

Microbiological assessment and Multiple Antimicrobial Resistance Profile of *Enterobacteriaceae* recovered from kulikuli sold at selected markets in Ife-North Local Government Area

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ABSTRACT

The native West African population eats a groundnut-based snack called *kulikuli* (groundnut cake). The study was carried out in order to evaluate, characterize, and determine multiple antibiotic resistance index (MAR-I) of isolated pathogen isolated in ready-to-eat *kulikuli* snacks. 52 samples of *kulikuli* in total were collected for this investigation from Odeomu market and its surroundings in Osun State, Nigeria. Using the pour plate technique, bacteriological analysis of the samples was performed. Total coliform counts were counted on MacConkey agar, total bacterial counts on Nutrient agar, and total fungal counts were counted on potato dextrose agar. Phenotypic and biochemical assays were used in identifying the bacterial isolates. Disc diffusion techniques were used to test the isolates' antibiotic susceptibility, and the MAR index of the isolates was determined. The total bacterial count, total coliform count and the total fungal count of the samples varied from 1.5 to 8.8×10^3 cfu/g, 0.3 to 6.6×10^3 cfu/g and 1 to 7.3×10^4 cfu/g respectively. The organisms recovered were enteric, Gram-negative members of the Enterobacteriaceae family. Multiple antibiotic resistance (MAR) to four to seven different antibiotics was developed by the isolates. The research area has a high overall rate of multiple drug resistance pathogens isolated from the *kulikuli* samples. The MAR Index was higher than 0.2, however a MAR index that is greater than 0.2 suggested high-risk sources, like places where bacteria have been repeatedly exposed to antibiotics or where the use of antibiotic is common. In conclusion, in order to avoid consuming these pathogens and harmful toxins together with the products, *kulikuli* should be sold in hygienic conditions and environments, be sealed, and not be exposed to air. This is because the organisms identified in this study indicated health risks that would be harmful to consumers.

Keywords: Antimicrobial resistance, Ready-to-eat, enterobacteria, MAR-Index, *Kulikuli*

Introduction

The protein-rich snack food *kulikuli* is native to the coast of West Africa (groundnut cake). A byproduct of the oil extraction process is a

substance consumed as a snack by humans, utilized as a flavoring agent in other dishes, and used as animal feed (Obojiofor *et al.*, 2022). Typically, once the oil is extracted from groundnut and fried, *kulikuli* are made from

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groundnuts (Adjou *et al.*, 2012). It is a common snack in Nigerian that is usually eaten with meals like "Gari" and "Akamu" and sporadically used in salads. In current society, "kulikuli" has become much more in demand. In addition to being a staple of the diets of people of all ages, kulikuli snacks is most frequently consumed by those in the range of 18-35 years of age, especially students. Because the snacks are always available and less expensive, groundnuts with their derivatives are sometimes categorized as "street food," which satisfies a necessity for the population in the urban area (Boli *et al.*, 2014). Moreover, it is a significant key component used in the formulation of feeds for chicken (Ademola *et al.*, 2015).

Kulikuli is protein-rich snacks albeit lesser in comparison to groundnut the source material (Kolapo *et al.*, 2012; Ejoh and Ketiku, 2013). The plant named as groundnut which the scientific name is *Arachis hypogea* Linn belongs to member of the *Fabaceae* family (Eke-Ejiofor *et al.*, 2012). It is a plant that is generally known as nut and contain the same profile of nutrient with that of the nuts tree (Ros, 2010). In terms of global production, this annual plant, which is often found in tropical, subtropical, and temperate regions, is second only to soybeans. (Ayoade & Adegbite 2016).

It is discovered that groundnuts have more plant protein per 100g than any other nuts or legumes when their fundamental makeup is compared to that of other types of nuts (Settaluri *et al.*, 2012; Sibte-Abbas *et al.*, 2015). Furthermore, because of its accessibility and low cost, groundnut is frequently known to be a poor man's major source of protein. Nigerian *kulikuli* snack is always at risk to contamination by varieties of microorganisms including several bacterial species that can range from basic commensals to pathogenic microorganisms and fungi, because of its nutrient richness. This yearly plant that is commonly located in different regions such as sub-tropic, temperate, and tropic, is second to

soybeans only, in order of legume significance in the world, in terms of overall production (Ezekiel *et al.*, 2014).

