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Original Article

Assessment of microbiological quality and safety of fermented and non-fermented Khmer Rice Noodles in Cambodia

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Fermented and non-fermented Khmer rice noodles is produced basically from *indica* rice, a popular food item and most important nutrition sources along with an integral part of rural household's food security of Cambodian population. This study was done to evaluate the microbiological quality of fermented and non-fermented Khmer rice noodle samples sold throughout the country. In total, 75 Khmer rice noodle samples, (23 fermented and 52 non-fermented) were collected from five local varieties (*Nom-Banhchok*, *Nom-Banhhoy*, *Koyteavkat*, *Lotchhar* and *Koyteav*) and three locations (Kandal province, Siem Reap province and Phnom Penh city) of Cambodia. The study results showed that irrespective of sample location, all the fermented and non-fermented rice noodle samples analyzed were either contaminated with faecal origin bacteria (*Enterococcus* spp., and coliform), or faecal indicator bacteria including *Escherichia coli* and pathogenic bacteria including, *Staphylococcus* spp., and *Bacillus* spp. Of the fermented rice noodles about 74% and 98% of non-fermented rice noodles was found contaminated with all the above mentioned bacteria. In addition, 30% samples were found contaminated with *Klebsiella pneumonia spp*. Furthermore, among the *Staphylococcus aureus* isolates, three isolates TM-K5-3, OM-L4 and DM-KK3-2 was seen producing protein A (aggregated polysaccharide specific immune latex strongly), positive clumping factor production and harbored type six coagulase gene and among the *B. cereus* strains, 12 *B. cereus,* isolates was seen produced enterotoxin, which may cause severe diarrhea and one *B. cereus (DM-NB1)* isolate was found harbored CRS gene, which may produce emetic toxin, unsafe for human consumption. Therefore, strong regulatory monitoring should be established in order to improve the quality of food to ensure public health.

Keywords: Khmer rice noodle, microbiological quality, fermented and non-fermented food, foodborne pathogen; spoilage bacteria, and food safety.

Introduction

Rice is a staple food providing major nutrition and an itegral part of food security of rural households of Cambodian[7]. Rice has many bland taste in diet that may have unique attributes including ease of digestion, a mild flavor, and hypoallergenic properties[10]. The quality of rice is not always easy to define as it depends on the consumer preferences, choices, and intended use and besides cooking, rice can be processed differently to make convenience food, snack, and dessert to satisfy people's desire.

Khmer rice noodles made from local *Nom Banhchok, Nom Banhhoy, Koyteavkat, Lotchhar.* and *Koyteav* rice varieties and all these rice varieties originally generated from *indica* rice varieties, which is suitable for rice noodle preparation than japonica rice varieties, because of more amylose content[15]. However, *Nom Banhh* rice varieties has a long history and commonly consumed in Cambodia, most popular till today to prepare various food items and serve frequently for breakfast menu. Traditionally, Khmer rice noodles are invariably produced on a small, labour-intensive scale due to the short shelf life (<1.0 day) and the quality standards and operation control depends heavily on the artisan's skill. The quality varies with processing conditions, but the operations were similar to the traditional extruded rice noodles. These noodles become unsafe if contaminated with fecal matters anytime in between the preparation and consumption. Presence of higher number of total aerobic bacteria indicates the use of poor quality raw material. On the other hand, presence of higher number of coliform bacteria indicates the poor processing environment; and presence of higher number of faecal coliform or *Escherichia coli* indicates the faecal contamination, and presence of higher number of *Staphycoccus aureus* and *Bacillus cereus* indicates the possibility of the presence of toxin in the food[13]. Although there was no study on the impact of consuming unsafe food in Cambodia, the public has become increasingly concerned about the food they eat. Incidents of people getting ill after eating unsafe food have frequently been posted and shared on social media. In 2016, there were about 1,000 reported cases of food poisoning throughout

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Cambodia and dealing with the unsafe food is a real challenge, because Cambodia's food industry is characterized by thousands of local SME operated traditionally by households that typically located in rural areas close to agricultural production zones[13]. Due to the limited resources, they used cheap food additive to control unsuitable bacterial growth in food.

