Culture and molecular identification of fungal contaminants in edible bird nests

Jennifer Xiao Jing Chen, Shew Fung Wong*, Patricia Kim Chooi Lim and Joon Wah Mak

School of Medical Sciences, International Medical University, Kuala Lumpur, Malaysia

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Widespread food poisoning due to microbial contamination has been a major concern for the food industry, consumers and governing authorities. This study is designed to determine the levels of fungal contamination in edible bird nests (EBNs) using culture and molecular techniques. Raw EBNs were collected from five house farms, and commercial EBNs were purchased from five Chinese traditional medicine shops (companies A–E) in Peninsular Malaysia. The fungal contents in the raw and commercial EBNs, and boiled and unboiled EBNs were determined. Culturable fungi were isolated and identified. In this study, the use of these methods revealed that all EBNs had fungal colony-forming units (CFUs) that exceeded the limit set by Standards and Industrial Research Institute of Malaysia (SIRIM) for yeast and moulds in EBNs. There was a significant difference ($p < 0.05$) in the number of types of fungi isolated from raw and commercial EBNs, but no significant difference in the reduction of the number of types of fungi after boiling the EBNs ($p > 0.05$). The types of fungi isolated from the unboiled raw EBNs were mainly soil, plant and environmental fungi, while the types of fungi isolated from the boiled raw EBNs, unboiled and boiled commercial EBNs were mainly environmental fungi. Aspergillus sp., Candida sp., Cladosporium sp., Neurospora sp. and Penicillium sp. were the most common fungi isolated from the unboiled and boiled raw and commercial EBNs. Some of these fungi are mycotoxin producers and cause opportunistic infections in humans. Further studies to determine the mycotoxin levels and methods to prevent or remove these contaminations from EBNs for safe consumption are necessary. The establishment and implementation of stringent regulations for the standards of EBNs should be regularly updated and monitored to improve the quality of the EBNs and consumer safety.

Keywords: edible bird nest; fungi; contaminants; molecular techniques

Introduction

Edible bird nests (EBNs) are made from the regurgitated saliva of the swiftlets Aerodramus species (formerly known as Collocalia species) (Marcone 2005). EBNs have been popularly consumed for centuries as an expensive delicacy and a tonic believed to have many beneficial health effects (Hobbs 2004). EBNs are either boiled with rock sugar as a sweet soup or boiled with chicken and abalone as a savoury dish. The Aerodramus swiftlets originally breed in mountain or seaside caves. However, due to the high demand for their nests, these swiftlets are now domestically bred in man-made house farms or abandoned shop houses in suburban areas (Sia 2012). The nests are then harvested and processed before being packaged and sold in traditional Chinese medicinal shops or supermarkets.

The industry was greatly affected by the ban by China in 2011, the main importer of Malaysia’s EBNs, as high nitrite levels were detected in some of the red EBNs from Malaysia. Some of the nests were found to contain between 200 and 350 mg kg$^{-1}$ of nitrite, which was far above the 34 mg kg$^{-1}$ permissible level set by WHO for all food products. Following this incident, many standard operating procedures and regulations have been implemented and the Malaysia Bird Nest Alliance was established to monitor the quality of EBNs in Malaysia. The most recent requirement for exported EBNs has been established between the Agriculture and Agro-Based Industry Department of Malaysia and the Chinese authorities in September 2012. The maximum limits for heavy metals, and microbial contents such as bacteria, yeast and mould in EBNs were set by the Standards and Industrial Research Institute of Malaysia (SIRIM) to monitor the standards and quality of EBNs in Malaysia (Department of Standards of Malaysia 2010, 2011, 2012). These regulations have been implemented and enforced by the Food Safety and Quality Division of the Ministry of Health, Malaysia.

The microbial and allergen contents in the EBNs remain undetermined. Hence, this study was designed to quantify, isolate and identify the fungi associated with EBNs. The tropical climate in Malaysia promotes fungal growth. Research has been conducted to isolate fungi from the feathers, body and nests of other birds such as pigeons, ducks and parrots, but not on Aerodramus swiftlets (Pugh & Evans 1970). Fungi cause food spoilage and may...
produce mycotoxins which are harmful to consumers and pose health risks to swiftlet farmers through the inhalation of spores or direct contact. The sources of fungi in EBNs could primarily be from the salivary product, feathers or body of the swiftlet itself or from the surrounding environment. Fungi could also be introduced during the storage of these EBNs before consumption. The clinical implications of fungal contamination on human health drive the need for this study.