As a results of inappropriate processing and storage conditions, contamination of groundnut and *kulikuli* with microbes may occur during processing, handling, transporting, and storage, which increases the introduction and risk of aflatoxin in groundnuts and their products (Polixeni and Panagiota, 2008; Mutegi *et al.*, 2012). Enterobacteriaceae which are also called enterotoxigenic bacteria contributes a lot to causing many illnesses such as diarrheal and many different types of infections in humans, which could be challenging in causing a serious problem in the healthcare industry and difficulties in controlling food safety problems in sub-Saharan Africa (Atanda *et al.*, 2013).

Nonetheless, for the production of *kulikuli*, traditional peasant processing and packing techniques are still frequently used. The latter subject the groundnut cake to microbial contamination, particularly when it is being sold and frequently exposed and or packaged in thin hand-knotted polythene bags. Despite the fact that mycotoxicoses and infections are rapidly being linked to the humans and animal pathology, there are no use of labels that provide essential information like the name of the maker and address or location of production site, the nutritional content, and the best before date for human use. Consumer refusal to dispose pretty attractive mouldy foods, such as groundnut cake because of its alluring flavor, could make the situation worse. Despite the rate of consumption and widespread distribution of *kulikuli* snacks, few information is known about their safety, hygienic state, and level of contamination. This study evaluates the antimicrobial susceptibility profile of the pathogens, the multiple antibiotic resistance (MAR-I) index, and the level of microbiological contamination of the kulikuli sold in Osun State, Nigeria.

Materials and Methods

Selection and Samples collection

Samples of *kulikuli* were bought in various places, including popular markets within Osun state, South-West Nigeria, and produced in various shapes. The samples collected were drawn at random from Ipetu, Odeomu, Sekona, and Akinola markets and they were transported within one hour of collection to the laboratory for evaluation and analysis of microbiological contaminants and further analysis.

Microbiological analysis of kulikuli samples

Ten grammes of the samples each was then aseptically transferred in 90 ml of diluent (sterile distilled water) in a sterile mortar and pestle and were homogenized. The solution was agitated properly before a 1 to 10 fold serial dilution was performed on to the power of 10^6 (Thonda *et al.*, 2014).

Serial Dilution of Samples

A sterile pipette was used to transfer 9 ml of the diluent (sterile distilled water) into seven testtubes and plugged with cotton wool, which was autoclaved and allowed to cool. The solution was shaken vigorously after which 1 ml was pipetted into one of the five test tubes containing the sterile distilled water and all samples were diluted serially according to the method of Oluduro *et al.* (2015).

One (1) mL aliquots of each dilutions was then introduced in triplicates onto nutrient agar using pour plating techniques, selective media (MacConkey agar and potato dextrose agar (PDA)) were used to isolate bacteria and fungi respectively. The nutrient agar plates was then used in determining the Total bacterial count (TBC) while the MacConkey plates was used for total coliform counts (TCC) and potato dextrose agar (PDA) was used for total fungi count (TFC) in spores forming unit (sfu/g). The agar plates were all incubated at 37°C for 24 hours, except PDA plates which was incubated for 3-6 days at

30°C before taking the results. Thereafter plates were counted and recorded as total bacterial count TBC, TCC and total fungal count (TFC). Agar plates with greater than 300 colonies were recorded as too numerous to count (TNTC). Each distinct colony on the plates were selected and sub-cultured severally to purify the isolates, and to ascertain morphological consistency. The pure cultures obtained was stored at 4°C for further use.

Identification and Characterization of Samples

Presumptive identification of isolates was done using colonial morphology and Gram staining reaction. According to Bergeys Manual of Determinative Bacteriology, the bacteria isolated were then identified based on their morphological traits and biochemical characteristics which include Indole, methyl red/Vogues-Proskauer, Motility, Oxidase, Simmon Citrate, Urease activity, Catalase, and Sugar fermentation tests. (Cheesbrough, 2006).

