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Molecular characterization of culturable bacteria in raw and commercial edible bird nests (EBNs)

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Abstract

Edible bird nests (EBNs) are highly demanded globally. The industry was recently affected by an import ban to China due to high nitrite levels.Subsequently, many concerns have been raised. In this study, the microbial composition of both raw and commercial EBNs was investigated. The raw EBNs were purchased from swiftlet farms: Kuala Sanglang (Perlis), Pantai Remis (Perak), Kluang (Johor), Kajang (Selangor) and Kota Bharu (Kelantan). The commercial nests were purchased from five different Chinese traditional medicinal shops (Companies A-E) in Malaysia and one from Indonesia (Medan). A total of 123 and 34 isolates were successfully identified from unboiled raw and commercial EBNs respectively. The highest average CFU (1.77×10^4) was associated with raw EBNs obtained from Kluang, while for the commercial EBNs, those obtained from Company M1 had the highest CFU (5.50×10^4). *Bacillus* sp. accounted for the highest isolated species from both unboiled raw and commercial EBNs. *Bacillus* sp. and *Brevibacillus* sp. were mainly isolated from the boiled EBNs. *Bacillus* spp. were the dominant bacterial groups in all the raw EBNs except for those obtained from Kajang. The average number of bacteria isolated from the raw EBNs (average = 7) was higher compared with those isolated from the commercial EBNs (average = 4). The highest average number of bacterial isolates was reported in the raw EBNs obtained from Kota Bharu. Among the commercial EBNs, one EBN sample each from Companies A and M1 showed the highest number of isolates (n = 10). In general, there was a significant reduction in the number of bacteria isolated after boiling the EBNs. Raw EBNs obtained from Kajang had a distinct pool of bacterial species where the majority of the isolated species belonged to *Staphylococcus* species. The associated health impacts of these microorganisms to the consumers and public need to be addressed.

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Introduction

Edible bird nests (EBNs) are consumed mainly by the Chinese worldwide as a tonic which promotes health. This functional food consists of the regurgitated saliva of four different species of swiftlets of the genus *Aerodramus* (formerly called *Collocalia*). Raw or unprocessed EBNs are harvested from natural caves (cave nests) and swiftlet farms or abandoned shops in town (house/farm nests). The raw nests go through the process of soaking, cleaning, bleaching, moulding and packaging before they are sold (Ma and Liu, 2012).

The medical benefits and nutritional contents of EBNs have been reported recently. EBN is mainly composed of protein (60-65%), carbohydrate (8-31%), ash (2.1%) and lipid (0.14-1.28%) (Marcone, 2005; Saengkrajang *et al.*, 2013; Hamzah *et al.*, 2013). EBN also contains minerals, the top four are calcium, sodium, magnesium and potassium (Marcone, 2005; Saengkrajang *et al.*,2013; Chen *et al.*, 2014). EBNs exhibit anti-influenza virus activities (Guo *et al.*, 2006);epidermal growth factor-like effects (Kong *et al.*, 1987); antioxidant properties (Yida *et al.*, 2014); neuro-protective effects against oestrogen deficiency-associated senescence via modification of redox system and attenuation of advanced glycation end-products (Houet *et al.*, 2015); ameliorate oxidative stress-induced apoptosis in SH-SY5Y human neuroblastoma cells (Yew *et al.*, 2014); demonstrate chondro-protective abilities on human articular chondrocytes *in vitro* via reduced catabolic activities and increased cartilage extracellular matrix synthesis (Chua *et al.*, 2013); prevent high fat diet-

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induced insulin resistance in rats (Yida *et al*., 2015); induce proliferation of corneal keratinocytes (Zainah *et al.*, 2011) and possess many more properties.

