

Article

Microbial quality of ready-to-eat food contact surfaces in local restaurants of Patuakhali district, Bangladesh

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Abstract: Cross-contamination of food contact surfaces can impose crucial barriers to food safety. Ready-to-eat (RTE) food items' have the possibility of contamination during handling and serving from the terminal contact surfaces like hands, serving plates and tables, spoons, knives, and glasses. Considering these, this study was aimed to investigate the microbial load of these contact surfaces. Swab samples were collected from four crucial RTE foods contact surfaces for instance food handlers' hands (HHS), plates (PS), tables (TbS), and spoons (SS) surfaces of twenty randomly selected local restaurants. To quantify the microbial load of these surfaces, total viable count (TVC) count was assessed using nutrient agar (NA), and total coliform (TC) count and fecal *Escherichia coli* (*E. coli*) were identified based on colony characteristics on chromocult coliform agar (CCA) media using standard methodologies described in previous studies. *E. coli* colonies from all contact surfaces and bacterial colonies isolated from HHS samples were confirmed by biochemical tests. Substantial number of bacterial presence was observed in all the samples, where TbS (1.93×10^5 CFU/cm²) and HHS (6.77×10^4 CFU/cm²) and SS (2.80×10^2 CFU/cm²) and HHS (2.88×10^2 CFU/cm²) had the highest TVC and TC count respectively. 65% of all samples were found to be contaminated with fecal *E. coli* while HHS samples harbored major foodborne pathogens like *Salmonella*, *Shigella*, *Klebsiella* spp., and *Bacillus cereus*. The study indicates poor microbial quality of these food contact surfaces which could implicate cross-contamination of the food items and incidences of foodborne illness among the consumers of these restaurants. These scenarios urge further studies on routes of these microbial contamination and limiting ways to enhance food safety in these restaurants.

Keywords: ready foods; non-food contact surfaces; microbiological evaluation; hygiene; Bangladesh

1. Introduction

Like other developing countries the overall food preparation and serving in restaurants in Bangladesh are vulnerable, especially in peripheral regions. Most of these restaurants in local markets have minimalistic investment causing inadequate facilities for hygienic management (Nizame *et al.*, 2019), and the personnel do barely have any training on proper hygiene and good food preparation (Banna *et al.*, 2021). Moreover, lack of knowledge and practices are highly prevalent among the workers (Hasan *et al.*, 2022). These attributes make the foods prepared in these restaurants susceptible to contamination.

Food safety can be compromised sporadically from breach of any aspects of the food chain. Food handlers in this chain serve as a potent source of microbial contamination of both raw and finished food products. The food handlers' hand-palm and fingernails are more liable to cause food contamination than other areas of hand (Stein-Zamir *et al.*, 2009). A bacterial assessment study in Iran showed food handlers fingernails harbor so many different microorganisms starting from commensal, opportunistic pathogen *Staphylococcus aureus* to multidrug resistance *Pseudomonas aeruginosa*, as well as *Escherichia coli*, Coliforms and other enteric pathogens. This study also suggested fast food handlers harbor the second highest microorganisms among the seven selected common food handlers i.e. butcher, baker, vegetable sellers and others (Nasrolahei *et al.*, 2017). Furthermore, foods can be subjected to cross-contamination by bacteria and foodborne viruses from clothing (apron) of the food handlers, and utensils or crockeries used in food preparation (Alves *et al.*, 2021). Food contact surfaces that are not directly associated with food preparation but with serving and consumption have also been implicated to be contaminated with numerous microorganisms at higher levels like the cleaning clothes for the cleaning of serving tables or wiping plates, seating chairs and tables, serving trays, food holding spatulas, drinking glasses, and spoons (Patel *et al.*, 2017; Giwa *et al.*, 2021). An estimated 27% of outbreaks by foodborne pathogens are resulted from contaminated kitchen utensils, while 97% of foodborne illnesses are due to improper food handling practices in restaurants and at home (Tenna *et al.*, 2023).

In Bangladesh, many studies on knowledge, attitudes, practices, and training of restaurant workers, street vendors and other food service providers have reported poor to average knowledge level but serious lacking in training and hygiene practice, (Banna *et al.*, 2022; Ali *et al.*, 2023; Hashanuzzaman *et al.*, 2020). Unacceptable level of microbial contamination and presence of specific pathogens in restaurant foods, as well as workers' overall hygienic condition especially contamination in hand have been reported in several studies (Younus *et al.*, 2019; Nizame *et al.*, 2019; Ema *et al.*, 2022). These previous studies suggest that the immediate contact surfaces of RTE foods may be heavily contaminated because of cross-contamination during handling (displaying, serving, and storing). Foods prepared in local restaurants are usually high heat treated for prolonged time, which considered sufficient to eradicate common pathogens, but recontamination at these points can be crucial to gain entrance of pathogens, and more importantly there is no further processing that can eliminate or reduce these microorganisms. Considering these scenarios, we investigated the kinds and numbers of bacterial contamination of these terminal food contact surfaces using swab samples from the food serving personnel's hands, food serving utensils including plates, and spoons, and table's surface (on which food is served). This study will suggest the extent of contamination occurring on these RTE foods contact surfaces in the restaurants of Patuakhali district, Bangladesh.

2. Materials and Methods

2.1. Ethical approval

This study did not require ethical approval.