On the contrary, the long-awaited food safety law yet to be passed and the country is still relied on inter-ministerial "prakas" to regulate food and beverage industry[11]. Foodborne pathogens can cause severe diarrhea or debilitating infections including meningitis. Food with chemical contamination can lead to acute poisoning or long-term diseases, such as cancer and long-lasting disability and even death[1]. Although it is hard to estimate the cost of unsafe food, it is generally agreed that the burden of foodborne diseases to public health and welfare and to the economy is substantial. Thus, this study was done to evaluate the microbiological quality and safety of fermented and non-fermented Khmer Rice Noodles sold throughout Cambodia.

Material and Methods

Sample collection:

Total 75 Khmer rice noodle samples, (23 fermented and 52 non-fermented) were purchased from retail open market of Kandal province, Siem Reap province and Phnom Penh city and were collected in sterilized zip lock bags and kept in cool box and transported to the laboratory for analysis.

Sample preparation:

Ten (10) grams of each rice noodles samples were weighted into a stomacher bag and 90 ml of phosphate buffer saline (PBS) solutions were added and stomached for 90 seconds. The diluted and non-diluted samples were surface plated onto selective and non-selective agar plates and the plates were incubated at various temperatures for 18-48 h depending on the bacteria to be determined before being counted.

Isolation and Identification of bacteria:

Ten (10) grams of each of the rice noodles samples were mixed with 90 ml of PBS, stomached for 90 seconds and serial dilutions were made and100 µl of diluted and non-diluted samples were surface plated onto Plate Count Agar (PCA), and Standard Desoxycholate Agar (DESO) medium for total aerobic bacterial count and total coliform count respectively. For pre enrichment-each stomached samples were incubated at 35^0C for 18-24 h, after incubation, 1.0 ml of pre-enrichment culture were transferred to 9.0 ml of Brilliant green lactose bile (BGLB) agar in Durham fermentation tube for the determination of *E. coli* and faecal coliforms, 2 x AC medium for identification of *Enterococcus spp.* and 2 x NB medium for identification of *B. cereus* and *S. aureus*, and incubated at 35^0C, 24h. For the confirmation of *E. coli* and *Enterococcus spp.* one loopful of respective bacterial cultures were transferred into a 10 ml of *Escherichia coli* (EC) Broth and All Culture (AC) agar medium, incubated at 44.5^0C, 24h, and were streaked on Eosin Methylene Blue (EMB) Agar, coliform and EF agar plates. For the confirmation of *coliforms, B. cereus* and *S. aureus* one loopful from respective were streaked onto MacConkey, Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB), NGKG (NaCl-Glycine-Kim-Goepfect) agar and mannitol salt agar plate, and incubated at 35 ^{0}C for 24h. The typical colonies were recorded and were further characterized by biochemical tests using Catalase/ Oxidase/ VPOF/ DNA test and Gram staining and were confirmed by API 20E, API 20 Strep, API 50 CHB (BioMérieux, Marcy l'Etoile, France), coagulate test for the examination of toxin production of *Bacillus cereus*.

Identification and characterization of the presence of foodborne pathogens.

The bacterial culture was streaked onto nutrient agar plate to isolate typical colonies of bacteria. Isolated bacteria were identified by microscope observation and biochemical tests. Finally were confirmed by API kits

- PCR Test for coagulase positive *S. aureus*: *S. aureus* were grown in Mannitol salt broth and then centrifuged and supernatant was discarded, the pellets were suspended in PBS and were subjected to agar well diffusion assay and used for PCR reaction of *S. aureus*[12].
- Identification of *Bacillus cereus* toxin genes by PCR method: The *Bacillus cereus* toxin genes were identified using PCR and well diffusion assay. The first will used the CRET-RPLA for enterotoxin and second PCR reaction testing method[14].

Data Processing and Analysis

Data analyses were done using descriptive statistics using computer based program on Minitab version 16 of Minitab-star-tables-cross tabulation and chi-square and Microsoft Excel to see the logarithmic values. For data analyses, both quantitative and qualitative methods were used. In quantitative techniques, description and analytic statistics such as charts, tables, graphs, frequency, percentage, analysis of variance, and other appropriate methods such as preference ranking and indexing were also used.