Swiftlet farming has expanded rapidly over the past decades in Southeast Asia (Koon, 2011; Thorburn, 2014) due to increased global demand for EBNs. Malaysia is the second largest exporter of EBNs where eighty percent of the EBNs are exported to China. There are many reports of allergic symptoms and food-induced anaphylaxis following the ingestion of EBNs (Kemp *et al.*, 2010; Goh *et al*.,1999; 2000; 2001; Thong *et al.*, 2005; 2007; Hon *et al.*, 2006; 2009). Obviously, there are many more unreported EBN-related food safety issues. The safety alarm was triggered in year 2011 when China banned the import of EBNs from Malaysia due to high level of nitrites. The nitrite levels ranged from 5.7 µg/g (or 5.7 ppm) for house nests to 843.8 µg/g (or ppm) for the cave nests (Quek *et al*., 2015) which are way above the permissible level (30 ppm) set by Department of Standards Malaysia (MS 2334: 2011).

Structural analysis of both raw and commercial EBNs revealed the presence of mites, fungi, bacteria and feather strands (Kew *et al*., 2014). Mite eggshells and faecal pellets, and body parts of other arthropods were seen on the raw nests. The commercial nests had a variety of unidentified structures and substances coated on the nests' surfaces that were not found on the raw nests. These could be the adulterants (karaya gum, red seaweed, tremella fungus, and gelatin) that are added to the EBNs in order to increase the weight of the commercial EBNs (Marcone, 2005; Tukiran *et al.*, 2015).The presence of nitrites, heavy metals and other contaminants may jeopardise the quality of EBNs and pose health risks to consumers. Furthermore, no documentation to date has reported the microbial diversity despite the concerns on safe consumption of EBNs. Hence, this study was designed to examine whether bacteria are associated with raw and commercial EBNs using both culture and molecular identification techniques.

Materials and Methods

Collection and processing of raw and commercial EBNs

The unprocessed (raw and un-cleaned) EBNs were purchased from house farms in five different localities in Malaysia: Kuala Sanglang (Kedah; 6° 16' 0"N, 100° 12' 0"E), Pantai Remis (Perak; 4° 27' 0" N, 100° 38' 0" E), Kluang (Johor; 02° 01′ 30″N 103° 19′ 58″E), Kajang (Selangor; 2° 59' 0"N, 101° 47' 0"E) and Kota Bharu (Kelantan; 6° 8' 0"N, 102° 15' 0"E). The commercial EBNs were purchased from five different Chinese traditional medicine shops (Companies A-E) and another commercial sample purchased from Medan, Indonesia (Company M1). Three to six nests were purchased from each locality/shop. The EBNs were sent to the laboratory for bacteria isolation and identification.

Culture and isolation of the bacteria associated with EBNs

Approximately 1.0 g of EBN was mixed with 10 mL of sterile ultra-pure water and was divided into two equal portions for boiled and un-boiled sample preparation. The boiled EBN portion was subjected to double boiling at 100°C for 3 hours. Both the boiled and un-boiled samples were diluted with molten nutrient agar, followed by plating in petri dishes at different dilutions. All the plates were allowed to solidify, inverted and incubated overnight in an incubator at 25°C. The total number of colonies formed for each plate was counted on the following day. The bacteria were isolated and sub-cultured based on gross morphological appearance until pure colonies were obtained.

DNA extraction, amplification and sequencing

Genomic DNA of the pure bacteria was extracted according to manufacturer's instruction (Qiagen DNeasy kit, Qiagen, Germany). Genomic DNA was amplified with PCR using the conserved primers for bacteria: 27F (forward; 5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (reverse; 1492R 5-GGTTACCTTGTTACGACTT-3) as described previously (Liu *et al.*, 2013). DNA sequencing and alignment analysis of these regions were performed.

Statistical analysis

Student t test or non-parametric Mann-Whitney test was used to determine the statistical differences between each group. P values were considered statistically significant if they were less than 0.05.