Materials and methods

Collection of raw and commercial EBNs from different localities in Malaysia

The unprocessed (raw and uncleaned) EBNs were purchased from house farms in five different localities in Malaysia: Kuala Sanglang (Perlis; 6°16’0″ North, 100°38’0″ East), PantaiRemis (Perak; 4°27’0″ North, 100°12’0″ East), Kluang (Johor; 02°01’30″ North, 100°15’0″ East), Kajang (Selangor; 2°59’0″ North, 101°47’0″ East) and Kota Bharu (Kelantan; 6°8’0″ North, 102°15’0″ East). The commercial EBNs were purchased from five different Chinese traditional medicine shops (companies A–E). Three to six nests were purchased from each locality/shop. Each nest was sealed in a sterile plastic bag and transported to the laboratory.

Processing of the EBN samples

Upon arrival at the laboratory, each sample (1 g) was weighed and placed into a 50 ml centrifuge tube. Under sterile conditions, 10 ml of sterile ultrapure water were added into each sample. The mixture was shaken vigorously for 3 min, forming a viscous gelatinous-like substance. Then, the gelatinous mixture was manually split into two equal parts using a spatula into two new tubes. One part of the mixture (approximately 5 ml; boiled EBNs) was placed into a beaker containing boiling water on a heating block (Thermal Bath Dual ALB128 Fine PCR) for 30 min. The mixture was transferred into 2 ml polystyrene sample tubes containing 1.0 mm diameter glass beads (Tomy Digital Biology, Tokyo, Japan) and was frozen at −80°C for 5 min before being subjected to mechanical disruption with a MicroSmash™ mechanical homogenising system (MS-100, Tomy Digital Biology) for 2 min at 5000 rpm. The samples were then frozen at −80°C for 5 min and the disruption cycle was repeated for 10–30 cycles until about 80% of the fungal cells were disrupted when checked under a compound microscope using a wet mount. The homogenate was transferred into a new microcentrifuge tube and centrifuged at 2300 rpm for 10 min. The supernatant was discarded and the pellet was resuspended with 600 μl of sorbitol buffer. Lyticase from Arthrobacter luteus (200 units, Sigma-Aldrich) was added and the mixture was incubated at 30°C using a heating block (Thermal Bath Dual ALB128 Fine PCR) for 30 min. The mixture was transferred into 2 ml polystyrene sample tubes containing 200 μl of sorbitol buffer and lyticase (200 units, Sigma-Aldrich) was added and the mixture was incubated at 30°C using a heating block (Thermal Bath Dual ALB128 Fine PCR) for 30 min. The mixture was transferred into 2 ml polystyrene sample tubes containing 1.0 mm diameter glass beads (Tomy Digital Biology, Tokyo, Japan) and was frozen at −80°C for 5 min before being subjected to mechanical disruption with a MicroSmash™ mechanical homogenising system (MS-100, Tomy Digital Biology) for 2 min at 5000 rpm. The samples were then frozen at −80°C for 5 min and the disruption cycle was repeated for 10–30 cycles until about 80% of the fungal cells were disrupted when checked under a compound microscope using a wet mount. The homogenate was transferred into a new microcentrifuge tube and centrifuged at 2300 rpm for 10 min. The DNA of the fungi was extracted using the supernatant and the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

DNA amplification with polymerase chain reaction (PCR)

