic productivity **[9]**. In most of the cases of foodborne illness, the pathogenic effect occurs in the alimentary tract giving rise to symptoms of diarrhoea and vomiting. Since it is a natural product, all pollen constituent can be degraded by bacteria and fungi. Unscientific methods of harvesting, inappropriate drying and purification, unsuitable packing, storage and transportation, inadequate hygiene of producers and congenital climatic conditions render the raw material prone to infestations and exposed it to many microbial contaminants. Raw plant materials are most often degraded by microorganisms before harvesting, during handling and after prolonged storage **[10, 11]**. The presence of sufficient numbers of microorganisms can be harmful to consumers. As a result of fungal contamination, the risk of mycotoxin production, especially aflatoxins, should be taken into consideration in the manufacturing process because of the proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive activities **[12, 13, 14, 15]**. From a legal point of view, only limited countries worldwide have a legislation code for pollen manufacturing **[16]**. Thus, mush more studies for understanding the microbial load and critical quality control levels for pollen and pollen products are necessary. In this study, 15 samples of marketed pollen were analysed for their microbial content. Isolates of microbial contamination indicators were subjected for identification and antibiotic susceptibility testing.

## MATERIALS AND METHODS

### *Sampling*

A total of 15 samples of pollen were collected from local public markets. 13 samples were packed in glass or plastic containers without vacuum and 2 hand-collected other samples were freshly obtained from bee-keepers. All the samples (150 g each) were transported to the laboratory and stored at 4°C, until testing. They were analysed within 24 h of sampling. Prior to analysis, 25 g of each sample was homogenized for 10 min with 225 ml of 0.1% sterile peptone water ($10^{-1}$ dilution). Serial dilutions were performed as required. The pH of the food samples was measured using a digital pH meter (Hanna 8417, Italy) in a 1:10 (w/v) mixture of the homogenate in sterile distilled water. Moisture of each sample was measured after drying three subsamples (1 g each) at 95°C until the obtention of constant weight.

### *Total aerobic plat counts*

The pollen samples of which dilution has been prepared were plated on plate count agar (PCA). Plates were incubated at 30°C for 72 hours aerobically.

*Enumeration and identification of molds*

From each dilution in peptone water, 0.1 mL was spread onto potatoes Dextrose Agar (PDA). The plates were incubated at 25°C for 5 days. Each distinct mould colony was observed microscopically for morphological characterization and identification **[14, 15]**.

### *Enumeration of Enterobacteriaceae*

Enterobacteria were counted by transferring a 100 µL aliquot of the appropriate dilution into Petri plates and poured with Mac Conkey agar and Brilliant Green Lactose Bile broth (BGLB). Plates were incubated at 35°C and typical colonies were counted after 24 h of incubation and subjected for biochemical identification.

### *Isolation of Salmonella* spp.

*Salmonella* spp. was detected in 4 steps. Pre-enrichment (25 g of pollen in 225 mL in buffered peptone water) at 37°C for 16-20 h, was followed by enrichment in Rappaport-Vassiliadis (RV) broth incubated at 42°C for 24 h. The isolation was done on xylose lysine desoxycholate (XLD) agar at 37°C for 24 h. The colonies on the XLD agar plate were transferred to a triple sugar iron agar slant (TSI, Becton, Dickinson and Company) and incubated at 35°C for 24 h. The colonies on the TSI agar slant were chosen for identification based on morphological and biochemical tests.

***Isolation and identification of Staphylococcus aureus***

Enrichment of the bacteria was done by adding one gram (1g) of the sample into peptone water and incubated for 18 h at 37°C. Isolation of the *Staphylococcus aureus* was achieved by streaking the pre-enriched culture from the peptone water on to a selective differential agar plate of Baird-Parker Agar (BPA) which was freshly prepared following manufacturer's instructions. The plates were then incubated at 37°C for 24 h under aerobic conditions. Suspected colonies of being *S. aureus* (circular, smooth, convex, moist, and gray to jet-black, frequently associated with an outer clear zone) were subjected to biochemical tests.

***Detection of Listeria* spp.**

Twenty-five grams of pollen was homogenized in 225 mL of Listeria-Enrichment Broth (LEB) for 48 h at 37°C. Loopful of culture was streaked on to Listeria-Selective Agar (LSA). Characteristic positive colonies were picked up and subcultured in Brain Heart Infusion Broth (BHI) at 37°C for 24 h and conserved in the same broth containing glycerol (15%, v/v) at 5°C for further biochemical confirmation.