Results

Colony forming units (CFU) of bacteria and number of bacteria isolates

The CFU for all the raw and commercial EBNs was counted and summarized in Table 1. The highest average CFU was associated with raw EBNs obtained from Kluang, Johor, followed by that obtained from Kuala Sanglang, Kedah. Bacteria growth (120 CFU) was observed after double-boiling in one of the raw EBNs obtained from Kuala Sanglang, Kedah. As for the commercial EBNs, the EBNs obtained

Table 1. Bacteria counts (colony forming units, CFU) and number of isolated bacteria from boiled and un-boiled (a) raw and (b) commercial EBNs

(a) Raw EBNs

Source	No	Type of nest	Weight of sample (g)	Bacteria count (CFU)per 0.5 g			No of isolated bacteria (gross morphology)		
				Un-boiled	Boiled	TOTAL	Un-boiled	Boiled	Total
Kuala Sanglang Kedah	1	Very old and dirty, found on ground	2.76	3.1×10^4	1.2×10^2	31120	4	3	7
	2	After 1 hatching	2.64	3.0×10^4	0	3000	3	0	3
	3	No hatching	4.01	1.6×10^4	0	1600	2	0	2
	4	After multiple hatchings	4.69	3.3×10^4	0	33000	3	0	3
	5	After multiple hatchings, and fell on ground	7.22	4.1×10^4	0	4100	3	0	3
	6	No hatching	2.05	2.4×10^4	0	2400	5	0	5
Average						12537			3.8
Pantai Remis Perak	1	No hatching	3.27	3.0×10^3	0	3000	9	0	9
	2	After 2 hatchings	6.75	3.4×10^4	0	3400	7	0	7
	3	After 1 hatching	7.26	9.4×10^4	0	9400	5	0	5
Average						5267			7.0
Kluang Johor	1	No hatching	2.92	1.3×10^3	0	1300	9	0	9
	2	After 1 hatching	3.39	8.0×10^2	0	800	3	0	3
	3	After 2 hatchings	2.83	5.1×10^4	0	51000	9	0	9
Average						17700			7.0
Kajang Selangor	1	No hatching	1.32	8.4×10^2	0	840	9	0	9
	2	After 1 hatching	1.57	3.4×10^4	0	3400	7	0	7
	3	After 2 hatchings	1.30	3.0×10^2	0	300	7	0	7
Average						1513			7.7
Kota Bharu Kelantan	1	No hatching	1.11	3.5×10^3	0	3500	21	0	21
	2	No hatching	1.30	6.0×10^2	0	600	11	0	11
	3	No hatching	1.25	3.2×10^2	0	320	7	0	7
Average						1473			13.0

(b) Commercial EBNs

Source	No	Weight of sample (g)	Bacteria count (CFU)per 0.5 g			No of isolated bacteria (gross morphology)		
			Un-boiled	Boiled	TOTAL	Un-boiled	Boiled	Total
Company A	1	3.01	7.5×10^4	0	75000	10	0	10
	2	3.01	2.6×10^4	0	2600	0	0	0
Average					38800			5.0
Company B	1	2.23	5.0×10^2	0	500	3	0	3
	2	2.19	8.0×10^2	0	800	2	0	2
Average					650			2.5
Company C	1	1.45	2.0×10^1	0	20	1	0	1
	2	1.47	4.0×10^1	0	40	0	0	0
Average					30			0.5
Company D	1	2.21	5.5×10^3	1.2×10^2	5500	5	3	8
	2	2.21	5.2×10^4	4.0×10^1	5200	5	1	6
Average					5350			7.0
Company E	1	1.57	4.6×10^2	0	460	1	0	1
	2	1.89	1.8×10^2	4.0×10^1	180	2	1	3
Average					320			2.0
Company M1	1	1.57	5.5×10^4	0	55000	10	0	10

from Company M1 had the highest number of colony forming units while the EBNs obtained from Company C had the least number of colonies (Table 1b).

Molecular characterization of the bacteria isolated from the raw and commercial EBNs

The bacteria colonies were isolated from the primary EBN-inoculated agar plates based on the colony morphology until single pure colonies were obtained. The colonies were stained with Gram stain for microscopic examination and further characterized using molecular techniques.