DNA with sufficient concentration and acceptable purity (A260/280 ratio = 1.8–2.0) was amplified with PCR. The volume of extracted DNA used for the PCR amplification was adjusted individually to a final concentration of 50 μg ml⁻¹. For each PCR reaction, a PCR mixture
containing 5 µl of 5× Hotstar HiFidelity PCR buffer (Qiagen), 5 µl of Q solution (for amplification of GC-rich targets), 0.5 µl of Hotstar HiFidelity DNA polymerase (2.5 units µl⁻¹) and 0.125 µl (0.5 µM) of each oligonucleotide primer (ITS1 and ITS4) was prepared. The nucleotide sequences for the forward primer (ITS1) and reverse primers (ITS4) were 5’-TCC GTA GGT GAA CCT GCG G-3’ and 5’-TCC TCC GCT GGT TAT GGA GC-3’ respectively (Luo & Mitchell 2002). DNase-free water was added in accordance to the volume of DNA used, bringing the whole PCR mixture to a total volume of 25 µl. For the negative control, the DNA template (50 µg µl⁻¹) was then substituted with DNase-free water. For the positive control, a fungal DNA sample with known successful PCR products was included for each PCR amplification cycle. The reaction mixtures were subjected to 35 cycles of amplification for 1 min at 95°C for denaturation, 1 min at 55°C for primer annealing and 2 min at 72°C for elongation using MyCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Electrophoretic separation of the PCR products on 2% agarose was performed using an electrophoresis system (Mini Sub-Cell GT, Bio-Rad) and Tris/borate/EDTA (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA) buffer at 80 V for 1 h. After completion of the electrophoresis, the gel was stained with ethidium bromide and visualised using a gel documentation system (Biovision, Milpitas, CA, USA) and photographed with the Biovision 1000 super-bright transilluminator software. The amplified PCR products were sent for PCR purification and DNA sequencing (Tech Dragon Ltd, Shatin, Hong Kong). Following which, alignment analysis of the internal transcribed spacer (ITS) regions was performed using the Basic Local Alignment Search Tool (BLAST) software from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) to determine the species of the fungi.

**Statistical analysis**

Statistical analysis was performed using the SPSS Software (PASW 18). A Student’s *t*-test was used to compare the number of fungal types found in raw and commercial EBNS. A paired *t*-test was used to compare the difference in the number of types of fungi isolated from EBN extracts before and after boiling the EBNS. The difference was considered statistically significant if *p* < 0.05.

**Results**

The colony-forming units (CFUs) for each EBN-inoculated agar plate were counted daily up to a maximum of 28 days to monitor fungal growth. The colonies were counted based on gross observation of distinctively separate and individual colonies. Some plates had rapidly growing or slow-growing fungal colonies. Some even had a mixture of both fast and slow growers. Colonies were counted based on their defined colony circumferences, regardless of their size. Some fungal colonies were very large and occupied large areas of the agar, and inhibited the growth of other colonies; such plates had low numbers of CFUs. On the other hand, some fungal colonies were very small or pinpoint, widely spread and scattered on the whole agar plate, with a larger number of CFUs.

The CFUs per plate on the 14th day after inoculation of the EBNS extract varied between samples of the same and between different locations (Table 1). For some of the agar plates, the overgrowth of large colonies caused an accurate colony count to be almost impossible. Raw EBNS obtained from Kajang (KJ 1–3) were found to have the highest number of CFUs on day 14 at 176, 389 and 452 respectively. All three agar plates inoculated with EBNS obtained from Pantai Remis (PR 1–3) showed an overgrowth of certain fungal colonies which occupied the entire surface of the agar, causing colony counts to be determined based on records of the last highest CFUs number on day 14.

### Table 1. Colony-forming units (CFUs) of fungi in the raw and commercial edible bird nests (EBNs) (dilution factor = 40×).

| Sample | Colonies per plate | CFU g⁻¹ | Sample | Colonies per plate | CFU g⁻¹ |
|--------|--------------------|---------|--------|--------------------|---------|
| KE1    | 6                  | 240     | A1     | 1                  | 40      |
| KE2    | 4                  | 160     | A2     | 9                  | 360     |
| KE3    | 3                  | 120     | Average| 5                  | 200     |
| KE4    | 11                 | 440     | B1     | 70                 | 2800    |
| KE5    | 10                 | 400     | B2     | 34                 | 1360    |
| KE6    | 10                 | 400     | Average| 52                 | 2080    |
| Average| 7.3                | 293.3   | C1     | 0 (66⁶)            | 2640⁶   |
| PR1    | 12                 | 2080    | C2     | 0 (30⁶)            | 1200⁶   |
| PR2    | 18                 | 480     | Average| 48                 | 1920    |
| PR3    | 18                 | 720     | D1     | 13                 | 520     |
| Average| 27.3               | 1093.3  | D2     | 11                 | 440     |
| KLG1   | 19                 | 760     | Average| 12                 | 480     |
| KLG2   | 10                 | 160     | E1     | 1                  | 40      |
| KLG3   | 11                 | 440     | E2     | 7                  | 280     |
| Average| 11.3               | 453.3   | Average| 4                  | 160     |
| KJ1    | 176                | 7040    |        |                    |         |
| KJ2    | 389                | 15560   |        |                    |         |
| KJ3    | 452                | 18080   |        |                    |         |
| Average| 339                | 13560   |        |                    |         |
| KB1    | 10                 | 400     |        |                    |         |
| KB2    | 1                  | 40      |        |                    |         |
| KB3    | 2                  | 80      |        |                    |         |
| Average| 4.3                | 173.3   |        |                    |         |