Antibiotic susceptibility Testing (AST)

The bacteria isolated were tested against commonly used antibiotics. Antibiotic susceptibility of isolated bacteria was carried out using Kirby-Bauer antibiotic disk diffusion method on Mueller Hinton agar in accordance to Clinical and Laboratory Standards Institute (2016). Briefly, the antibiotics (oxoid, UK) used were Augmentin (30µg), Cefotaxime (30µg), cefuroxime (25µg), gentamycin (10µg); cefixime, (10µg); ofloxacin (15µg), nitrofurantoin (20µg), ciprofloxacin (25µg) were gently placed on the Mueller Hinton agar plates already inoculated with standardized test organisms (0.5 McFarland standard) and were incubated at 37°C for 24hrs. Clear zones of inhibition was measured in milliliter using a calibrated ruler. The diameters of zones of inhibition were interpreted according to the CLSI standard (Thonda *et al.*, 2014; CLSI, 2017).

Multiple Antibiotic Resistance Index of the Isolates

The multiple antibiotic resistance index (MAR-index) of the isolates recovered was determined by following the procedures of Krumpferman (1983) (Datok *et al.*, 2021). MAR index of an isolate can be calculated as: Number of antibiotics that the isolate showed resistant to divided by the total number of antibiotics used against isolates.

Statistical analysis

Experiments were carried out in triplicates. Results obtained was analysed using Microsoft Excel™ 2016, T-test and statistical differences was compared using $P \leq 0.05$.

Results and Discussion

This study determined the presence of enteric bacteria and multiple resistance isolates recovered in *kulikuli* samples sold at some selected markets in Ife-North LGA, Nigeria. The bacteria recovered from location HIJ has the highest number with 48.3% as represented in Figure 1. The total bacterial counts (TBC), total coliform counts (TCC) and the total fungal counts (TFC) of the samples varied from 1.5 to 8.8×10^3 cfu/g, 0.3 to 6.6×10^3 cfu/g and 1 to 7.3×10^4 cfu/g respectively (Table 1). The identity of the microorganisms are *Yersinia intermedia* (16.1%) which has the highest prevalence, followed by *Salmonella* sp and *Klebsiella ornithinolytica* (12.9%), *K. pneumoniae* and *Escherichia coli* (9.7%), *Proteus* sp (6.5%), *Klebsiella planticola* (6.5%), *Enterobacter* sp, *Shigella soonei* (6.5%) while the least was found in *Serratia marcescens* and *Salmonella bongori* (3.2%) as depicted in table 2.

Antibiotic Susceptibility Pattern of Bacteria Isolated from kulikuli samples

The antibiotic sensitivity of the isolated pathogens in this study indicated that all the isolates showed 100% resistance to ceftazidime, cefuroxime, augmentin while they are 90%

resistance to cefixime and all are 58% resistance to gentamicin. The isolates were 67% and 64% susceptible to ofloxacin and ciprofloxacin respectively as depicted in table 3. The distribution of the bacteria isolates is shown in figure 2.

The multiple antibiotic resistance pattern of the isolates and the MAR index was shown table 4. The MAR index varied from 0.5 to 0.75 for *Klebsiella* species, 0.63 to 0.88 for *Salmonella* sp, 0.5 - 0.88 for *Yersinia intermedia*, while *Pseudomonas flourescens* and *Serratia marcescens* have the highest value of MAR ranging from 0.88 - 1.0. The antibiotic sensitivity of isolates to the tested antibiotics is shown in Plate 1.