The average number of bacteria isolated from the raw EBNs (average = 7) was higher compared with those isolated from the commercial EBNs (average = 4) (Figure 1). There was no significant difference in the number of bacteria isolated from raw and commercial EBNs (p = 0.06, Mann-Whitney U test). The highest average number of bacteria isolates was reported in the raw EBNs obtained from Kota Bharu, Kelantan. Only three types of bacteria were isolated from one of the double-boiled raw EBNs obtained from Kuala Sanglang, Kedah. All of these heat-resistant bacteria were of *Bacillus* species (Table 2).

Among the commercial EBNs, one EBN sample each from Companies A and M1 showed the highest number of bacteria isolated (n = 10, Table 1). A total of five isolates was successfully cultured from boiled commercial EBNs purchased from Companies D and E. All the isolated heat-resistant bacteria from Company D were of *Brevibacillus* species while those isolated from boiled EBNs of Company E were of *Bacillus* species (Table 2).

In general, it was observed that there was a significant reduction in the number of bacteria isolated after boiling the EBNs (p = 0.000; Mann-Whitney U test). The genus of bacteria isolated from the raw and commercial EBNs differed from each other (Tables 2 and 3). The majority of the bacteria associated with the raw EBNs were of *Bacillus* species (> 50% of total isolates) especially for those obtained from Kuala Sanglang, Kluang and Kota Bharu compared with the commercially purchased EBNs except for Companies C and E (Table 2). Raw EBNs obtained from Kajang had distinct pools of

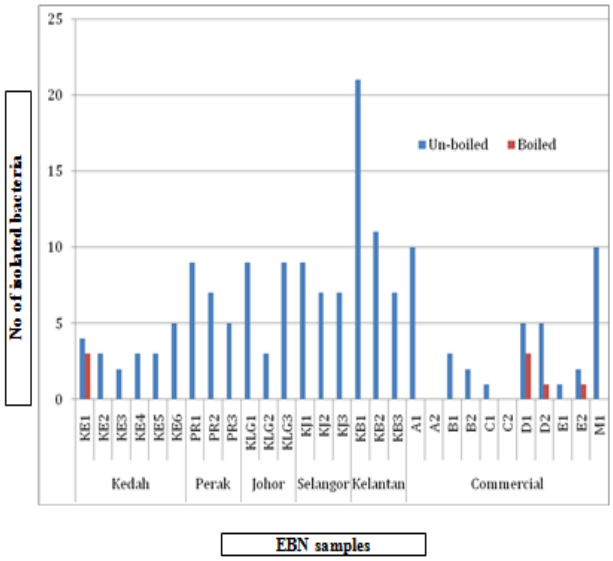

Figure 1. Number of bacteria isolated from the raw and commercial EBNs

bacterial species compared with the rest of the raw EBNs obtained from other locations. The identified bacteria that were commonly associated with these nests belonged to *Staphylococcus* species. Other least commonly identified species which included *Enterococcus, Paenibacillus, Brevibacterium, Listeria, Acinetobacter* and *Virgibacillus* species. *Enterobacter, Exiguobacterium, Brevibacillus, Caryphanon, Solibacillus* species were found exclusively in the commercial EBNs.

Discussion

EBNs are consumed as double-boiled soup together with rock sugar by Chinese for centuries for many perceived health benefits. Recently, there have been several scientific reports on their effectiveness in improving health, and skin and face textures. However, there is no report on the microbial contents associated with these nests and their significance to the health of the consumers, public and workers in this industry.

With reference to the guidelines issued by Food Safety and Quality Division of Ministry of Health Malaysia (2012), two and one raw EBNs obtained from Kuala Sanglang and Kluang respectively had higher than 30000 CFU/g based on the assumption that the protein levels of these nests were higher or equal to 4%. Similarly, one of the commercial EBNs purchased from Company A and those of Company M1 had higher total CFU counts compared with the reference standard set by Ministry of Health Malaysia. The Ministry of Health Malaysia specified that the total plate count should be ≤ 30000 CFU/g when protein ≥ 4% or ≤ 1000 CFU/g when protein < 4% (according to the latest Standard Operating

Table 2. Identities of bacteria isolated from the (a) raw and (b) commercial EBNs according to locations