Notes: *Overgrowth of some colonies on the whole plate inhibited growth of other colonies.

*Growth on 28 days.
Among the commercial EBN samples it was found that EBN samples from company B (B1 and B2) had the highest number of CFUs on day 14 at 70 and 34 respectively. On the other hand, no growth was observed on the agar plates inoculated with EBNs from company C up to the 14th day. The colonies took longer than 2 weeks to grow, and CFUs recorded on day 28 were 66 for sample C1 and 30 for sample C2.

The CFU g$^{-1}$ for all the EBNs in this study were found to exceed the permissible limits set by SIRIM for yeast and mould ($\leq 10$ CFU g$^{-1}$).

**Types of fungi isolated from the raw and commercial EBNs**

The fungal colonies were isolated from the primary EBN-inoculated agar plates initially based on their morphologies until a single pure colony was obtained. It was found that the average number of fungal types isolated from the raw EBNs (average = 22) was higher compared with the number of fungi isolated from the commercial EBNs (average = 9). Among the raw EBN samples, EBN samples from Perak showed the highest number of types of fungi, followed by Kedah, Johor, Selangor and Kelantan.

Among the commercial EBNs, EBN samples from company B showed the highest number of types of fungi (14 different types), followed by companies D, A, C and E respectively. There was a significant difference ($p < 0.05$) in the number of types of fungi isolated from the raw and commercial EBNs (Student’s t-test, $p = 0.03$).

Identification using molecular techniques was successful only for some of the fungal types (up to 49 types) despite many attempts of DNA extraction and PCR amplification. For the isolates that were successfully identified using both morphological and molecular methods, it was found that several isolates that appeared to be different morphologically were in fact of similar species after DNA sequencing and analysis. Some were found to be different strains of the same genus of the fungi.

For isolates without molecular data, the identification based on morphological methods could not be confirmed. Thus, the number of types of fungi isolated from EBNs based on their confirmed identifications could not be determined.

**Table 2** shows the different fungi genera (up to 49 types) isolated from the raw and commercial, and boiled and unboiled EBNs, which were divided into three groups to aid comparison, namely fungi generally found in the soil, on plants and in the environment.

| Soil fungi | Commercial EBNs | UNBOILED EBNs | BOILED EBNs |
|------------|----------------|---------------|-------------|
| Blastobotrys sp. | Chrysosporium sp. | Blastobotrys sp. | Phialosimplex sp. |
| Lichtheimia sp. | Nigrospora sp. | Chrysosporium sp. | |
| Nigrospora sp. | Paecilomyces sp. | Eurotium sp. | |
| Perenniporia sp. | Phialosimplex sp. | Lichtheimia sp. | |
| Phialosimplex sp. | Syncephalatrum sp. | Nigrospora sp. | |
| Sagenomella sp. | Stephanoascus sp. | Paecilomyces sp. | |
| Talaromyces sp. | |
| Plant fungi | | |
| Coprinellus sp. | Fomitopsis sp. | Coprinellus sp. |
| Fomitopsis sp. | Lasiodiplodia sp. | Fomitopsis sp. |
| Lentinus sp. | Lenzius sp. | Lasiodiplodia sp. |
| Letendrea sp. | Nigrospora sp. | Lenzius sp. |
| Polyporales sp. | | Nigrospora sp. |
| Rigidoporus sp. | | Polyporales sp. |
| Environmental fungi | | |
| (soil, food, air, dust, storage) | | |
| Aspergillus sp. | Aspergillus sp. | Aspergillus sp. |
| Candida sp. | Candida sp. | Candida sp. |
| Cladosporium sp. | Cladosporium sp. | Cladosporium sp. |
| Neurospora sp. | Neurospora sp. | Neurospora sp. |
| Penicillium sp. | Penicillium sp. | Penicillium sp. |
| Eurotium sp. | | Eurotium sp. |
Greater varieties of fungi genera were found in the raw EBNs compared with the commercial EBNs. Generally, more soil and plant fungi were found in the raw EBNs compared with the commercial EBNs. The fungi found in the commercial EBNs were mostly environmental fungi. Some of these soil and plant fungi were found to be wild mushrooms, e.g., *Paecilomyces* sp., *Lenzites* sp., *Fomiptosis* sp. and *Polyporales* sp. For the commercial EBNs, no plant fungi and only four types of soil fungi were isolated, namely *Nigrospora* sp., *Sebacinales* sp., *Chrysosporium* sp. and *Sagenomella* sp. *Sebacinales* sp. and *Chrysosporium* sp. were isolated in the commercial EBNs, but not from the raw EBNs.

