

Microbial diversity and microbiological quality of edible-birdnest (EBN) during processing and storage

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Abstract

The microbial quality of 45 EBN consisting of raw unclean and raw clean Edible-birdnest (EBN) from two different premises were analyzed for 8 weeks. Using 3M™ Petrifilm, Total Aerobic Count (TAC) of raw clean EBN and Total Coliform Count (TCC) for raw unclean, 3 days, 2 weeks and 4 weeks EBN in both premises exceeded the limit stated in by the Malaysian Standard (MS 2334:2011; SIRIM/DVS 3:2015). A total of 264 bacteria and 208 fungi species were isolated. Bacteria isolated was identified using bioMérieux's API® kit and a series of biochemical tests. Fungi were identified using techniques described by the techniques of James and Natalie (2001). The most common bacteria encountered in both premises were *Staphylococcus* sp., *Corynebacterium* sp. and *Actinomyces* sp. While in premise B, *Aeromonas* sp. and *Ochrobactrum anthropi* were also found to be prevalent. The difference in occurrence of bacteria in EBNs in both premises at all stages were found to be non-significant ($P>0.05$). As for fungi, *Aspergillus niger* was the most encountered fungi in premise A. While in premise B, *Candida* sp. was the most frequently isolated in that premise. The most common fungi encountered in both premises were *Aspergillus niger* and *Mucor* sp. Differences in occurrence of the fungi were only significant in the raw unclean EBN and non-significant in the rest of the stages. The bacteria and fungi mostly found in this study were environmental microorganisms. In one of the premise practices, Good Manufacturing Practice (GMP), however the results indicate that the cleaning process of both premises are similar as the source of contamination could be caused by mishandling, cleaning, processing step and equipment.

Keywords: Bacteria, Edible-birdnest (EBN), Fungi, Good Manufacturing Practice (GMP)

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Introduction

The edible-birdnest (EBN) swiftlet farming industry is one of the major contributors to Malaysia Gross National Income (GNI) with a total value of RM 4.5 billion as reported by The Malaysia Economic Transformation Program (ETP) in 2011. Unfortunately, in 2011 a high level of nitrites in exported EBNs from Malaysia was reported, which led China to ban their imports from Malaysia resulting in a huge economic loss to the Malaysia EBN industry. This caused the demand for EBN from Malaysia to drop approximately 20% to 30%, while the prices depreciated by 20% (Ramli and Azmi, 2012). Thus, the food safety aspect of EBNs is crucial for the viability of the Malaysia EBN industry (Kew *et al.*, 2014). The World Health Organization (WHO) reported that the cause of the most widespread health problem and a drop in economic productivity in the today's world are due to illnesses caused by contaminated food. Throughout the world, microbial contamination of foods continues to be a major concern for public health, consumers, regulatory agencies and food industries. Foodborne pathogens cause gastrointestinal illnesses, which affect millions of population, mostly children, pregnant women, babies, the elderly and vulnerable people with diseases, and in many cases it can lead to death (WHO, 2017). To ensure the sustainability of the EBN industry, stringent enforcement is crucial. Hence, the government has come out with a comprehensive master plan for the development of the EBN industry. One of the strategies highlighted is the introduction of Good Manufacturing Practices (GMP) (Mohd Rashid and Shauqi, 2015). This Malaysian Standard provides guidelines to EBN processors for the production of quality and safe EBN for human consumption (Dept Standard Malaysia, 2011). The majority of birdhouses are not registered with the Department of Veterinary Services (DVS). There are an estimated 60,000 birdhouses operating in the country but less than 20 per cent have voluntarily registered (ETP Annual Report, 2013). In a local newspaper, DVS reported that swiftlet farming premises must be registered in the DVS system as it is a fully traceable system to ensure the EBNs from Malaysia are of good quality and can be traced to the bird house they came from. To date, DVS has registered more than 10,000 swiftlet premises and it is expected to be able to register at least 90 per cent if not all of the swiftlet premises by 2020 (The Star, 2016). Food contamination with antibiotic-resistant bacteria can be a major threat to public health. As a popular delicacy in promoting health it is important to ensure EBN is safe for consumption by screening the antibiotic sensitivity of bacterial isolates from EBN and identify any potential hazards that might affect the safety of consumers. There is no doubt that EBN is a valuable source of income to the country, hence maintaining its quality is crucial in order to ensure the sustainability of its production. Hence, the present study was designed with the objective of assessing the microbial quality of EBN during processing and storage at two types of EBN processing premises.

Materials and Methods

EBN sample preparations: A total of 90 EBN samples were used in this study with 45 EBN samples being obtained from each premise; premise A and premise B. The EBN samples from both premises were collected in Melaka state at five sampling stages which consisted of raw unclean EBN, raw clean EBN at 3 days, 2 weeks, 4 weeks and 8 weeks old with nine samples at each sampling stage. The raw unclean EBN were harvested from the edible-nest swiftlet ranch. The raw unclean EBN underwent cleaning processes which included but were not limited to sorting, picking feathers and impurities, molding, drying, grading and packing. Prior to sampling, the EBN samples were kept at room temperature (27°C) at the respective premises. All of the samples were transported in a cool box (4°C) to the Bacteriology Laboratory at the Faculty of Veterinary Medicine, University Putra Malaysia for further processing.