(a) Raw EBNs

Location	Bacteria	Unboiled EBNs, % n = 20	Boiled EBNs, % n = 3
Kuala Sanglang (Kedah)	*Bacillus subtilis*	10.00	33.33
	***Bacillus* sp.**	**45.00**	**66.67**
	Bacillus megaterium	5.00	
	Bacillus pumilus	10.00	
	Bacillus circulans	10.00	
	Bacillus aryabhattai	5.00	
	Staphylococcus nepalensis	5.00	
	Staphylococcus kloosi	5.00	
	Enterococcus faecalis	5.00	
Location	**Bacteria**	**n = 21**	**n = 0**
Pantai Remis (Perak)	***Bacillus* sp.**	**19.05**	
	Bacillus cereus	4.76	
	Bacillus flexus	4.76	
	Bacillus shackletonii	4.76	
	Staphylococcus nepalensis	4.76	
	Staphylococcus kloosi	4.76	
	Staphylococcus sp.	4.76	
	Staphylococcus sciuri	4.76	
	Staphylococcus sp. Y3	9.52	
	Enterococcus faecalis	4.76	
	Enterococcus sp.	4.76	
	Paenibacillus sp. 23-13	4.76	
	Paenibacillus agglomerans	4.76	
	Paenibacillus alvei	14.29	
	Brevibacterium sp.	4.76	
Location	**Bacteria**	**n = 20**	**n = 0**
Kluang (Johor)	***Bacillus* sp.**	**75.00**	
	Bacillus pumilus	4.76	
	Bacillus aryabhattai	4.76	
	Bacillus flexus	4.76	
	Enterococcus faecalis	4.76	
	Microbacterium sp.	4.76	
Location	**Bacteria**	**n = 23**	**n = 0**
Kajang (Selangor)	*Bacillus* sp.	8.70	
	Staphylococcus nepalensis	21.74	
	***Staphylococcus* sp.**	**34.78**	
	Paenibacillus sp.	4.35	
	Brevibacterium sp.	8.70	
	Listeria fleischmannii	13.04	
	Virgibacillus halophilus	4.35	
	Acinetobacter sp.	4.35	
Location	**Bacteria**	**n = 39**	**n = 0**
Kota Bharu (Kelantan)	***Bacillus* sp.**	**51.28**	
	Bacillus megaterium	2.56	
	Bacillus pumilus	5.13	
	Bacillus aryabhattai	12.82	
	Bacillus cereus	5.13	
	Staphylococcus nepalensis	15.38	
	Staphylococcus sp. Y3	2.56	
	Acinetobacter sp.	2.56	
	Deinococcus sp.	2.56	

(b) Commercial EBNs

Location	Bacteria	Unboiled EBNs, % n = 10	Boiled EBNs, % n = 0
Company A	*Acinetobacter radioresistens*	20.00	
	Acinetobacter calcoaceticus	20.00	
	***Exiguobacterium* sp.**	**40.00**	
	Enterobacter cloacae	10.00	
	Enterobacter hormaechei	10.00	
Location	**Bacteria**	**n = 2**	**n = 0**
Company B	*Bacillus badius*	50.00	
	Staphylococcus sp.	50.00	
Location	**Bacteria**	**n = 1**	**n = 0**
Company C	*Bacillus cereus*	100.00	
Location	**Bacteria**	**n = 10**	**n = 4**
Company D	***Brevibacillus* sp.**	10.00	**75.00**
	Brevibacillus agri		25.00
	***Bacillus* sp.**	**30.00**	
	Bacillus lichniformis	10.00	
	Staphylococcus pasteuri	10.00	
	Sporosarcina saromensis	10.00	
	Caryphanon sp.	10.00	
	Deinococcus sp.	10.00	
	Solibacillus silvestris	10.00	
Location	**Bacteria**	**n = 3**	**n = 1**
Company E	*Bacillus* sp.	33.33	100.00
	Bacillus badius	33.33	
	Bacillus flexus	33.33	
Location	**Bacteria**	**n = 8**	**n = 0**
Company M1	*Bacillus* sp.	25.00	
	Acinetobacter sp.	25.00	
	Staphylococcus sp.	12.50	
	Staphylococcus saprophyticus	12.50	
	Staphylococcus sciuri	12.50	
	Brevibacterium sp.	12.50	