Results and Discussion

Total aerobic bacterial count and coliform count in Khmer Rice noodle

Each Khmer rice noodles were diluted 2 x using PBS and plated onto respective selective agar plates and incubated at respective temperature for specified period. After the incubation, colonies appeared in the respective agar plates were counted and recorded. Irrespective of sample location, total aerobic bacterial count was found higher than that of *coliform* bacteria was recorded and low level of *coliform* bacteria was recorded in fermented rice noodles than that of non-fermented rice noodles (Fig 1). Nonetheless, in both fermented and non-fermented rice noodles the aerobic bacterial count was crossed the redline indicating the unacceptable quality of rice noodles.

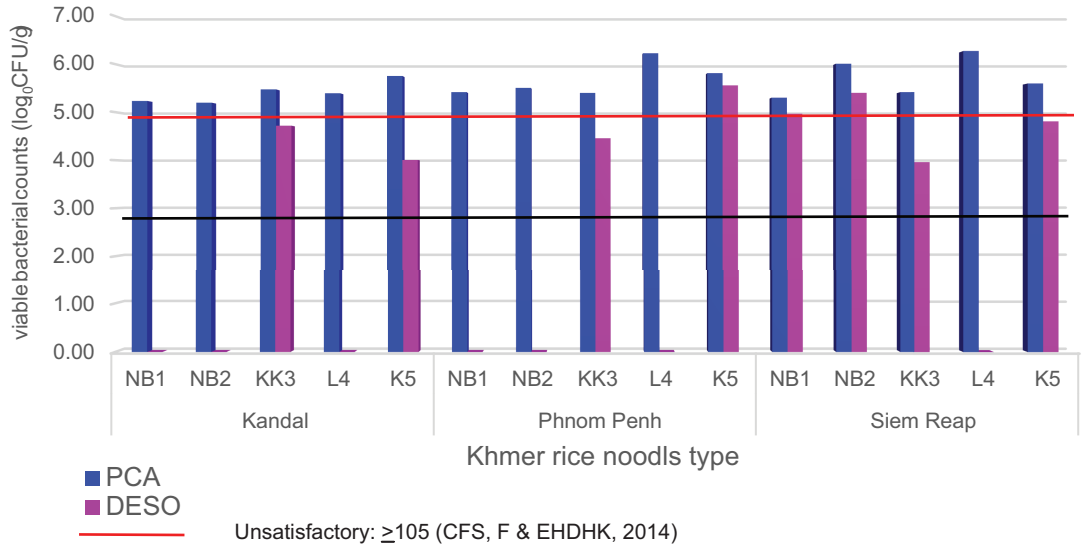

Figure 1: *Comparison of viable bacterial count and coliform bacterial count of fermented and non-fermented Khmer rice noodles of in two provinces and one city.*

Note: Fermented = Nom bachchok (NB1), Nom bachhoy (NB2), Non-Fermented = Kuytaykat (KK3), Lout char (L4), Kuytyv (K3).Under the black line: in the satisfactory; Between the black, and red line: in the acceptable; Above the red line line: in the unacceptable[2].

Although highest aerobic bacterial count was recorded in L4 samples of the open market of Phnom Penh city and Siem Reap province, but non-detectable level of coliform was detected in those samples meaning that the processing environment of those open market was good. However, except L4 sample, all other rice noodles sample showed the presence of higher number of coliform bacteria in Siem Reap markets compared to Phnom Penh city and Kandal province. About 33% of both fermented and non-fermented rice noodles were found contaminated with coliform bacteria.

Irrespective of fermented and non-fermented rice noodles, 40% samples were found contaminated with coliform bacteria, 4% with *Escherichia coli*, 17% with *Enterococcus* spp, and 12% samples were contaminated with both *Staphylococcus spp.,* and *Bacillus cereus*. However, among the fermented rice noodles (23 samples), only one sample of Siem Reap was found contaminated with *E.coli*, 36% samples were contaminated with *Bacillus cereus, 30%* samples were contaminated with *Enterococcus* spp, and none of the fermented rice noodles samples were found contaminated with contaminated with Staphylococcus spp. On the contrary, among the non- fermented rice noodles (52 samples), 40% samples were found contaminated with coliform bacteria, 5 % with *Escherichia coli*, 23% with *Staphylococcus* spp. and 11.5% with *Bacillus cereus.* Therefore, Khmer rice noodles were found grossly contaminated with pathogenic bacteria and may cause severe health problem if appropriate measure was not taken because, food poisoning arises from sanitation problems or vertical transmission, and the microorganisms includes total aerobic bacteria, total coliform bacteria and fecal coliform that represented sanitary quality and *E. coli, Staphylococcus aureus* and *Bacillus cereus* determined the presence of foodborne pathogens[13].