## RESULTS

***Moisture and pH***

Fifteenth pollen samples purchased from local Algerian markets were subjected for microbiological analysis. Moisture and acidity (pH) of the samples were also measured (Table 1), pH values varied from 4.55 (from Mila sample) to 6.29 (from Egypt sample and Biskra sample). Statistical analyses indicate that there was no significant difference between pH values of analysed pollen samples ($P > 0.05$). In addition, moisture content of pollen ranged from 18.11% to 36.29% (samples from Egypt and Constantine respectively). Raw pollen obtained from Biskra and Constantine has the highest moisture content (3.036 and 36.29% respectively, Table 1). A two-way ANOVA test revealed that there was a significant difference ($P < 0.05$) between relative humidity of pollen samples PS-Alg1/PS-Egy, PS-Alg2/PS-Cons, PS- Chi1/PS-Egy, PS-Alg3/PS-Cons, PS-Alg4/PS-Cons, PS-Bli/PS-Cons, PS-Msi/PS-Cons, PS-Bis/PS-Egy, PS-BBA/PS-Cons, PS-Syr/PS-Egy, and PS-Egy/PS-Chi2. Furthermore, extremely significant difference between moisture of pollen from Egypt and Constantine ($P < 0.001$) was observed.

**Table 1: Moisture, pH and microbial count for 15 pollen samples. Results are expressed as Log CFU/g of wet pollen and presence (+) or absence (-) for *Salmonella* and *Listeria***

| Pollen samples | TAMC[a] | TMYC[b] | *Staphylococcus aureus* | Enterobacteria | *Salmonella* spp. | *Listeria* spp. | pH | Moisture (%) |
|---|---|---|---|---|---|---|---|---|
| **PS-Alg1** | 3.30 | 2.770 | 2.477 | 7.18 | + | + | 4.72 | 28.42 |
| **PS-Alg2** | 3.30 | 2.690 | 2.903 | ND | - | - | 5.11 | 25.79 |
| **PS-Chi1** | 3.60 | 3.450 | ND | ND | - | + | 6.03 | 27.92 |
| **PS-Sét** | 3.95 | 3.230 | 3.301 | 8.016 | + | - | 5.86 | 26.85 |
| **PS-Alg3** | 3.85 | 2.300 | 2.903 | 5.38 | + | + | 4.97 | 25.43 |
| **PS-Alg4** | 3.60 | 2.300 | 2.903 | 6.96 | + | - | 5.33 | 25.48 |
| **PS-Bli** | 5.48 | 2.850 | 2.602 | 5.72 | + | + | 5.18 | 26.58 |
| **PS-Mil** | 4.56 | 4.090 | 3.204 | ND | - | + | 4.55 | 27.57 |
| **PS-Msi** | 3.70 | 2.480 | 2.301 | ND | - | + | 5.30 | 25.14 |
| **PS-Bis** | 5.49 | 6.920 | 7.890 | 4.18 | + | + | 6.30 | 30.36 |
| **PS-BBA** | 3.30 | 3.080 | 2.477 | ND | - | + | 5.19 | 26.18 |
| **PS-Syr** | 3.57 | 3.000 | 2.477 | 4.32 | - | - | 5.51 | 28.36 |
| **PS-Egy** | ND[c] | 6.990 | 8.320 | 7.67 | + | + | 6.29 | 18.11 |
| **PS-Chi2** | ND | 4.480 | 2.778 | 4.20 | - | - | 6.14 | 27.90 |
| **PS-Cons** | ND | 4.210 | 6.420 | 6.41 | - | + | 5.38 | 36.29 |
| **Safety criteria (Campos *et al.* 2008)** | < 5 Log CFU/g | < 4.7 Log CFU/g | Absent/1 g | < 2 Log CFU/g | Absent/10 g | / | / | / |

*[a] TAMC. Total aerobic mesophylic count; [b] TMYC. Total molds and yeasts count; [c] ND. not detected; +. presence; -. absence. PS: pollen sample, PS-Alg1: Algiers1, PS-Alg2 : Algiers2, PS-Alg3 : Algiers3, PS-Alg4 : Algiers4, PS-Chi1 : China1, PS-Chi2 : China2, PS-Sét : Sétif, PS-Bli: Blida, PS-Mil: Mila, PS-Msi: M'sila, PS-Bis: Biskra, PS-BBA: Bordj Bou-Arreridj, PS-Syr: Syria, PS-Egy: Egypt, PS-Cons: Constantine.*

***Total Microbial Load***

Total microbial count indicates that all pollen samples with the exception of pollen from Egypt, second sample from China and sample from Constantine (CFU=0), have less or more microbial load (Log CFU/g = 3.00 for pollen from Syria to Log CFU/g = 5.48 for pollen from Blida, Table 1). Furthermore, height fungal loads was observed for the pollen sample from Biskra and from Egypt sample (Log UFC/g = 6.92 and 6.99 respectively, Table 1), an intermediate fungal content (Log UFC/g=2.3 to 4.48) for the other samples. Statistical analysis (Two-way ANOVA

test) indicates that there were no significant differences between values total microbial count as well as fungal count for all pollen samples ($P > 0.05$).