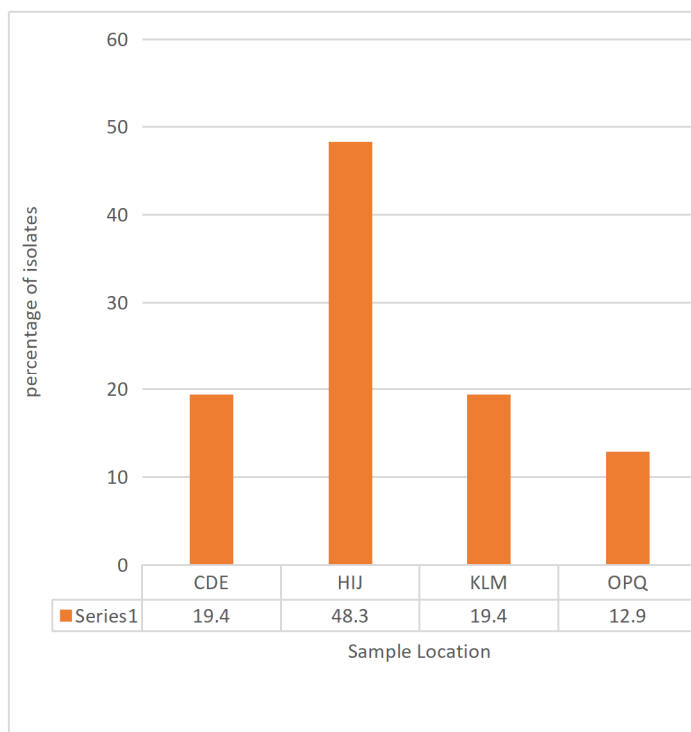


Figure 1: Percentage distribution of bacteria isolates based on the location

Table 1: The TBC, TCC, and TFC count of isolated bacteria

Sample code	Location	TBC (cfu/g)	TCC (cfu/g)	TFC (cfu/g)
A	CDE	1.7 x 10 ⁴ ±0.12	3.1 x 10 ² ±0.06	4.2 x 10 ⁴ ±0
		1.5 x 10 ³ ±0.12	0.3 x 10 ³ ±0.15	1.7 x 10 ⁴ ±0
		7.7 x 10 ⁵ ±1.15	0.0 ± 0.00	2.4 x 10 ³ ±0.52
B	HIJ	1.2 x 10 ³ ±0.10	1.3 x 10 ² ±0.12	2.1 x 10 ⁶ ±0.0
		1.4 x 10 ⁶ ±0.10	0.8 x 10 ³ ±0.12	7 x 10 ² ±0.0
		8.8 x 10 ² ±0.10	6.6 x 10 ³ ±0.10	0±0.0
C	KLM	1.4 x 10 ³ ±0.06	2.6 x 10 ² ±0.00	7.3 x 10 ² ±0.58
		2.4 x 10 ⁴ ±0.15	2.3 x 10 ³ ±0.10	1.7 x 10 ³ ±0.58
		2.6 x 10 ³ ±0.15	1.4 x 10 ⁶ ±0.06	2 x 10 ² ±0.0
D	OPQ	3.5 x 10 ³ ±0.06	1.8 x 10 ² ±0.06	3 x 10 ² ±0.0
		4.2 x 10 ³ ±0.10	3.3 x 10 ³ ±0.06	2 x 10 ⁵ ±0.0
		4.2 x 10 ⁵ ±0.26	5.7 x 10 ⁴ ±0.58	1 x 10 ³ ±0.0
		8.4 x 10 ³ ±0.06	2.3 x 10 ² ±0.15	2 x 10 ⁵ ±0.0
		8.4 x 10 ⁴ ±0.06	1.0 x 10 ³ ±0.00	3.7 x 10 ³ ±0.58

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KEY: TBC: Total Bacteria Count, TCC: Total Coliform Count, TFC: Total fungal Count

Table 2: Frequency distribution of bacteria isolated from kulikuli samples

Identified Organisms	Frequency (%)
<i>Klebsiella ornithinolytica</i>	4 (12.9)
<i>K. pneumoniae</i>	3 (9.7)
<i>K. planticola</i>	2 (6.5)
<i>Salmonella bongori</i>	1 (3.2)
<i>Salmonella sp</i>	4 (12.9)
<i>Yersinia intermedia</i>	5 (16.1)
<i>Escherichia coli</i>	3 (9.7)
<i>Proteus vulgaris</i>	2 (6.5)
<i>Pseudomonas flourescens</i>	2 (6.5)
<i>Enterobacter absuriae</i>	2 (6.5)
<i>Serratia mercescens</i>	1 (3.2)
<i>Shigella sonnei</i>	2 (6.5)
Total	31