Table 1. *Number of pathogenic bacterial contamination in 75 Khmer rice noodle samples*

Isolated bacteria	Province						Numbers of contaminated sample	Percentages
	Kondal		Phnom Penh		Siem Reap			
	Fermented	Non Fermented	Fermented	Non Fermented	Fermented	Non Fermented		
Coliforms	0	5	2	6	7	10	30	40
Escherichia coli	0	0	0	2	1	1	4	5
Enterococcus spp.	1	2	3	5	3	3	17	23
Staphylococcus spp.	0	2	0	4	0	6	12	16
Bacillus cereus	3	2	2	3	1	1	12	16
Total	4	11	7	20	12	21	75	100

Note: Kind of Khmer rice noodles, in which Fermented (NB1, NB2), Non-fermented (KK3, L4, K5)

Isolation and characterization of *S. aureus* and *B. cereus* in Khmer rice noodles

Staphylococcus aureus strains were isolated from MSA agar on the basis of color changes in MSA medium. *Staphylococcus aureus* showed rods shape yellow colonies in MSA but other than *S. aureus*, did not change the color. In addition, *S. aureus* bacteria were able to ferment and other biochemical testing includes catalase (+Ve), aerobic and anaerobic growth (+Ve), VP (+Ve), and salt tolerant properties (6.5% NaCl). In total 22 *Staphyloccous* spp was detected and 3 of the isolated *Staphyloccous* spp were determined as *S. aureus*. These results were further confirmed using DNase and immune PS latex testing

In total, 27 *Staphylococcus* spp, 17 isolates out of 27 *S. aureus* isolates gave positive DNase tub agar (63%), and only 3 *S. aureus* isolates gave positive Immune latex testing (11%), respectively. *S. aureus* isolates were given 3 PCR positive coagulase genes (*S. aureus TM-K5-3* (7), *OM-L4* (12) and *DM-KK3-2* (13) strains), and a base pair of 267bp genes produce Type 6 coagulase similar to that of positive *S. aureus* IFO 13276(control). Type 6 coagulase positive characteristics showed correlation with positive DNase and Immune latex test.

Table 2. *Biochemical reactions and Gram stains of Staphylococcus spp.*

Test or substrate	Bacterial isolates				
		S. aureus			*S. aureus* (+)
Chang colored in MSA		Yellow	Yellow		Yellow
Colonies Color in MSA		Yellow	Yellow		Yellow
Gram reaction	+	+	+	+	+
Shapes	Rods	Rods	Rods	Rods	Rods
DNA agar	+	-	-	+	+
Immune latex	-	- (+)	+(-)	+	+
Catalase	+	+	+	+	+
Oxidation test	-	-	-	-	-
Aerobic growth	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
OF	F	F	F	F	F
VP	+	+	+	+	+
6.5%NaCl	+	+	+	+	+
Total	4	11	9	3	1