Some genera of fungi known to be common in the environment were isolated from both raw and commercial EBNs, such as *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Neurospora* sp. and *Penicillium* sp. Another fungi, *Eurotium* sp., known to be commonly present in the environment, was isolated only from the raw EBNs but not from the commercial EBNs.

**Types of fungi isolated from the unboiled and boiled EBNs**

The types of fungi isolated from the unboiled and boiled EBNs based on morphological identification are compared in Figure 1. In general, it was observed that there was a reduction in the number of fungal types isolated after boiling the EBNs, with the exception of EBN sample KE3, where there was an equal number of fungal types found before and after boiling, and in EBNs of companies A (A2) and C (C1), where there were higher numbers of fungal types isolated after boiling the EBNs. However, there was no significant difference in the reduction of fungal types after boiling the EBNs (paired *t*-test = 0.310; *p* > 0.05).

It was found that there was a larger variety of fungal genera isolated from the unboiled EBNs compared with the boiled EBNs (Table 2). None of the soil and plant fungi genera isolated from the unboiled samples was found in the boiled samples, except for *Phialosimplex* sp. The majority of the fungi isolated in boiled samples were environmental fungi, such as *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Eurotium* sp., *Neurospora* sp. and *Penicillium* sp.

**Types of fungi isolated from raw EBNs according to locations, and commercial EBNs according to companies**

This study found that the types of fungal genera isolated from the raw EBNs varied between the locations from which the nests were collected (Table 3). *Aspergillus* sp. was isolated from the raw EBNs obtained from all the different locations studied. In fact, *Aspergillus* sp. was the only genus identified in all the EBN samples from Kelantan (KB). *Penicillium* sp. was also common in all the raw EBN samples from all locations, except for those from Kelantan (KB). *Eurotium* sp. was isolated from the raw EBNs obtained from three out of five studied locations.

This study also found that the types of fungi isolated from the commercial EBNs varied among the companies from which they were purchased (Table 3). *Penicillium* sp. was found to be most common isolated fungi among the commercial EBN samples from all the different companies.

![Figure 1](image-url)
companies, while *Aspergillus* sp. was isolated from the EBN samples purchased from three out of five companies (B, D and E).

**Identification of fungi isolated from EBNs**

Altogether, 66 different types of fungi were isolated from the raw and commercial EBNs in this study see the Online Supplementary material. (Figures 2a–g). Of these 66 different types, only 49 were successfully identified with both morphological and molecular methods, while 17 could not be identified due to problems with both staining and molecular methods.

**Discussion**

The CFU g⁻¹ for all the EBNs in this study were found to exceed greatly the permissible limits for CFUs of yeast and mould per g in EBNs set by SIRIM (yeast and mould ≤ 10 CFU g⁻¹). A few reasons could be drawn to explain this finding. Firstly, the standards set by SIRIM did not state when this CFU should be measured. In this study, the CFU was determined on the 14th day after inoculation for standardisation and comparison except for two commercial EBNs in view of the variation in size, overgrowth of fast-growing fungi and difficulty in counting accurately after that. In this study, daily monitoring and counting of the plates were performed. These may not be a good practice and is not recommended as it can cause dislodging of spores from already developed colonies, where such spores can form new colonies on the agar plate resulting in overestimation of counts. This is one limitation and should be addressed in future studies.