Table 3. Identities of bacteria isolated from the raw and commercial EBNs

Bacteria	Raw EBNs	
	Unboiled, % (n = 123)	Boiled, % (n = 3)
Bacillus subtilis	1.63	33.33
***Bacillus* sp.**	**40.65**	**66.67**
Bacillus megaterium	1.63	
Bacillus pumilus	4.07	
Bacillus circulans	1.63	
Bacillus aryabhattai	5.69	
Bacillus cereus	2.44	
Bacillus flexus	1.63	
Bacillus shackletonii	0.81	
Staphylococcus nepalensis	**10.57**	
Staphylococcus kloosi	1.63	
***Staphylococcus* sp.**	**7.32**	
Staphylococcus sciuri	0.81	
Staphylococcus sp. Y3	2.44	
Enterococcus faecalis	2.44	
Enterococcus sp.	0.81	
Paenibacillus sp.	0.81	
Paenibacillus sp. 23-13	0.81	
Paenibacillus agglomerans	0.81	
Paenibacillus alvei	2.44	
Brevibacterium sp.	2.44	
Microbacterium sp.	0.81	
Listeria fleischmannii	2.44	
Virgibacillus halophilus	0.81	
Acinetobacter sp.	1.63	
Deinococcus sp.	0.81	
Bacteria	**Commercial EBNs**	
	Unboiled, % (n = 34)	Boiled, % (n = 5)
***Brevibacillus* sp.**	2.94	**60.00**
Brevibacillus agri		20.00
***Bacillus* sp.**	**17.65**	20.00
Bacillus badius	5.88	
Bacillus cereus	2.94	
Bacillus lichniformis	2.94	
Bacillus flexus	2.94	
Acinetobacter radioresistens	5.88	
Acinetobacter calcoaceticus	5.88	
Acinetobacter sp.	5.88	
***Exiguobacterium* sp.**	**11.76**	
Enterobacter cloacae	2.94	
Enterobacter hormaechei	2.94	
Staphylococcus sp.	5.88	
Staphylococcus pasteuri	2.94	
Staphylococcus saprophyticus	2.94	
Staphylococcus sciuri	2.94	
Sporosarcina saromensis	2.94	
Caryphanon sp.	2.94	
Deinococcus sp.	2.94	
Solibacillus silvestris	2.94	
Brevibacterium sp.	2.94	

Procedure for monitoring of raw clean edible bird's nest issued by Food Safety and Quality Division of Ministry of Health Malaysia, 2012).

The bacteria isolated from the raw EBNs of both locations (Kuala Sanglang and Kluang) are mainly *Bacillus* species. There are many species under the genus of *Bacillus* with *B. anthracis* and *B. cereus* being the most clinically important species causing anthrax and food poisoning respectively (Farrar and Reboli, 2006). *B. cereus* was found in the raw EBNs from Pantai Remis and Kota Bahru, and commercial EBNs from Company C. *B. cereus* produces heat resistant endospores and is able to form biofilm. The enterotoxins released by *B. cereus* cause nausea, vomiting, abdominal cramps and/or diarrhoea with an incubation period of 1 to 16 hours (Jeβberger *et al*., 2015; Farrar and Reboli, 2006). *B. cereus* can cause other infections e.g. conjunctivitis, keratitis, orbital abscess, secondary infections in normal or immunocompromized (traumatized, cancer and diabetic) patients.