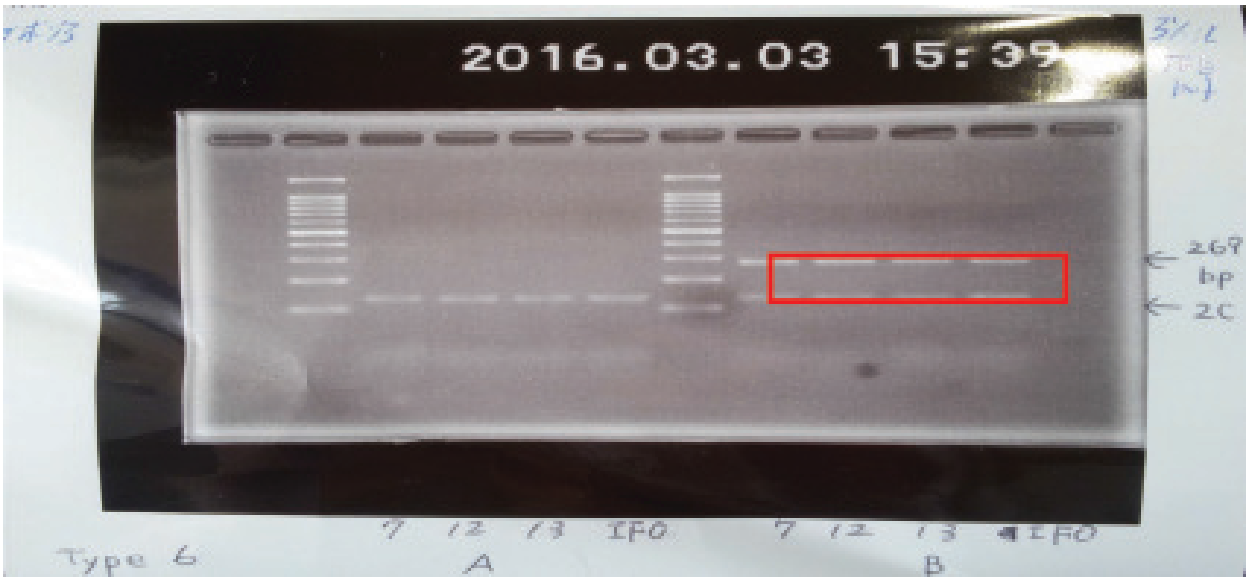

Figure 2. Show *S. aureus produced Type 6 coagulase* by PCR method

In summary, positive yellow colonies on mannitol salt agar were isolated (27 strains) and 17 of these 27 isolates produced DNase, and only 3 of these isolates aggregated polysaccharides specific immune latex strongly (protain A and clumping factor production positive).These 3 isolates (*S. aureus* TM-K5-3, OM-L4 and DM-KK3-2) harbored Type-6 coagulase gene.

Potential of toxin production of isolated *B. cereus* strains

Bacillus cereus a gram positive round presents a red and white and slightly thick colonies in NGKG agar plates and basically confirmed by biochemical reactions. All the isolated bacteria tested showed rods shaped and able to ferment. To test with gram reaction, oxidase, catalase, aerobic growth, anaerobic growth, and VP resulted positive. Other biochemical parameters including starch hydrolyze test revealed that only 7 isolates were *Bacillus cereus* negative, yet *Bacillus cereus*, which were compared with positive indicator of *Bacillus cereus* presented positive.

Bacillus cereus toxin genes were determined using PCR and well diffusion assay methods. There were 2 steps conducted in PCR and well diffusion assay. The first will use PCR methods to determine *Bacillus cereus* toxin genes and the second will use Enterotoxin-Reverse Passive Latex sensitized (CRET-RPLA) test kit to confirm the toxicity. The cell-free culture supernatants of *B. cereus* isolates were subjected to detect for enterotoxin production by *B. cereus*. Enterotoxin-Reverse Passive Latex sensitized (CRET-RPLA) test kit.

The results as reported in the Figure 3 showed that 12 of 27 *B. cereus* isolates gave positive sensitized (44%) compare with control enterotoxin by Lyophilized. The 27 of *B. cereus* isolates, which gave multiplex PCR positive with only one genes, showed no correlation with RPLA test. So that, *B. cereus* isolate DM-NB1-1, which gave multiplex PCR positive with 227bp genes similar positive control *Bacillus cereus* (C= Control Cereus rid Toxin produce) and higher than negative control still 134bp (L= Internal Control), but also difference showed negative Latex sensitized (CRET-RPLA) test. *B. cereus* strains isolated from Khmer rice noodles samples were Gram-positive, rod-shaped, heat resistant & spore forming bacteria. All the isolated strains exhibited positive results in VP reaction, and catalase production. Twelve (12; 44%) isolated *B. cereus* were produced enterotoxin, may cause diarrhea. However, one (1; 4%) isolated *B. cereus* harbored CRS gene, may produce emetic toxin.