**Table 2: Fungal species isolated from pollen samples**

| Molds | Samples | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PS-Alg1 | PS-Alg2 | PS-Chi1 | PS-Sét | PS-Alg3 | PS-Alg4 | PS-Bli | PS-Mil | PS-Msi | PS-Bis | PS-BBA | PS-Syr | PS-Egy | PS-Chi2 | PS-Cons |
| *Aspergillus flavus* | | + | | | | | | | + | | + | + | | | |
| *A. niger* | + | | + | | | | | | | | + | + | | | |
| *A. alliaceus* | | | + | + | | | | | | | | | | + | + |
| *Penicillium* sp. 01 | | + | | | | + | | | | + | | | | | |
| *Penicillium* sp. 02 | | + | | | + | | | | | | | | + | | |
| *Penicillium* sp. 03 | | | | + | | | | | | | | | | | |
| *Penicillium* sp. 04 | | | | + | | | | | | | | | | | |
| *Penicillium* sp. 05 | | | | | + | | | | | | | | | | |
| *Penicillium* sp. 06 | | | | + | | | | | | | | | | | |
| *Alternaria alternata* | | | | + | | | | + | | | | | | + | |
| *Alternaria* sp. 01 | | | | + | | | + | | | | | | | | |
| *Alternaria* sp. 02 | | | | + | | | + | | | | | | | | + |
| *Monila sitophilia* | | | | | | | + | | | | | | | | |
| *Cladosporium werneckii* | | | | + | | | | | | | | + | | | |
| *Drechslera tritici-repentis* | | | | + | | | | | | | | | | | |
| *Verticillium albo-atrum* | + | | | | + | | | | + | | | | + | + | |
| *Rhisomucor pusillus* | + | | | | + | | | | | | | | | | |
| *Mucor hiemalis* | | | | | + | | | | | | | | | | |
| *Sepedonium chrysospermum* | | | | + | | | | + | + | | | | | | |
| *Phialophora verrucosa* | + | + | | | | + | + | + | | | | | | + | |
| *Monascus ruber* | | | | | | | | + | | | | | | | |
| *Geotrichum candidum* | | | | | | | + | | | | | | | | + |

***Pathogen Content***

Considering now *Staphylococcus aureus*, the absence of these bacteria only in one pollen sample imported from China. The bacterial density of the other samples varied from 2.30 to 8.32 Log CFU/g. The highest was for pollen imported from Egypt (8.32 Log CFU/g) followed by 7.89 Log CFU/g for pollen from Biskra (Table 1). The average of *Staphylococcus aureus* density for the other samples ranged from 2 to 4 Log CFU/g (Table 1). A significant statistical difference ($P < 0.05$) exists between Log CFU of one sample imported from China and pollen sample from Biskra. However, a very significant difference ($P < 0.01$) was also observed between values the same china's pollen sample and that for the sample imported from Egypt. No statistical difference was observed for values of enterobacteria count ($P > 0.05$).

The average of enterobacteria count ranged from 4.18 to 8.016 Log CFU/g for ten samples. In opposite, counts of enterobacteria for the other five samples were negative. It seems that samples from Sétif, Egypt, Constantine, Blida and Algiers were very contaminated (5.38 to 8.016 Log CFU/g). As another indicator of fecal contamination, *Salmonella* was recovered from seven samples (Table 1). Elevated bacterial density was observed. Only one sample of imported pollen (from Egypt) contains *Salmonella* spp., the other analyzed samples which contain these bacteria were locally produced. Also, *Listeria* was detected in 10 samples, from which 2 were imported, one from China and another from Egypt (Table 1).

***Microbial identification***

Twenty-two fungal isolates were assigned to species level based on morphological characteristics. They were characterized to 16 different species (*Aspergillus flavus, A. niger, A. alliaceus, Penicillium* sp., *Alternaria alternate, Alternaria* sp., *Monila sitophilia, Cladosporium werneckii, Drechslera tritici-repentis, Verticillium albo-atrum, Rhisomucor pusillus, Mucor hiemalis, Sepedonium chrysospermum, Phialophora verrucosa, Monascus ruber* and *Geotrichum candidum*) (Table 2). Furthermore, From Mac Conkey agar plats, several and morphological different colonies derived from the culture of each pollen sample were subjected for biochemical characterization. Table 3 shows results of the identified isolates belonging to enterobacteria.