Secondly, transferring the remainder of the extract–agar mixture to the pour plate was not efficient as the whole amount of the extract may not be transferred, resulting in underestimation of mould counts in this study. If this aspect was taken into consideration, the CFUs determined may be higher.

Thirdly, the accuracy of CFUs is questionable and subjective as it has been shown that different technicians (all skilled and competent) frequently observed different counts on the same sample (Sutton 2011). This variation in counting can be minimised if the colonies on the counted plates are separated and well defined. It is more difficult to count colonies in the pour plate and hence for mould enumeration surface plating is recommended for future studies. Colony counts are also inaccurate because they only represent the colonies that happened to be well separated on the plate and can be distinguished after growth. In this study, the counting of colonies was difficult as different fungal colonies grew at different rates and to different sizes and forms. Some were very large and rapidly growing, overgrowing onto the entire surface of the agar. The large colonies inhibited or limited the growth of other colonies due to competition of nutrient and space. Thus, agar plates with large fungal colonies will then reflect a very small number for CFUs. In addition, due to the different growth period of each fungus, the rapidly growing fungi would have overgrown on the agar plates before the slow-growing ones could even have the chance to grow. To avoid this, selective media (e.g. dichloran-glycerol 18 (DG18), dichloran rose-Bengal chloramphenicol (DRBC)) can be used to slow down the growth of fast-growing moulds (King et al. 1979; Hocking & Pitt 1980).

Another limitation of this study was not taking into consideration the non-culturable fungi. Other molecular

| Genus of isolated fungi |
|-------------------------|
| **Raw EBNs**            |
| Kuala Sanglang, Kedah (KE) | *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Eurotium* sp., *Lentendreae* sp., *Lasiodiplodia* sp., *Neospora* sp., *Paecilomyces* sp., *Penicillium* sp., *Perenniporia* sp., *Sagenomella* sp., *Talaromyces* sp. |
| Pantai Remis, Perak (PR) | *Aspergillus* sp., *Eurotium* sp., *Letendreae* sp., *Penicillium* sp., *Stephanoascus* sp. |
| Kluang, Johor (KLG)      | *Aspergillus* sp., *Coprinellus* sp., *Eurotium* sp., *Fomitopsis* sp., *Penicillium* sp., *Polyporales* sp., *Rigidoporous* sp. |
| Kota Bharu, Kelantan (KB)| *Aspergillus* sp. |
| Kajang, Selangor (KJ)    | *Aspergillus* sp., *Blastobotrys* sp., *Candida* sp., *Cladosporium* sp., *Penicillium* sp., *Phialosimplex* sp. |

| **Commercial EBNs**      |
|--------------------------|
| Company A                | *Candida* sp., *Penicillium* sp. |
| Company B                | *Aspergillus* sp., *Penicillium* sp., *Sebacinales* sp. |
| Company C                | *Cladosporium* sp., *Nigrospora* sp., *Penicillium* sp. |
| Company D                | *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Sagenomella* sp. |
| Company E                | *Aspergillus* sp., *Chryosporium* sp., *Penicillium* sp. |
methods, e.g. metagenomics, can be used to perform genomic analysis of microorganisms by direct extraction and cloning of DNA of all culturable and non-culturable microorganisms present in a sample (Handelsman 2004).

Many challenges will be faced during the culture, isolation and identification of fungi using morphological and molecular methods. Most of the time, fungal identification is based on the morphology of the fungal reproductive structures. Some of the fungi could not be stained adequately for identification by microscopy. Some fungi require special culture media, different culture condition and duration for growth and induction of sporulation for the identification.