Table 3. *Biochemical reactions and Gram stains of Bacillus cereus*

Test or substrate	Bacterial isolates		
	Bacillus cereus	*Bacillus cereus*	*Bacillus cereus* (+)
Chang colored in NGKG		Medium around colonies presents a red	
Colonies Color in NGKG		White and slightly thick	
Gram reaction	+	+	+
Shapes	Rods	Rods	Rods
Oxidase test	+	+	+
Catalase	+	+	+
Aerobic growth	+	+	+
Anaerobic growth	+	+	+
OF	F	F	F
VP	+	+	+
Starch Hydrolyse	-	+	+
Total	7	20	1

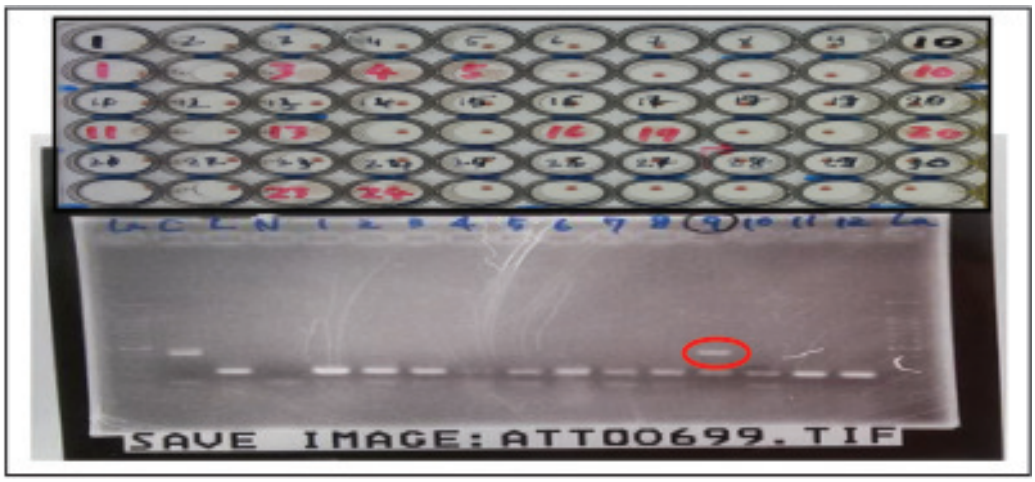

Figure 3. *Show Bacillus cereus toxin genes by CRET-RPLA and multiplex PCR method*

Table 4. *Comparison of contamination rate of bacterial strains in Khmer rice noodle sample in two provinces and one city of Cambodia*

Bacterial Type	Kandal				Phnom Penh				Siem Reap					
	Fermented		Non fermented		Fermented		Non fermented		Fermented		Non fermented		P-value	Total contm
	(+) /Total	(%)	(+)/Total	(%)	(+)/Total	(%)	(+)/Total	(%)	(+)/Total	(%)	(+)/Total	(%)		Rate (%)
Citrobacter freundii	0/6	0	0/9	0	0/12	0	1/18	6	0/12	0	0/18	0	1.000	1
Cronobacter spp	0/6	0	2/9	22	0/12	0	0/18	0	0/12	0	0/18	0	0.155	3
Enterobacter amnigenu	0/6	0	0/9	0	0/12	0	3/18	17	0/12	0	0/18	0	0.080	4
Enterobacter cancerogenus	0/6	0	2/9	22	0/12	0	0/18	0	0/12	0	0/18	0	0.155	3
Enterobacter cloacae	0/6	0	2/9	22*	0/12	0	6/18	33*	1/12	8*	4/18	22*	<0.001	17
Enterobacter gergoviae	0/6	0	1/9	11	1/12	8	0/18	0	0/12	0	1/18	6	0.560	4
Enterrobacter aerogene	0/6	0	0/9	0	0/12	0	1/18	6	0/12	0	2/18	11	0.080	4
Escherichia coli	0/6	0	0/9	0	0/12	0	3/18	17	1/12	8	1/18	6	0.172	7
Klebsiella pneumoniae	0/6	0	10/9	111*	2/12	17*	18/18	100*	16/12	133*	12/18	67*	<0.000	77
Pseudomonas aeruginosa	0/6	0	0/9	0	0/12	0	1/18	6	0/12	0	0/18	0	1.000	1
Rahnella aquatilis	0/6	0	1/9	11	0/12	0	0/18	0	0/12	0	0/18	0	1.000	1
Raoultella ornithinolytica	0/6	0	0/9	0	0/12	0	1/18	6	0/12	0	0/18	0	1.000	1
Serratia liquefaciens	0/6	0	0/9	0	0/12	0	1/18	6	0/12	0	2/18	11	0.080	4
Serratia rubidaea	0/6	0	0/9	0	0/12	0	0/18	0	1/12	8	0/18	0	1.000	1
Enterococcus faecium	1/6	17*	4/9	44*	4/12	33*	10/18	56*	2/12	17*	10/18	56*	<0.001	41
Enterococcus faecalis	0/6	0	2/9	22	1/12	8	4/18	22	3/12	25	2/18	11	0.229	16
Staphylococcus aureus	0/6	0	7/9	78*	0/12	0	10/18	56*	0/12	0	10/18	56*	<0.000	21
Bacillus cereus	3/6	50	5/9	56	7/12	58	7/18	39	4/12	33	1/18	6	0.832	36