**Table.3: Bacterial isolats belonging to enterobacteria recovered from pollen samples**

| Number of isolats | Pathogen identity | Pollen samples in which pathogen was recovered |
|---|---|---|
| **12** | *Salmonella* sp. | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét |
| **25** | *Shigella* sp. | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1 |
| **30** | *Proteus mirabilis* | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2 |
| **28** | *Citrobacter diversus* | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2 |
| **14** | *Klebsiella* sp. | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2 |
| **26** | *Escherichia coli* | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2 |
| **19** | *Providencia* sp. | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Chi2, PS-Mil, PS-Msi |
| **29** | *Enterobacter cloacae* | PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2 |

## DISCUSSION

Plant products have been identified and confirmed as a significant source of pathogens and chemical contaminants that pose a potential threat to human health worldwide. There has been a growing interest in developing and applying microbiological criteria to the sanitary control of beehive products especially pollen. Ideally such standards should be based on bacterial counts associated with disease. Instead, a standard related to sanitation practices might specify the permissible number of microorganisms in an acceptable food product derived from CFU counts obtained in a large-scale study on representative pollen samples. Usefulness of these study data is limited since the study was designed to survey microbial counts in only fewer samples **[17]**.

In the present study, 15 pollen samples were analysed. Their acidity, moisture, microbial load and pathogen content were determined. Enormous total aerobic mesophylic count (TAMC) was recorded for 12 samples (>3 Log CFU/g). In three samples total aerobic mesophylic microorganisms was nil even their pH values were near neutrality and they have low moisture. It seems that microorganisms in these samples were in dormant stat and needs longer period than that of incubation time for adaptation to the laboratory chemical and physical growth conditions. Furthermore, total molds and yeast count (TMYC) was also important. All pollen samples have remarkable fungal density. Comparing our results with that recommended by Campos *et al*. **[16]** (TAMC not exceed than 5 Log CFU/g and a TMYC less than 4.7 Log CFU/g), three pollen samples (PS-Bli, PS-Bis, and PS-Egy) were out of safety recommendations. In addition, according to the same safety criteria (Absent of *Staphylococcus*/1 g and enterobacteria should be less than 2 Log CFU/g); all pollen samples (except that imported from China, PS-Chi1) were of pour microbiological quality. Ten samples contain more than 2 Log CFU of enterobacteria/g, and 14 samples contain more than 8 Log CFU of staphylococci per gram of pollen. More importantly, the detection of *Salmonella* in seven pollen samples is a direct evidence for a fecal contamination, and renders the food material a potentially product and indicates poor food handling practices.

In fact, the test for *Enterobacteriaceae* has replaced the tests for coliforms that traditionally have been used as indicators of hygiene and contamination after processing. The major problems with the coliform tests are the variability in definition of the term coliforms (they are defined usually by the method used for their detection) and the fact that only lactose fermenting organisms are detected. In comparison the family *Enterobacteriaceae* is well defined taxonomically and methods for their enumeration are based on common properties. Furthermore, the methods also detect important non-lactose fermenting organisms such as *salmonellas* **[18]**. Also, *Listeria* spp. other than *L. monocytogenes* are rarely implicated in illness. They are indicators for the likely presence of *L. monocytogenes*. Furthermore, this pathogen is widely distributed in the environment and is able to multiply slowly at 4°C. The shelf life of foods varies enormously, and the presence of *L. monocytogenes* at any level may be of significance due to its potential for growth during storage. The use of an enrichment procedure, in addition to enumeration, should therefore be considered to ensure that the organism is absent from the product.

From another point of view, the slightly acidic aspect of the analyzed pollen samples favors the development of fungi and mycotoxin production. *Aspergillus, Penicillium, Alternaria, Mucor* and others were recovered from pollen. They are involved in various diseases such as allergic illness, mycotoxicosis, and aflatoxicosis **[19]**. In addition, the recovery of potent pathogenic bacteria belonging to the family *Enterobacteriaceae* in another proof of unsatisfactory and unacceptable pollen set for human consumption in local markets. A greatly intension should be taken developing legislation considering pollen and pollen extracts as well as standards for microbial quality control

of this type of products. More importantly, expanded studies about pollen processing and preservation to reduce microbial load pathogen elimination are necessary.

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