Other *Bacillus* species isolated from the EBNs in this study, which cause human diseases include *B. subtilis, B. pumilus* (cutaneous infection, food poisoning or sepsis in infants)(Tena *et al.*, 2007; From *et al.*, 2007; Kimouli *et al*., 2012), B. circulans (endocarditis; Gatermann *et al*., 1991) and *B. megaterium* (cutaneous infection; Farrar and Reboli, 2006; Duncan and Smith, 2011). *B. subtilis* is an aerobic Gram positive endospore-forming bacterium that is commonly found in soil, water and on plants. Spores of *B. subtilis* are resistant to heat, chemicals and UV radiation, and this is consistent with our ability to isolate *B. subtilis* and other *Bacillus* species from the EBNs after boiling (Setlow, 2006). *B. subtilis* is a well-known producer of antibiotics such as lantibiotics, polyketides, amino sugar and phospholipid (Stein, 2005). *B. subtilis* is rarely associated with infection (such as food poisoning) (Cote *et al.*, 2015) but inhalation of the derivatives of *B. subtilis* (e.g. proteolytic enzyme) may illicit pulmonary and allergic diseases (Flindt, 1969).

As expected, double-boiling of the EBNs purchased from Companies D and E did not kill all the nest-associated bacteria. *Bacillus cereus* and *Brevibacillus* species were isolated and identified. *Brevibacillus* species was reported to cause peritonitis in a patient with hepatocellular carcinoma (Parvez *et al.*, 2009). Both *Bacillus* and *Brevibacillus* species may pose health threats to those who consumed the double boiled EBN soup which contains these heat-resistant bacteria. On the other hand, both species may serve as probiotics which benefit the consumers (Sanders *et al*., 2003). Double boiling with an interval of a few hours apart for spore germination to remove heat resistant spores could be considered. However, this will not be practically convenient as the duration for the spore germination varies among different species of microorganisms.

Other than *Bacillus* species, *Staphylococcus nepalensis*, *Staphylococcus* species, *S. sciuri, Staphylococcus sp*. Y3 and *S. kloosi* were isolated from the raw EBNs. *S. nepalensis* was reported in the guano of bats (Vandzurova *et al*., 2013) but has not been reported to cause infection in human. *S. sciuri* was isolated from a skin wound of a patient with infective endocarditis (Hu *et al.*, 2015). *S. kloosii* was isolated from the respiratory tree of *Holothuria leucospilota* (sea cucumber) from Teluk Nipah,

Pangkor Island, Malaysia (Kamarudin *et al*., 2013) and is reported to produce orange pigment. For the commercial EBNs, *S. pasteuri, S. saprophyticus* and *S. sciuri* were isolated. *S. pasteuri* is emerging as one of the causative agents of nosocomial infections and blood derivative contaminants with increasing resistance to several classes of antibiotics including methicillin, oxacillin, tetracyclins, chloramphenicol, streptomycin etc. (Savini *et al*., 2009). *S. saprophyticus* is the second most prevalent species causing acute community-acquired urinary tract infections after *Escherichia coli* (Ferreira *et al.*, 2012) and is mainly isolated from the urine of the sexually active young women. Similarly *S. sciuri* is also gaining more attention due to its association with hospital settings and increasing clinical significance including urinary tract infections, endocarditis, septic shock, wound infections and pelvic inflammatory diseases (Dakic *et al*., 2005). The association of these species with commercial EBNs is of public health concern. More frequent occurrence of *S. aureus* in the raw EBNs from Kajang may be associated with the surrounding environmental habitat of the swiftlets where they obtain their food from.

Acinetobacter species were isolated from commercial EBNs (Company A) and one of the raw EBNs obtained from Kajang and Kota Bahru respectively. The natural habitats for *Acinetobacter* species are soil and water and its presence in both commercial and raw EBNs could be due to contamination from soil, water and even from the environment. Its presence in the commercial EBNs could also be introduced during the soaking, cleaning, bleaching, moulding and packaging of the raw EBNs. They can survive environmental desiccation for weeks and contaminate the respiratory-therapeutic and ventilation equipment, and hands of healthcare workers. They cause ventilator-associated pneumonia and bloodstream infections. They can colonize the skin, wound, respiratory and gastrointestinal tracts of the patients (Munoz-Price and Weinstein, 2008).