Bacteria identification: The entire exposed surface of each EBN was swabbed with a sterile moist cotton swab. Each swab was directly cultured onto blood and MacConkey agar (Difco USA) and incubated for 24 hours at 37°C. Colonies of different types were selected and further purified by sub culturing. Initial identification of the unknown isolates was done through Gram staining to identify the morphological appearance of the bacteria (cocci or bacilli, Gram negative or positive). The unknown isolates were identified using bioMérieux's API® 20E for Enterobacteriaceae and other non-fastidious Gram-negative bacteria and API® 20NE for identification of Gram-negative non Enterobacteriaceae. Briefly, each discrete colony was picked using a sterile loop and homogenized with a sterile 0.85% NaCl (5ml). The suspension was immediately inoculated into API strip according to the manufacturer's instructions. Other unknown bacteria were subjected to a series of standard biochemical tests. The biochemical tests performed were Catalase test, Oxidase test, Slide coagulase test, Triple Sugar Iron agar test (TSI), Sulphide Indole Motility test (SIM), Citrate utilization test, Urease test, Methyl Red test, Voges-Proskauer test (MRVP) as described in the Manual of Veterinary Clinical Bacteriology and Mycology (2008).

Fungi identification: Each EBN swab was directly cultured on Sabouraud dextrose agar (SDA) (Difco) and incubated at room temperature for 3 to 5 days. Different colonies were picked and the purification step was done by sub culturing each of the different colonies onto fresh SDA plates and incubated at room temperature for 3 to 5 days. The identification of the unknown fungal strain was done using Lactophenol cotton blue stain following the techniques of James and Natalie (2001). The procedure was done by placing a drop of the stain on a clean slide with the aid of a pipette. A small portion of the mycelium from the fungal cultures was removed and placed in a drop of Lactophenol. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted on a light microscope and observed with x10 ocular lens and x40 objective lenses respectively. The

fungus isolates were identified on macroscopic and microscopic morphology. Such characteristics included the colour of the top and reverse site of the culture plate, shape, texture and consistency of the growth, septation of hyphae, shape, size, texture and arrangement of the conidia following the method of Cheesbrough (2002).

Microbial enumeration: Enumerations of Total Aerobic Count (TAC), Total Coliform Count (TCC) and Total Yeast and Mold Count (TYMC) on EBN were carried out using 3M™ Petrifilm. Dilution of each EBN sample was done by measuring the weight of the EBN. Sodium chloride (0.85%) was used as a diluent and added to the EBN to form a 10^{-1} dilution depending on the EBN weight. The EBN was then homogenized using a stomacher and was further serially diluted (10^{-2} – 10^{-6}) with 0.85% NaCl. Using a pipette, 1 ml of each diluted sample was then placed on the petrifilm and incubated following the manufacturer's instruction. The colonies were enumerated and expressed in CFU/g.

Antibiotic susceptibility test: A total of 32 isolates representing the bacteria from 8 weeks old raw EBN samples were subjected to antibiotic susceptibility test. The period of two months was chosen as it is the average duration EBN is stored at the premises before it reaches the consumer or being exported. The antibiotic susceptibility was conducted on Mueller–Hinton agar (MHA) plates (Difco) using the agar diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2010). An inoculum suspension was prepared by picking several colonies from a pure culture on a nutrient agar (Difco) with a sterile loop and suspending them in nutrient broth (Difco) to the density of McFarland 0.5 standard. The inoculum was spread evenly over the entire surface of the agar plate by swabbing in three directions using a sterile cotton swab. Antibiotic discs were applied firmly on the agar surface within 15 mins of inoculation and incubated at 37°C for 24 hours. The susceptibility of the bacteria was tested against 12 antibiotics discs (µg/disc) (Oxoid Company, Britain); Amikacin (30), Ampicillin (10), Amoxycillin/clavulanic acid (30), Chloramphenicol (30), Ciprofloxacin (5), Doxycycline (30), Erythromycin (15), Gentamicin (10), Imipenem (10), Penicillin G (10 units), Tetracycline and Vancomycin (30). The bacteria were indicated as sensitive or resistant towards the antibiotic based on the zone of inhibition following the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2010).

Data Analysis: Mann-Whitney *U* test was done to test the significance difference of the CFU count and the microbial quality between premise A and premise B. The use of this statistical test is appropriate for studies with a small sample size and when the variables of interest do not show normal distribution. All analyses were carried out on SPSS version 22.0 and a *P* value of <0.05 was considered significant.

Results

Enumeration of TAC, TCC and TYMC in EBN: The average microbial load of 90 EBN samples collected from premise A and premise B at five sampling stages are summarized in Figure 1. The data obtained for TAC showed that among the samples, the highest TAC was found in 8 weeks old raw clean EBN (5.15×10^7 CFU/g) from premise A and the lowest TAC was found in raw unclean EBN (3.77×10^5 CFU/g) from premise B. The TCC result showed that in premise A, no coliforms were found in raw unclean, 4 weeks and 8 weeks old raw clean EBN. The highest TCC was found in 3 days old raw clean EBN from premise B (5.3×10^3 CFU/g). As for TYMC, the highest number of TYMC were found in 4 weeks old raw clean EBN (1.43×10^3 CFU/g) from premise B and the lowest from 2 weeks old raw clean EBN belonging to premise A (1.1×10^2 CFU/g).