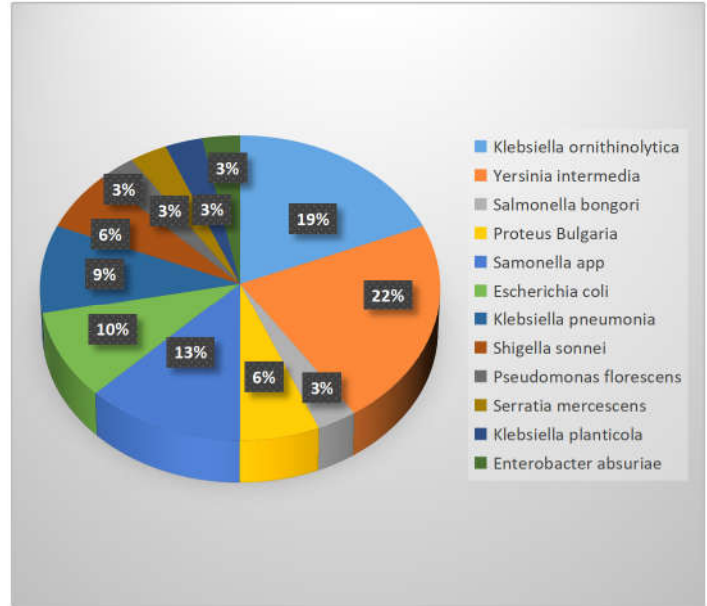


Figure 2: Percentage distribution of bacterial isolates from kulikuli samples



Plate 1: Antibiotic Sensitivity of pathogenic isolates to the tested antibiotics

Table 3: The Resistant and Susceptibility Rate of Bacteria Isolates

Antibiotics	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR
Resistant Isolates n (%)	31 (100)	31 (100)	18 (58.0)	27 (90.0)	08 (25.0)	31 (100)	13 (41.9)	06 (19.0)
Susceptible Isolates n (%)	0 (0)	0 (0)	03 (9.6)	02 (6.5)	21 (67.0)	0 (0)	15 (48.0)	20 (64.0)

(P<0.05)

KEY:-CAZ: Ceftazidine, CRX: Cefixime GEN: Gentamycin, OFL: Ofloxacin, AUG: Augmentin, CRX: Cefuroxime, NIT: Nitrofurantoin, CPR: Ciprofloxacin

Table 4: Multiple Antibiotics Resistance Profile of the Isolate and MAR Index

Isolates	Resistance Pattern	Frequency	MAR Index
<i>Yersinia intermedia</i> (5)	Crx, Caz, Cxm, Aug	1	0.5
	Crx, Caz, Gen, Cxm, Aug	1	0.63
	Crx, Caz, Gen, Aug, Nit	1	0.63
	Crx, Caz, Gen, Cxm, Aug, Nit	1	0.75
	Crx, Caz, Cxm, Ofi, Aug, Cpr	1	0.75
<i>Salmonella sp</i> (5)	Crx, Caz, Cxm, Ofi, Aug, Niit, Cpr	1	0.88
	Crx, Caz, Gen, Cxm, Ofi, Aug	2	0.75
	Crx, Caz, Gen, Aug, Nit	1	0.63
	Crx, Caz, Gen, Aug	1	0.5
<i>Proteus vulgaris</i> (2)	Crx, Caz, Gen, Aug, Nit,	1	0.63
	Crx, Caz, Cxm, Aug	1	0.5
<i>Shigella sonnei</i> (2)	Crx, Caz, Gen, Aug, Nit	1	0.63
	Crx, Caz, Cxm, Aug	1	0.5
<i>Klebsiella sp</i> (10)	Crx, Caz, Gen, Cxm, Nit, Aug	1	0.75
	Crx, Caz, Gen, Cxm, Ofi, Aug,	3	0.75
	Crx, Caz, Gen, Cxm, Aug	2	0.63
	Crx, Caz, Cxm, Aug	3	0.5
<i>Escherichia coli</i> (3)	Crx, Caz, Cxm, Aug	1	0.63
	Crx, Caz, Gen, Cxm, Aug	2	0.63
<i>Enterobacter absuriae</i> (2)	Crx, Caz, Cxm, Aug	2	0.5
<i>Serratia mercescens</i> (1)	Crx, Caz, Cxm, Gen, Ofi, Aug, Nit, Cpr	1	1.0
<i>Pseudomonas flourescens</i> (1)	Crx, Caz, Gen, Cxm, Ofi, Aug, Nit, Cpr	1	1.0

KEY:- CRX: Cefixime CAZ: Ceftazidine, GEN: Gentamycin, OFL: Ofloxacin, AUG: Augmentin, CRX: Cefuroxime, NIT: Nitrofurantoin, CPR:Ciprofloxacin