In addition, DNA extractions for some of the fungi were also unsuccessful with good yields and satisfactory ratios, albeit with many attempts. Perhaps in future different fungal genomic extraction kits and conventional methods, e.g. using Trizol or Tr reagent and phenol–chloroform extraction, and also lysis methods, may be used to overcome this problem. For this study, both mechanical (tissue grinder and glass beads) and biochemical (lyticate, tissue lyser) methods were used to break the rigid cell wall of the fungi. Then DNA was extracted using the Qiagen DNA extraction kit. Other conventional DNA extraction and lysis methods can be applied in future for those samples with poor yield for better recovery of DNA with the optimum quality and quantity (Cubero et al. 1999; Haugland et al. 2002). Among these are glass bead pulverisation with vortexing, grinding with a mortar and pestle followed by glass bead pulverisation, glass bead pulverisation using 1% hydroxyacetyl trimethyl ammonium bromide (CTAB) buffer in a water bath sonicator, water bath sonication in CTAB buffer, grinding followed by incubation with CTAB and lyticase enzymatic cell lysis (Burik et al. 1998; Löffler et al. 1997). One of the studies concluded that the use of glass beads with extended vortexing is optimal for extraction of microgram amounts of DNA from filamentous fungal cultures (Burik et al. 1998). However, from this study it was found that different types of fungi would require different cycles of mechanical homogenisation with glass beads, with some taking up to 30 cycles of homogenisation at 5000 rpm for 120 s.

In this study DNA extraction was carried out using a single kit, which was the Qiagen DNeasy Blood and Tissue Kit. This is a column-based method recommended for DNA extraction of multiple samples. However, it is possible that some of the fungi produced different inhibitory enzymes that might have inhibited the buffers used in this kit, thus the unsuccessful attempts at extracting the DNA. On the other hand, some of the extraction gave good and satisfactory DNA yield but could not be amplified with PCR, producing no desired product band on agarose gel. In addition, some of the PCR products sent for DNA sequencing were found to have no significant similarities upon alignment analysis with the NCBI Database. This finding requires further investigation.

Despite the above challenges, 66 different types of fungi were isolated from the raw and commercial EBNs in this study. Out of these, only 49 were successfully identified with both morphological and molecular methods, while 17 could not be identified due to problems with morphological method and unsuccessful extraction of fungal DNA, PCR amplification or DNA sequencing. The problems faced with DNA extraction were low DNA concentrations, or poor 260/280 nm ratio (< 1.8 or > 2.0). For fungi with successful DNA extraction, some of the PCR amplification for these DNA was unsuccessful, showing no band when visualised after gel electrophoresis.

Of the isolated fungi successfully identified, more than half were associated with plants and soil. Among them were Lenzites sp., Lasiodiplodia sp., Rigidoporus sp., Polyporales sp., Fomitopsis sp., Letendraea sp., Coprinellus sp., Paecilomyces sp., Perenniporia sp., Lichtheimia sp., Syncephalatrum sp., Sagenomella sp., Talaromyces sp., Stephanoascus sp., Nigrospora sp., Phialosimplex sp. and Blastobotrys sp. Some of these fungi were found to be common plant pathogens, or mushrooms that grew on trees, such as Paecilomyces sp., Lenzites sp., Polyporales sp. and Fomiopsis sp. Most of these fungi exhibit different morphology when they are free-living in the environment, and when they were cultured on SDA plates in the laboratory.

Most of these plant and soil fungi isolated from the raw EBNs are not known to be pathogenic in humans and most were found to be absent from the commercial EBNs, except for Sagenomella sp., Nigrospora sp., Sebanicalea sp. and Chrysosporium sp. The most possible explanation for this is that the raw EBNs have been harvested directly from the house nests of the swiftlets, and have yet to be cleaned or processed. Swiftlets live in the environment and are in contact with plants and soil while looking for food. Naturally, these plant and soil fungi will be on the bodies and feathers of the swiftlets, and possibly transferred to the EBNs produced. One of the fungal species, Chrysosporium sp., which was reported in a study on the fungi isolated from Malaysian soil sample, was also found in EBN samples in this study (Soon 1991). Although these species are not known to cause severe infections in immunocompetent humans, they may cause infections in animals. Sagenomella sp. is a filamentous fungus that causes systemic infection in dogs (Gene et al. 2003). Nigrospora sp. is known to cause superficial infection of the toes (onychomycosis) (Fan et al. 2009). Chrysosporium sp. was reported recently to cause invasive pulmonary infection in a 16-year-old male with acute T-cell lymphoblastic leukaemia (Suankratay et al. 2015). This species can cause endogenous endophthalmitis and allergic pneumonitis (Anstead et al. 2012; Shekhar et al. 2014).
For the commercial and boiled EBNs, most of the isolated fungi were those associated with the environment (air, food, dust and storage places). These fungi included *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Penicillium* sp., *Eurotium* sp. and *Neurospora* sp. This is probably because commercial EBNs have gone through a process of cleaning and bleaching, and the boiled EBNs have been boiled for 3 h at 100°C and these processes might have removed all the plant and soil fungi in the EBNs, except for the environmental fungi, which could most possibly be introduced from the environment after boiling or processing or during storage, or these genera may possess thermotolerant or thermoresistant properties.