Isolation and identification of bacteria in EBN: A total of 264 Gram-positive and Gram-negative bacteria were isolated from 90 EBN samples taken from both premises (Table 1 and 2), 27 species were isolated from premise A and 20 species from premise B. In premise A, the most prevalent bacteria found were *Staphylococcus* sp. (66.6%), *Actinomyces* sp. (66.6%), and *Corynebacterium* sp. (31.1%). In premise B, the most prevalent bacteria isolated were *Actinomyces* sp. (44.4%), *Staphylococcus* sp. (37.7%), *Corynebacterium* sp. (24.4%). Other isolates found were *Ochrobactrum anthropi* (26.6%) and *Aeromonas* sp. (26.6%). Table 3 shows that *Staphylococcus hyicus* is the most common bacteria found in raw unclean EBN, with a total of 77.8% and 88.9% in premise A and B respectively. In 3 days old raw clean EBN, the most encountered bacteria in premise A was *Actinomyces* sp. (100%) and *O. anthropi* (77.8%) in premise B. For 2 weeks old raw clean EBN, *Staphylococcus* sp. (77.8%) was found to be the most abundant bacteria in both premises. While in 4 weeks old raw clean EBN, *Corynebacterium* sp. was the most common bacteria found in both premises, 100% and 66.7% in premise A and B respectively. The most isolated bacteria in 8 weeks old raw clean EBN in premise A were, *Corynebacterium* sp. and *Staphylococcus* sp. (55.6%) each, and *Actinomyces* sp. in premise B (44.4%). The differences in the occurrence of EBN isolates both premises in all the stages were found to be non-significant.

Antibiotic susceptibility test: Antibiotic susceptibility of 32 bacteria isolated from 8 weeks old raw clean EBN was determined against 12 antibiotics. One hundred percent (100%) susceptibility was found to six antibiotics which were Ampicillin, Amoxycillin/clavulanic acid, Ciprofloxacin, Imipenem, Penicillin G and Tetracycline. The susceptibility decreased for Doxycycline (90.62%), Chloramphenicol and Vancomycin (87.5%) each, Amikacin and Erythromycin (75%) each and least susceptibility was found to Gentamicin (68.75%) (Table 4).

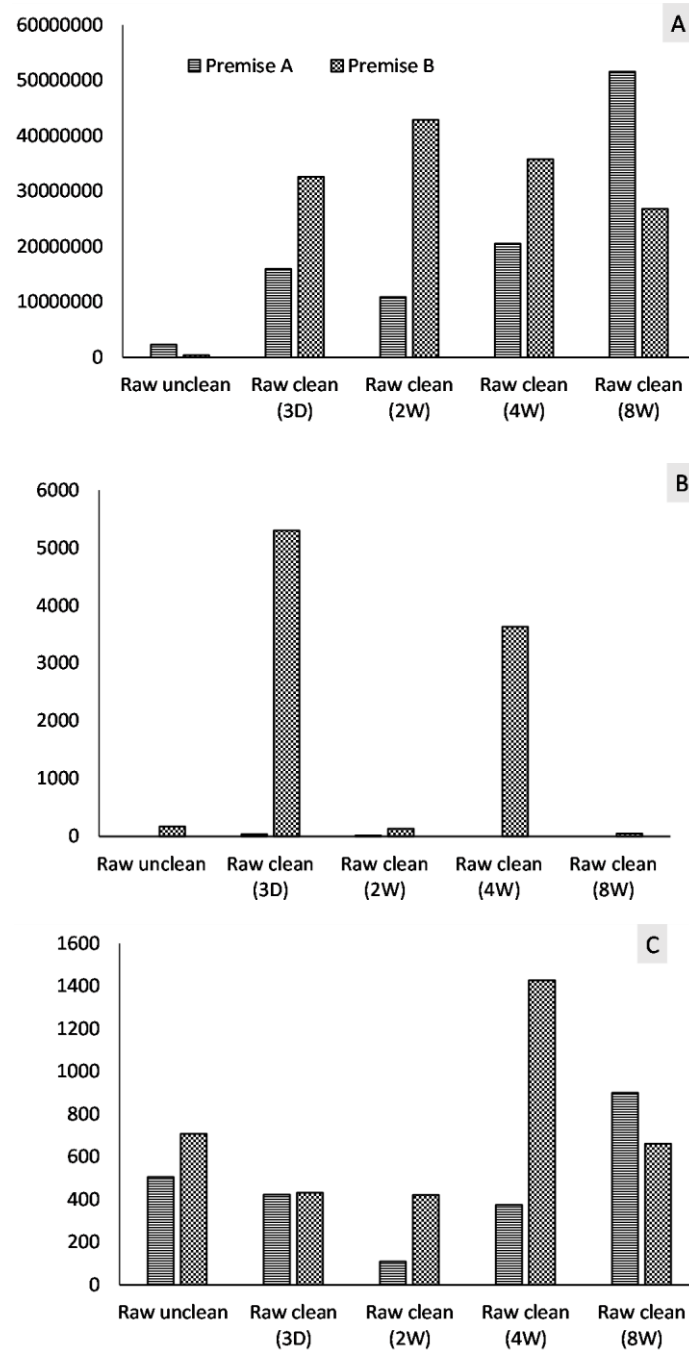


Figure 1 Bar chart of (A) Total Aerobic Count (B) Total Coliform Count (C) Total Yeast and Mold Count in EBN from premise A and B.

Table 1 The number of Gram-positive bacteria isolated from EBN in premise A and premise B

Gram-positive bacteria	Premise A		Premise B	
	No. of isolates	Frequency (%)	No. of isolates	Frequency (%)
<i>Actinomyces</i> sp.	30/45	66.6	20/45	44.4
<i>Bacillus</i> sp.	2/45	4.4	8/45	17.7
<i>Bacillus subtilis</i>	0	0	1/45	2.2
<i>Corynebacterium</i> sp.	14/45	31.1	11/45	24.4
<i>Corynebacterium diphtheriae</i>	2/45	4.4	0	0
<i>Corynebacterium auris canis</i>	3/45	6.6	0	0
<i>Proteus</i> sp.	0	0	1/45	2.2
<i>Rhodococcus</i> sp.	1/45	2.2	0	0
<i>Staphylococcus</i> sp.	30/45	66.6	17/45	37.7
<i>Staphylococcus hyicus</i>	7/45	15.5	8/45	17.7
<i>Staphylococcus intermedius</i>	1/45	2.2	0	0
Total	90	57.7	66	42.3