Discussion

The study showed that the kulikuli being sold to the larger population in the sampled locations were strongly polluted with Enterobacteriaceae namely, *Klebsiella pneumoniae*, *Serratia*

mercescens, *Proteus sp*, *Klebsiella sp*, and *Enterobacter sp* and so on. The organisms isolated and identified from this study is similar with the findings of Ezekiel, *et al.*, 2011 while it is contrary to Ayoade and Adegite study in 2016

who reported that *Serratia fonticola* and *Proteus* sp. *Klebsiella pneumoniae* and *Citrobacter freundii* are detected in the samples of *kulikuli* analysed in the region to be highly pathogenic organisms which indicated improper handling, inadequate sanitation procedures, and a lack of education among the local producers and food handlers that, if left unchecked, could pose a serious risk and result in food borne diseases and other illnesses. Other potential variables include the dusty surroundings in the location where the product is being sold and displayed on tables and the lack of a fly-net on the wheelbarrow or carrier.

The current study's identification of enteric bacteria in the food samples emphasizes how important it is for preserving general public health and again that the people in the sampled locations consumes *kulikuli* that is poor in quality. The family Enterobacteriaceae members are typically signs of fecal contamination and are been linked to a number of human illnesses (Ayoade & Adebite 2016). *Serratia* sp., *Proteus* sp., and *Proteus vulgaris*, which are members of this bacterial family discovered in the current study, are well known for their capacity to infect human lower respiratory tracts and cause significant illnesses (Wendel *et al.*, 2013). *Escherichia coli* developed multiple antibiotic resistance (MAR) to four to five different antibiotics, and for *Salmonella* sp., four to seven different antibiotics.

The phenotypic resistance patterns observed in the isolates tested against the antibiotics showed that *Pseudomonas fluorescens* and *Serratia marcescens* had the highest phenotypic resistance. The P value was less than 0.5 ($P < 0.05$) which shows that there was a significant differences in the rate of resistance. Majority of the isolated organisms showed resistance to more antibiotics tested. The value of MAR index of all the isolates is more than 0.2 which ranged between 0.5-1.0 and this indicated that the organisms isolated are multiple drug resistant and the high risk source of

contamination is where antibiotics are frequently used.

The tested antibiotics were resistant to a variety of the pathogens. The widespread and careless use of antibiotics for medical treatment without consideration and other preventive purposes may be accountable for the greater rate of antibiotic resistance that has been reported. Antimicrobial resistance is increasingly developing and spreading among enteric bacteria (Sawant, 2007). The overall rate of antimicrobial drug resistance of the isolated pathogenic organisms was huge in the area of study, this is in agreement with the findings of Schroeder *et al.* (2002)

Eight different conventional antibiotics were tested against the pathogens isolated and different pattern resistant were observed as, CAZ (100%), CRX (100%), GEN (58%), CXM (90%) OFL (25%), AUG (100%), NIT (41.9%), CPR (19%). A major problem is that *kulikuli* may be exposed to microbial contamination, particularly during product hawking. Despite the fact that mycotoxicoses are being associated with human beings more and more, there are no printed labels to show important information like the name of the makers and addresses or location of the where the product was made, the nutrient content, the recommendation for storage, or the expiring date for human consumption. The quantity and kind of bacteria in product affect how quickly it deteriorates, therefore groundnut cake may be infected and cause several types of illnesses on humans.

Conclusion

In conclusion, *kulikuli* carries different type of enterobacteria as shown in the study and the rate of resistance is on the high side in the study location which is of great concern. The MAR index is higher than 0.2, indicating high-risk sources where bacteria have been repeatedly exposed to antibiotics or where antibiotic use is common. The groundnut cake is more susceptible

to microbial contamination due to exposure and packaging, particularly during product hawking, which is frequently exposed. Consumers' unwillingness to throw out food samples that are fairly mouldy, like *kulikuli*, could make the situation worse. Stewardship of antibiotics, monitoring of medication resistance, and educating producers about hygiene standards to be met both during and after production of the products are all strongly advised for all parties involved.

Recommendation

1. Maintaining a clean environment is very crucial and necessary to stop contaminants or hazardous substances from entering the *kulikuli*.
2. In order to prevent these diseases from infecting the *kulikuli* sold in the market, it should be carefully wrapped.

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Conflict of Interest

Authors declare no conflict of interest regarding the publication of this article.

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