Some fungi genera are known to be commonly found in the environment. The average concentration of fungal bioaerosols in indoor and outdoor air of the selected primary schools in Malaysia was 401 ± 235 CFU m⁻³ and the most frequently isolated fungal genera were *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and *Zygomycetes* (Hussin et al. 2011). These findings corresponded with the results in this study, where *Penicillium* sp. and *Aspergillus* sp. were the most common genera isolated from the EBN samples.

An interesting finding to note was the growth of fungi from plates inoculated with boiled EBNs. This suggested the possibility that these fungal isolates were heat tolerant, resistant or dormant especially for the spores. There have been many studies on heat-, baro- and preservative-resistant fungi that cause the spoilage of foods due to the formation of heat-resistant ascospores (Pitland & Christian 1970; Voldrich et al. 2004; Smits & Brul 2005; Yaguchi et al. 2012). Among the well-known thermotolerant food-spoilage fungi are *Byssoschlamys fulva*, *Aspergillus chevalieri*, *Aspergillus mangini*, *Talaromyces flavus*, *Talaromyces avellaneus*, *Xeromyces bisporus* and *Zygosaccharomyces bailii* (Pitland & Christian 1970; King 1997; Voldrich et al. 2004; Smits & Brul 2005; Yaguchi et al. 2012).

However, none of these species has been identified in this study, except for *T. flavus*, which was isolated from the raw and unboiled EBNs, but not in the commercial or boiled EBNs. Even though *T. flavus* was not isolated from commercial or boiled EBNs, its presence cannot be neglected. *Aspergillus*, *Penicillium* and *Paecilomyces* are known to be thermo-tolerant, with the maximum temperature for optimal growth at 55°C (Mouchacca 2000). However, the method employed in this study was boiling of EBNs up to 100°C for 3 h. So far there is no literature on fungi that can withstand such a high temperature for such a long duration, except for the recent discovery of Agni’s fungi in India the spores of which, it is claimed, can survive exposure to 115°C for 2 h (Suryanarayanan et al. 2011). Therefore, further studies are required to confirm the mechanism of survival of these fungi after boiling the EBNs.

Fungi have been known for centuries to be one of the causes of food spoilage. The persistence of fungal growth in heat-processed, high-pressure pasteurised, preservative-treated foods has baffled the food-processing industry, creating a great dilemma on the best way to process food. The common fungi genera isolated from the raw, commercial, unboiled and boiled EBN samples in this study were *Aspergillus* and *Penicillium*. These genera are known to cause super and the most frequently isolated fungal genera were *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and *Zygomycetes* (Hussin et al. 2011). These findings corresponded with the results in this study, where *Penicillium* sp. and *Aspergillus* sp. were the most common genera isolated from the EBN samples.

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often exposed to inhalation of fungal spores in the house farms.

Conclusions

Both the raw and commercial EBNs are contaminated with higher-than-permissible levels of fungi as determined using culture and molecular techniques. The types of fungi isolated from the unboiled raw EBNs were soil, plant and environmental fungi, while the types of fungi isolated from the boiled raw EBNs, unboiled and boiled commercial EBNs were mainly environmental fungi. Some of the fungi isolated such as Aspergillus sp. and Penicillium sp. may produce mycotoxins. These fungi were isolated from all EBN samples, even after boiling the EBNs. Therefore, relevant authorities like SIRIM and the Food Safety and Quality Division of Malaysia should take necessary actions to quantify the mycotoxin levels contained in EBNs and establish regulatory limits for mycotoxins levels in EBNs. This would perhaps serve as a better indicator on the fungal contamination in EBNs and its dangers to human health.

Some fungi found in the raw EBNs are known to cause respiratory infections, such as Cladosporium sp. and Eurotium sp. Therefore, swiftlet ranchers exposed to the frequent inhalation of these allergens might be at risk to hypersensitivity pneumonia and should take necessary precautions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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