Table 2 The number of Gram-negative bacteria isolated from EBN in premise A and premise B

Gram-negative bacteria	Premise A		Premise B	
	No. of isolates	Frequency (%)	No. of isolates	Frequency (%)
<i>Achromobacter xylosoxidans</i>	1 /45	2.2	0	0
<i>Aeromonas salmonicida</i>	2/45	4.4	1/45	2.2
<i>Aeromonas stutzeri</i>	0	0	3/45	6.6
<i>Aeromonas</i> sp.	1/45	2.2	12/45	26.6
<i>Acinetobacter baumannii</i>	0	0	2/45	4.4
<i>Bulkholderia cepacia</i>	1/45	2.2	0	0
<i>Chryseobacterium indologenes</i>	6/45	13.3	0	0
<i>Comamonas testosteroni</i>	0	0	1/45	2.2
<i>Delftia acidovorans</i>	2/45	4.4	0	0
<i>Enterobacter cloacae</i>	1/45	2.2	0	0
<i>Elizabethkingia mengioseptica</i>	1/45	2.2	0	0
<i>Enterobacter</i> sp.	0	0	1/45	2.2
<i>Klebsiella pneumoniae</i>	1/45	2.2	0	0
<i>Ochrobactrum anthropi</i>	0	0	12/45	26.6
<i>Pasteurella pneumotropica</i>	1/45	2.2	0	0
<i>Photobacterium damsela</i>	3/45	6.6	0	0
<i>Pseudomonas fluorescens</i>	2/45	4.4	9/45	20
<i>Pseudomonas oryzae</i> habitans	6/45	13.3	4/45	8.8
<i>Pseudomonas putida</i>	5/45	11	1/45	2.2
<i>Pseudomonas stutzeri</i>	6/45	13.3	10/45	22.2
<i>Serratia plymuthica</i>	1/45	2.2	0	0
<i>Shigella dysenteriae</i>	0	0	1/45	2.2
<i>Stenotrophomonas maltophilia</i>	0	0	3/45	6.6
<i>Tatumella</i> sp.	5/45	11	2/45	4.4
<i>Vibrio</i> sp.	1/45	2.2	0	0
Total	46	42.59	62	57.41

Table 3 The number of bacteria isolated in premise A and B at different sampling stages

Sampling stages	Bacteria	Premise A	Premise B
Raw unclean EBN	<i>Acinetobacter baumani</i>	0	2/9
	<i>Actinomyces</i> sp.	5/9	2/9
	<i>Bacillus subtilis</i>	0	1/9
	<i>Bacillus</i> sp.	0	3/9
	<i>Bulkholderia cepacia</i>	1/9	0
	<i>Corynebacterium diphteriae</i>	2/9	0
	<i>Corynebacterium</i> sp.	0	1/9
	<i>Comamonas testosteroni</i>	0	1/9
	<i>Klebsiella pneumoniae</i>	1/9	0
	<i>Proteus</i> sp.	0	1/9
	<i>Pseudomonas stutzeri</i>	1/9	3/9
	<i>Stenotrophomonas maltophilia</i>	0	3/9
	<i>Serratia plymuthica</i>	1/9	0
	<i>Staphylococcus hyicus</i>	7/9	8/9
	<i>Staphylococcus</i> sp.	7/9	2/9
	<i>Vibrio</i> sp.	1/9	0
3 days old raw clean EBN	<i>Achromobacter xylosoxidans</i>	1/9	0
	<i>Actinomyces</i> sp.	9/9	4/9
	<i>Aeromonas</i> sp.	0	3/9
	<i>Corynebacterium auris canis</i>	1/9	0
	<i>Bacillus</i> sp.	0	1/9
	<i>Corynebacterium</i> sp.	0	1/9
	<i>Delftia acidovorans</i>	2/9	0
	<i>Enterobacter cloacae</i>	1/9	0
	<i>Ochrobactrum anthropi</i>	0	7/9
	<i>Photobacterium damsela</i>	2/9	0
	<i>Pseudomonas fluorescens</i>	0	4/9
	<i>Pseudomonas stutzeri</i>	3/9	5/9
	<i>Staphylococcus intermedius</i>	1/9	0
	<i>Staphylococcus</i> sp.	6/9	3/9
2 weeks old raw clean EBN	<i>Actinomyces</i> sp.	6/9	6/9
	<i>Aeromonas</i> sp.	0	4/9
	<i>Aeromonas stutzeri</i>	0	3/9
	<i>Bacillus</i> sp.	0	4/9
	<i>Chryseobacterium indologenes</i>	6/9	0
	<i>Corynebacterium auris canis</i>	2/9	0
	<i>Corynebacterium</i> sp.	0	0
	<i>Enterobacter</i> sp.	0	1/9
	<i>Pseudomonas oryzae</i>	4/9	1/9
	<i>Pseudomonas putida</i>	5/9	1/9
	<i>Rhodococcus</i> sp.	1/9	0
	<i>Staphylococcus</i> sp.	7/9	7/9
	<i>Tatumella</i> sp.	5/9	1/9
4 weeks old raw clean EBN	<i>Actinomyces</i> sp.	6/9	4/9
	<i>Aeromonas salmonicida</i>	2/9	0
	<i>Aeromonas</i> sp.	1/9	5/9
	<i>Bacillus</i> sp.	2/9	0
	<i>Corynebacterium</i> sp.	9/9	6/9
	<i>Elizabethkingia mengioseptica</i>	1/9	0
	<i>Photobacterium damsela</i>	1/9	0
	<i>Pseudomonas fluorescens</i>	2/9	4/9
	<i>Pseudomonas stutzeri</i>	2/9	2/9
	<i>Ochrobactrum anthropi</i>	0	5/9
	<i>Staphylococcus</i> sp.	5/9	4/9
8 weeks old raw clean EBN	<i>Actinomyces</i> sp.	4/9	4/9
	<i>Aeromonas salmonicida</i>	0	1/9
	<i>Corynebacterium</i> sp.	5/9	3/9
	<i>Pasteurella pneumotropica</i>	1/9	0
	<i>Pseudomonas fluorescens</i>	0	1/9
	<i>Pseudomonas oryzae</i>	2/9	3/9
	<i>Shigella dysenteriae</i>	0	1/9
	<i>Staphylococcus</i> sp.	5/9	1/9
	<i>Tatumella</i> sp.	0	1/9