The superscript * represents a statistically significant difference in the detection rat (P<0.05) between fermented and non-fermented noodle in three located areas in Cambodia.
Note: Fermented (NB1, NB2), Non-fermented (KK3, L4, K5), Positive (+), Total number of samples size.

Conclusion

Food poisoning and foodborne diseases posed serious threat to both consumers and food industries and lead to death worldwide particularly in developing countries that poor in food safety, and quality[6]. The comparison of contamination rate of bacteria in fermented and non-fermented Khmer rice noodle is shown in Fig 1 & Table 1. In general, the contamination rates in non-fermented rice noodles were higher than that of fermented rice noodle. Most bacteria did not survive in fermented rice noodle; however, *Klebsiella pneumoniae* spp was evident in both fermented and non-fermented rice noodles.

There was substantial number of bacterial contamination observed in both fermented and non-fermented rice noodle which was presented in Table 4. It was found that the rice noodles of Siem Reap province were highly contaminated and four indicator bacteria (*Enterobacter cloacae*, *Klebsiella pneumoniae, E. faecium, S .aureus)*, showed higher significant difference (P<0.01). On the other hand, non-significant differences in fermented and non-fermented rice noodle in three locations. The general composition of fermented rice noodles is influenced by the microorganisms present during the manufacturing process and after the rice-soaking step[8]. Consequently, precluding illnesses associated with foodborne pathogenic microbes remain a foremost health challenge[3]. Therefore, it is worth while in exploring fermentation technology for expanding traditional production to an industrial scale and developing a new technology to improve the quality of production of rice noodles[5].

The superscript * represents a statistically significant difference in the detection rat (P<0.05) between fermented and non-fermented noodle in three located areas in Cambodia.

The Khmer rice noodles were grossly contaminated with faecal origin and other pathogenic bacteria such as *Bacillus spp*, *Enterococcos spp*, *Staphylococcus spp* and coliform bacteria were presence in NB1, NB2, KK3, L4 and K5. Furthermore, among the 75 samples analyzed, only K5 in Phnom Penh and NB2 in Siem Reap were acceptable compare with the standard foods. Fermentation processed rice noodles have less gram positive and gram negative bacterial species than khmer rice noodless made without fermentation. Especially, Siem Reap province and Phnom Penh city was explored the most gram positive and negative bacterial isolates. Furthermore, 3 *Staphylococcus aureus* isolates (TM-K5-3, OM-L4 and DM-KK3-2) aggregated PS immune latex strongly, protein A and positive clumping factor production and harbored Type-6 coagulase gene. For *B. cereus,* 12 isolates produced enterotoxin that may cause diarrhea, and one *B. cereus* (DM-NB1*)* isolates harbored CRS gene, may produce emetic toxin. Rice noodles sold in local markets in Cambodia have risk to cause foodborne illnesses especially caused by the toxin of *S. aureus and B. cereus*.

In conclusion, the present study demonstrated in Khmer rice noodle were contaminated with various microorganisms indicating the poor personnel hygiene of food handler throughout the value chain. Moreover, we found that the higher numbers of pathogens were observed in non-fermented rice noodles in Siem Reap province, Kandal province and Phnom Penh city. Therefore, improvement of food hygiene practices to be developed to aware the consequences of food contamination in their personnel hygiene and related to above mentioned, these issue caused from the low hygienic practice from producer and during display in the market that requires the sellers and producers take into account in hygienic. Basically, it was suggested that food Quality control in Cambodia is still limited and need to improve more.

This research study demonstrated that, Khmer rice noodles were contaminated with many type of micro-organisms from the low hygienic practice from production and manufacturing environment and during display in the market, that requires the sellers and producers take into account in hygienic awareness programme. Basically, it was suggested that food Quality control in Cambodia is still limited and need to improve more and more awareness should be increased through the training and awareness programme.

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