Enterobacter species were isolated from commercial EBNs (Company A) and belong to the Enterobacteriaceae family. *Enterobacter* species are Gram negative bacilli and were associated with the nationwide outbreak of septicemia in 1976 due to contaminated intravenous solutions (Maki *et al*.,1976). *Enterobacter* species are important nosocomial pathogens (after *Escherichia coli* and *Klebsiella* species) associated with bacteremia, pneumonia, lower respiratory tract infections, urinary tract infections, surgical site infections and so forth. One of the major concerns for this species is the induction of β-lactam antibiotic resistance via hyper-production of inducible chromosomal Amp C β-lactamase following broad-spectrum cephalosporins prescriptions in patients with septicemia (Cosgrove *et al.*, 2002). B-lactamases or extended-spectrum β-lactamases (ESBLs) are bacterial enzymes which hydrolyse the expanded-spectrum (or third-generation) cephalosporins (e.g. cefotaxime, ceftriaxone, ceftazidime) and monobactams (e.g. aztreonam). In 2010, two cases of infant bloody diarrhoea were associated with *E. hormaechei* and an unidentified *Enterobacter* species (Jackson *et al.*, 2016).

Exiguobacterium species were isolated from commercial EBNs (Company A) only. *Exiguobacterium* species were previously isolated from very diverse sources and ranges of temperature (-12 to 55^{o}C) including Greenland glacial ice, hot springs at Yellowstone National Park, plant's rhizosphere and the environment of food processing plants (Vishnivetskaya *et al*., 2009). This genus consists of psychrotrophic, mesophilic and moderate thermophilic species or strains. *Exiguobacterium* species are capable of neutralizing highly alkaline textile industry waste water (bioremediation), removing pesticide, and reducing arsenate to arsenite. Several enzymes like alkaline protease, catalase, guanosine kinase, ATPases, dehydrogenase and esterase have been isolated from this species.

According to the latest standard set by Ministry of Health Malaysia for the export of raw clean EBNs to China (Operating procedure for monitoring of raw clean edible bird's nest issued by Food Safety and Quality Division of Ministry of Health Malaysia, 2012) the EBNs should be free of contamination by *Escherichia coli, Salmonella* species and *Staphylococcus aureus*. No guideline was stated for other microorganisms. No *Escherichia coli* and other coliforms other than *Enterobacter* sp. were isolated from the EBN in this study despite the use of a general purpose nutrient agar. This was consistent with recent findings which reported that *Staphylococcus* sp. and *Bacillus* sp. were the most prevalent airborne bacteria as well as bacteria isolated from the faecal samples of swiftlets (*Aerodramus* species) in the swiftlet houses (Leong, 2015). The isolated bacteria were mainly Gram positive bacteria (97.5% of faecal bacteria and 93.5% airborne bacteria respectively). These faecal and airborne bacteria showed moderate to high level of resistance to penicillin and cephalosporin (Leong, 2015).

Fungal contamination of EBNs has been reported previously (Chen *et al*., 2015). 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. Among the commercial EBNs, EBN samples from Company B showed the highest number of types of fungi (14 different types). Greater varieties of fungal genera were found in the raw EBNs compared with the commercial EBNs. *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Neurospora* sp. and *Penicillum* sp. were the most common fungi isolated from the unboiled and boiled raw and commercial EBNs. Some of these fungi are mycotoxin producers and can cause opportunistic infections in humans.

The source(s) of these contaminants remains unknown. The possible source(s) include the saliva or feathers of the swiftlets, the insects ingested by the swiftlets, the environment, the microorganisms associated with the nests, arthropods (such as mites) which inhabit the swiftlets or their nests, the cleaning processes of the raw nests, the adulterants added to the commercial nests and/or the contaminants introduced, and the infestation during the moulding and storage of the nests.

Conclusion

There was a significant reduction in the number of bacteria isolated after boiling the EBNs. Raw EBNs obtained from Kajang had a distinct pool of bacterial species where the majority of the isolated species belonged to *Staphylococcus* species. In view of the different niches of bacteria present in EBNs obtained from different locations, it will be wise to look at the significance of these bacteria on the health of the workers who are closely associated with EBN industry, the public who stay adjacent to the EBN house farms and to those who consume the EBN soup.

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