Table 4 Percentage of susceptibility of 8 weeks raw clean EBN on antibiotic treatment

Antibiotic (µg/disc)	Percentage (%)
Amikacin (30)	75
Ampicillin (10)	100
Amoxycillin/clavulanic acid (30)	100
Chloramphenicol (30)	87.50
Ciprofloxacin (5)	100
Doxycycline (30)	90.62
Erythromycin (15)	75
Gentamicin (10)	68.75
Imipenem (10)	100
Penicillin G (10 units)	100
Tetracycline (30)	100
Vancomycin (30)	87.50

Isolation and identification of fungi in EBN: A total of 208 fungi isolates were obtained from all the tested EBN samples (Table 5), with 11 fungi species in EBN from premise A and 10 species isolates in EBN from premise B. The most common isolates from the premise A were *Aspergillus niger* (44.4%), followed by *Fonsecaea pedrosoi* (33.3%) and *Mucor* (33.3%). For EBN in premise B, the most isolated fungi were, *Candida* sp. (40%), *Mucor* sp. (26.67%), *A. niger* (17.77%) and *Penicillium* sp. (17.77%). The differences in occurrence of fungi isolates during 8 weeks of storage were assessed (Table 6). Raw unclean EBN showed the highest widespread of *Mucor* sp. (44.4%) in both premises. Meanwhile, 3 days old raw clean EBN showed the highest percentage of *A.*

niger (66.7%) and *Candida* sp. (66.7%) in premise A and B respectively. In 2 weeks old raw clean EBN, *Candida* sp. (55.6%) was frequently isolated in premise B while *Neurospora* sp. (55.6%) in premise A. In 4 weeks old raw clean EBN the most common fungi isolate in premise A were *Aspergillus niger* (44.4%) *Candida* sp. (33.3%) and *Aspergillus versicolor* (33.3%) and *Penicillium* sp. in premise B (44.4%). The most encountered fungi isolate in 8 weeks old raw clean EBN were *A. niger* (33.3%) and *Candida* sp. (55.6%) in premise A and B respectively. The difference in occurrence of fungi was only significant in raw unclean nest and non-significant in the rest of the stages.

Table 5 The number of fungi isolated from EBN in premise A and premise B

Fungi isolates	Premise A		Premise B	
	No. of isolates	Frequency (%)	No. of isolates	Frequency (%)
<i>Aspergillus flavus</i>	13/45	28.8	5/45	11.1
<i>Aspergillus niger</i>	20/45	44.4	8/45	17.77
<i>Aspergillus versicolor</i>	10/45	22.2	7/45	15.55
<i>Aspergillus</i> sp.	5/45	11.1	4/45	8.88
<i>Candida</i> sp.	13/45	28.8	18/45	40
<i>Cladosporium</i> sp.	13/45	28.8	3/45	6.66
<i>Fonsecaea pedrosoi</i>	15/45	33.3	3/45	6.66
<i>Penicillium</i> sp.	9/45	20	8/45	17.77
<i>Mucor</i> sp.	15/45	33.3	12/45	26.67
<i>Neurospora</i> sp.	8/45	17.77	0	0
<i>Rhodotorula mucilaginosa</i>	13/45	28.8	6/45	13.33
Total	134	64.4%	74	35.6%

Table 6 The number of fungi isolated from EBN in premise A and premise B

Sampling stages	Fungi	Premise A	Premise B
Raw unclean EBN	<i>Aspergillus flavus</i>	3/9	0
	<i>Aspergillus niger</i>	3/9	2/9
	<i>Aspergillus versicolor</i>	2/9	1/9
	<i>Aspergillus</i> sp.	3/9	1/9
	<i>Candida</i> sp.	3/9	1/9
	<i>Fonsecaea pedrosoi</i>	3/9	1/9
	<i>Mucor</i> sp.	4/9	4/9
	<i>Neurospora</i> sp.	1/9	0
	<i>Penicillium</i> sp.	3/9	0
	<i>Rhodotorula mucilaginosa</i>	3/9	0
3 days old raw clean EBN	<i>Aspergillus flavus</i>	3/9	1/9
	<i>Aspergillus niger</i>	6/9	2/9
	<i>Aspergillus versicolor</i>	3/9	3/9
	<i>Aspergillus</i> sp.	0	1/9
	<i>Candida</i> sp.	3/9	6/9
	<i>Cladosporium</i> sp.	5/9	0
	<i>Fonsecaea pedrosoi</i>	4/9	0
	<i>Mucor</i> sp.	5/9	2/9
	<i>Penicillium</i> sp.	2/9	1/9
	<i>Rhodotorula mucilaginosa</i>	3/9	1/9
2 weeks old raw clean EBN	<i>Aspergillus flavus</i>	4/9	1/9
	<i>Aspergillus niger</i>	4/9	1/9
	<i>Aspergillus versicolor</i>	2/9	2/9
	<i>Candida</i> sp.	2/9	5/9
	<i>Cladosporium</i> sp.	4/9	1/9
	<i>Fonsecaea pedrosoi</i>	4/9	1/9
	<i>Mucor</i> sp.	4/9	2/9
	<i>Neurospora</i> sp.	5/9	0
	<i>Penicillium</i> sp.	1/9	2/9
	<i>Rhodotorula mucilaginosa</i>	4/9	3/9
4 weeks old raw clean EBN	<i>Aspergillus flavus</i>	1/9	2/9
	<i>Aspergillus niger</i>	4/9	0
	<i>Aspergillus versicolor</i>	3/9	1/9
	<i>Aspergillus</i> sp.	1/9	0
	<i>Candida</i> sp.	3/9	1/9
	<i>Cladosporium</i> sp.	2/9	2/9
	<i>Fonsecaea pedrosoi</i>	2/9	1/9
	<i>Mucor</i> sp.	0	3/9
	<i>Neurospora</i> sp.	1/9	0
	<i>Penicillium</i> sp.	2/9	4/9
	<i>Rhodotorula mucilaginosa</i>	1/9	1/9
8 weeks old raw clean EBN	<i>Aspergillus flavus</i>	2/9	1/9
	<i>Aspergillus niger</i>	3/9	3/9
	<i>Aspergillus</i> sp.	1/9	2/9
	<i>Candida</i> sp.	2/9	5/9
	<i>Cladosporium</i> sp.	2/9	0
	<i>Fonsecaea pedrosoi</i>	2/9	0
	<i>Mucor</i> sp.	2/9	1/9
	<i>Neurospora</i> sp.	1/9	0
	<i>Penicillium</i> sp.	1/9	1/9
	<i>Rhodotorula mucilaginosa</i>	2/9	1/9

Discussion

This study was carried out to assess the microbiological quality of the edible-birdnest (EBN) in two premises. Premise A practiced EBN production following guidelines of Good Manufacturing Practice (GMP) and premise B did not follow any specific guidelines. GMP is a vital component of quality control which helps to ensure that products are consistently produced with the quality standards appropriate for their intended use. The differences of the premises are based on the standard of quality and safety of EBN produced for human consumption. Premises that practice GMP that runs with the compliance of Malaysia Food Act 1983 (Act 281), Food Regulations

1985 and Malaysian Standard (MS 2334:2011). The Malaysia Standard (MS 2334:2011) covers specifications for house raw unclean EBN during the sorting, grading and packaging stages. Both premises were expected to differ in their stringency and thoroughness of the cleaning processes applied to the raw nests. The sampling of EBN in this study started with the raw unclean EBN which were harvested from swiftlet ranches without undergoing any cleaning process. The sampling then continued with raw clean EBN that had undergone cleaning processes and was then stored at the period of 3 days, 2 weeks, 4 weeks and 8 weeks. These sampling intervals were selected to observe the microbial pattern of EBN over 8 weeks period, from raw unclean stage, cleaning stage, storage

and until the EBN reaches consumer. Based on Malaysia Standard (MS 2334:2011) and specification by Standard and Industrial Research Institute of Malaysia

(SIRIM)/ Department of Veterinary Services (DVS), EBN needs to meet the requirements shown in Table 7.

Table 7 Microbiological parameters of EBN

Guideline	Category	Microbiology Parameters
Microbiological parameters of raw EBN (MS 2334:2011)	Total aerobic count	$\leq 2.5 \times 10^6$ CFU/g
Specification for raw unclean EBN after pre-clean (SIRIM/DVS 3:2015)	<i>E. coli</i>	$\leq 1.0 \times 10^2$ CFU/g
	<i>S. aureus</i>	$\leq 2.5 \times 10^3$ CFU/g
	<i>Salmonella</i>	Nil
	TYMC	$\leq 2.5 \times 10^4$ CFU/g

Based on the findings (Fig 1), the Total Aerobic Count (TAC) was only acceptable in raw unclean EBN from both premises with a count below 2.5×10^6 CFU/g. However, after cleaning the number of bacteria increased tremendously in both premises but were not significantly different from each other throughout the sampling stages. This could be the result of the cleaning process, in which the strands of the nests were being separated during soaking causing the release of the bacteria. EBN that had been soaked for too long or not immediately dried would cause the bacteria to proliferate on the nest over time. Despite the washing and processing of the raw EBNs, mites and strands of feathers were still found on the surface of the commercial nests and also embedded within the strands of EBNs. They reported that those embedded within the strands of the nests were only visible after breaking the nest into small pieces (Kew et al., 2014). The pattern of the Total Coliform Count (TCC) in EBN was irregular but decreased with time. The TCC in raw clean EBN for 3 days (5.3×10^3 CFU/g) and 4 weeks (3.6×10^3 CFU/g) in premise B is high compared to the limit set by SIRIM/ DVS which only allows coliform abundance up until 1.0×10^2 CFU/g. The presence of coliforms could be explained by the improper cleaning of EBN that could result in a higher coliform count as the raw unclean nests consisted of impurities such as, faecal matter, feathers, grass, sand, twigs which some could remain in the nest even after washing. It is also crucial to separate the raw clean and raw unclean EBN to prevent cross contamination. Coliform bacteria can be isolated from polluted and non-polluted waters, soils and plants, as well as from the faeces of humans and warm-blooded animals (mammals and birds). Hence, it represents not only the intestinal bacteria but also other free-living coliforms and, therefore, could be understood in a simplified way to represent environmental bacteria (Von Sperling and de Lemos, 2005).

For Total Yeast and Mold Count (TYMC) in EBN, both premises depicted a lesser number of count as compared to the parameters set by the Malaysian Standard which requires the TYMC to be not more than 2.5×10^4 CFU/g. In both premises, the TYMC could be seen to decrease in the 2 weeks old EBN, however continue to increase until after 8 weeks. Both yeasts and molds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually on any type of food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage (Valerie et al., 2001). Higher levels of fungi exceeding the legal limits deteriorate the quality of

food and cause food borne diseases. Food decomposition occurs due to the presence of molds and many species of molds are known to produce mycotoxins (Gourama and Bullerman, 1997). In another study, total aerobic count (TAC), coliforms, *E. coli*, *S. aureus*, yeast and mold of raw clean EBN before and after irradiation were assessed. Before radiation treatment, raw clean EBN readings exceeded the permissible limit set by Standard Malaysia (Sani et al., 2015), the TYMC readings reported are higher compared to this study. Similarly, as reported by Chen et al. (2015), all EBNs had fungal CFU that exceeded the limit (100 CFU/g).

Isolation of bacteria in EBN from both premises shows premise A had a higher number of different species of bacteria compared to premise B with a total number of 27 and 20 isolates respectively. In Table 3, the most prevalent bacteria in raw unclean EBN was *S. hyicus* (77.8%) and *Staphylococcus* sp. (77.8%) in premise A. The same bacteria, *S. hyicus* (88.9 %) was also the most encountered bacteria in raw unclean EBN in premise B. *S. hyicus* has not previously been reported to cause human infections but is a well-known cause of diseases in a variety of animals, however it was found in a human wound after a donkey bite (Osterlund and Nordlund, 1997).

The most common bacteria in raw clean EBN obtained from premise A at 3 days, 2 weeks and 4 weeks old were *Actinomyces* sp. (100%), *Staphylococcus* sp. (77.8%) and *Corynebacterium* sp. (100%) respectively. In 8 weeks old raw clean EBN, the most encountered bacteria were *Staphylococcus* sp. (55.6%) and *Corynebacterium* sp. (55.6%). In premise B, the most frequently isolated bacteria in raw clean EBN from 3 days to 8 weeks old were *O. anthropi* (77.8%), *Staphylococcus* sp. (77.8%), *Corynebacterium* sp. (66.7%) and *Actinomyces* sp. (44.4%) respectively.

To summarize, the most prevalent bacteria in raw clean EBN from both premises were *Actinomyces* sp., *Staphylococcus* sp., and *Corynebacterium* sp. However, a report in another study shows that *Bacillus* spp. were the dominant bacterial groups in all the raw clean EBNs except for those obtained from Kajang (Selangor) which shows *Staphylococcus* sp. as the most prevalent bacteria in raw clean EBNs (Wong et al., 2018). Stringent monitoring of *Bacillus cereus* in food is crucial to avoid foodborne illnesses as these bacteria are highly resistant to processing and harsh conditions and can germinate to vegetative cells under any favourable conditions (Lv et al., 2019).

Actinomyces sp. are part of the normal flora of the oral cavity and respiratory tract but infection of organs and the bloodstream may happen upon disruption of

tissue barriers (Lakshamana Kumar *et al.*, 2005). *Actinomyces* sp. has been isolated in bottled spring and well waters. The presence of *Actinomyces* sp. are usually not a threat, some *Actinomyces* sp. are known to be opportunistic human pathogens and in rare cases, may cause actinomycosis which presents as abscesses in the mouth, gastrointestinal tract or lungs. *Staphylococci* are also isolated from a various range of foodstuff such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water (Heikens *et al.*, 2005; Kloos and Schleifer, 1976). Staphylococcal food poisoning is one of the most encountered food-borne diseases worldwide (Hennekinne *et al.*, 2009), resulting from the ingestion of staphylococcal enterotoxins preformed in food by enterotoxigenic strains of coagulase-positive staphylococci, mainly *Staphylococcus aureus*. As staphylococcal enterotoxins are heat stable, they may be present in food even when *S. aureus* are absent (Balaban and Rasooly, 2000). However, in this study, *S. aureus* were not to be found in any of the EBN samples.

There are more than 90 species of bacteria that have been described and over 50 of these bacteria are considered as rare human pathogens or transmitted by zoonotic contact while the remaining species seem to be isolated exclusively from animals, soil, water or food samples (Bernard, 2012). A number of *Corynebacterium* sp. are pathogens of humans or animals, whereas others are beneficial in industrial applications and in food production. *Corynebacterium* sp. are among the most commonly isolated pathogens linked with subclinical mastitis in dairy cows (Juliano *et al.*, 2016). *Corynebacterium* sp. of the human microbiome have regularly been assigned as opportunistic pathogens because they have occasionally been linked with human infections and have been viewed as contaminants in clinical specimens (Lewis *et al.*, 2012). These bacteria may be associated with spoilage or ripening of hygienic quality. However, this bacterium can be an important threat to a safe food supply. It may exist in a variety of foods and can be transmitted to human causing disease (Alibi *et al.*, 2017).

While only in premise B, *O. anthropi* and *Aeromonas* sp. were frequently isolated. *Ochrobactrum* sp. has been described as a free living opportunistic *alpha proteobacteria* which can be isolated in soil and water (Lebuhn *et al.*, 2000), as well as from plants (Tripathi *et al.*, 2007), from healthy and sick animals (Kampfer *et al.*, 2008) and humans (Holmes *et al.*, 1988). *O. anthropi* has been encountered in hospital and environmental water sources and is considered to be an emerging pathogen in immunocompromised patients. However so far, there has been no published report of *O. anthropi* infection in Malaysia (Rohani and Tzar, 2013). *Aeromonas* sp. are ubiquitous aquatic bacteria that have gained increasing attention as foodborne pathogens, and consumption of contaminated water and seafood are thought to be the main sources of human *Aeromonas*-related infections (Figuera and Beaz-Hidalgo, 2015). *Aeromonas* sp. are also dangerous human opportunistic pathogens that are able to cause intestinal, blood, skin and soft tissue and trauma-related infections, particularly in young children and the elderly. (Janda and Abbott, 2010; Real *et al.*, 1994).

These bacteria are responsible for food spoilage and may serve as vectors for disease transmission to humans (Tsai and Chen, 1996).

An antibiotic susceptibility test was done on 32 isolates representing the bacteria from 8 weeks old raw EBN. The antibiotic susceptibility test revealed that all the 32 bacteria tested were highly susceptible to antibiotics (Table 4). All tested bacteria were susceptible (100%) against six types of antibiotics which were Ampicillin, Amoxycillin/clavulanic acid, Ciprofloxacin, Imipenem, Penicillin G and Tetracycline. Most of the isolates showed sensitivity towards Doxycycline (90.62%), Chloramphenicol and Vancomycin (87.5%) each, followed by Amikacin and Erythromycin (75%) each. The bacteria were least susceptible to Gentamicin (68.75%). The high susceptibility to antibiotics shows that the birds were less exposed to the antibiotics tested. Antibiotics used was chosen according CLSI (2010) tables that lists the recommended antibiotics for testing specific genus of bacteria which also comprises different classes of antibiotics which includes Aminoglycosides, Carbapenems, Chloramphenicol, Glycopeptides, Macrolides, Penicillins, Quinolones, Tetracyclines.

Fungi are potentially important contaminants because they produce certain toxic metabolites that can cause severe health hazards to humans and animals (Al Gabr *et al.*, 2014). As for fungi (Table 5), the most found fungi isolated in EBN from both premises were *A. niger* (44.4%) and *Candida* sp. (40%) in premise A and premise B respectively. In different stages (Table 6), the most prevalent fungi in raw unclean EBN in both premises were *Mucor* sp. (44.4%). *Mucor* sp. is very widespread in nature, occurring in soils, decaying vegetation, dung and many other moist habitats where rapidly growing fungi has an advantage (Pitt and Hocking, 2009). In premise A, the most common fungi isolated in raw clean EBN for 3 days and 2 weeks old raw clean EBN were *A. niger* (66.7%) and *Neurospora* sp. (55.6%). The genus *Neurospora* has never been implicated in human disease or observed to cause disease in animals or plants (David and Rowland, 2000). In 4 weeks old raw clean EBN, most encountered fungi were *Aspergillus niger* (44.4%), *Candida* sp. and *A. versicolor* (33.3%) was the most frequently isolated in 8 weeks old raw clean EBN. In premise B, *Candida* sp. is the most common fungi isolated in raw clean EBN in the sampling period of 3 days, 2 weeks and 8 weeks, with a total of 66.7% of *Candida* sp. were found in 3 days old raw clean EBN, and 55.6% of *Candida* sp. in 2 weeks and 8 weeks old raw clean EBN respectively. In 4 weeks old raw clean EBN, the most encountered fungi were *Penicillium* sp. (44.4%). To summarize, the most prevalent fungi found in both premises were *A. niger* and *Mucor* sp. The difference in the occurrence was only significant in the raw unclean EBN stage, while non-significant for the rest of the stages. In another study, *Aspergillus* sp. and *Penicillium* sp. were found to be the most common fungi genera from the raw, commercial, uncooked and cooked EBN samples (Chen *et al.*, 2015). These genera have been known to be one of the most common food spoilage fungi, known to produce mycotoxins which are dangerous to human health. A different study also reported that *Aspergillus* sp., *Candida* sp., *Cladosporium* sp.,

Neurospora sp. and *Penicillium* sp. were found to be the most common isolated bacteria from raw clean EBN (Wong et al., 2018), however no specific conclusions have been reached on the source of contaminants. *Aspergillus* is known to produce aflatoxin (Pechanova et al., 2013), a mycotoxin related to causing toxic hepatitis leading to jaundice and associated with reduced levels of secretory immunoglobulin

Aspergillus and *Penicillium* are also known to produce ochratoxin A (OTA) (Frisvad et al., 2011; Hope and Hope, 2012), which is nephrotoxic, immunotoxic and causes other kidney diseases such as focal segmental glomerulosclerosis. OTA has been classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen to humans. While in commercial and boiled EBNs, *Candida* sp. were found to be abundant. *Candida* sp., *Aspergillus* sp., *Penicillium* sp. and *Neurospora* sp. are reported to be associated with the environment (air, food, dust and storage places) (Chen et al., 2015).

This study provides useful information about the microbiological quality of EBN. It is found that the most encountered bacteria and fungi are environmental based organisms which could be introduced back on the EBN even after cleaning. The contamination may have been caused by improper hygiene technique for the facilities which includes water supply, drainage, equipment and personal hygiene. Microbial contamination reduces shelf life and food quality which may lead to food infection and poisoning outbreaks, some of which are life threatening. There have been reports of allergic symptoms and food-induced anaphylaxis following the ingestion of EBNs due to the presence of allergens which could be caused by the microorganisms associated with the nests (such as fungi, bacteria and protozoa), arthropods (such as mites) which inhabit the swiftlets or their nests. Continuous monitoring of food processing is essential to avoid potential health problems. The establishment and implementation of regulations for EBNs should be regularly updated and monitored to improve the quality of the EBNs and consumer safety.

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Recommendation on future studies: In this study, EBN samples were taken from two premises. A better insight on the microbiological profile of EBN can be achieved by increasing the number of premises for EBN sampling all around Malaysia.

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