2.2. Study area and hygienic scenarios of selected restaurants

This study was conducted on twenty randomly selected local restaurants of Patuakhali district, mainly Patuakhali Sadar (six restaurants, blue color dots) and Dumki Upazilla (14 restaurants, green color dots) during July to December 2023 (Figure 1). Sample preparation and all microbial investigations and biochemical tests were carried out in the Food Microbiology Laboratory (FML), Patuakhali Science and Technology University, Bangladesh. These restaurants suffer from poor hygiene, no handwashing facilities for customers or staff, dirty serving utensils, lack of protective gear like aprons and gloves, staff handling food waste without washing hands, uncovered food, messy cooking areas, improper waste disposal, shared glasses, handling cash and food without cleaning hands, and unclean food preparation areas.

2.3. Sample collection

A total of 80 swab samples were collected randomly from these twenty selected restaurants, four samples from each restaurant, including handler's hand (HHS), tables (TbS), plates (PS), and spoons (SS). For the hand samples, either right or left hand were chosen, based on the worker's handedness, and swabbing was done on hand palm, fingers and nails, and between the fingers. A 100 cm² (10×10 cm) area was swabbed from each sample, and sterilized steel scale was used for measuring the area which was sanitized using 70% ethanol before every measurement. For the sample collection horizontal, vertical, and diagonal swabbing was performed. The swab tips were collected in tubes containing 10 ml sterile normal saline (0.95% NaCl) and placed in an ice box for transportation to the laboratory where it stored at 4°C temperature.

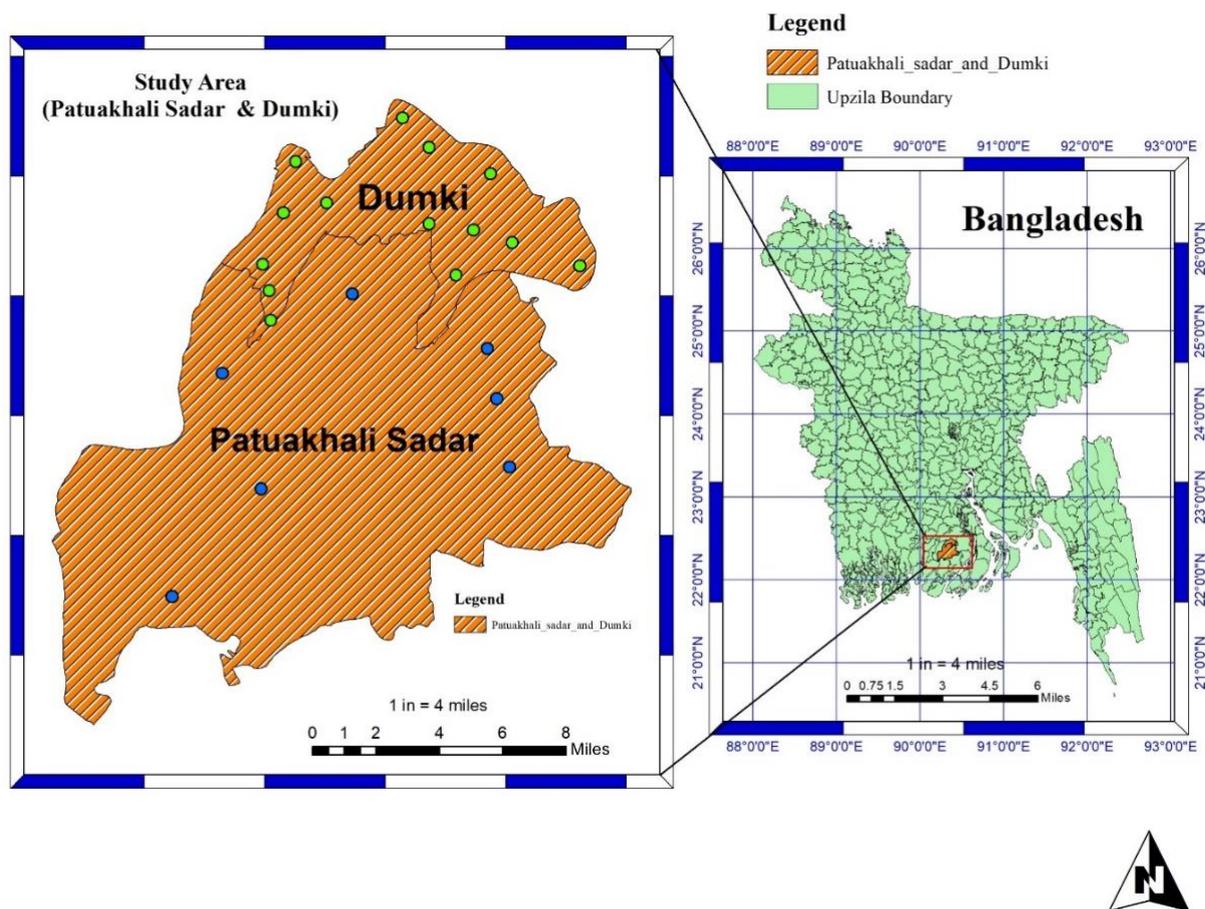


Figure 1. The study areas and sampling sites map (blue and green color dots denotes restaurants studied).

2.4. Sample preparation and microbiological methods

The refrigerated samples were vortexed for 2 minutes and serially diluted up to 10^{-4} for TVC and up to 10^{-2} dilution for total coliform (TC) count. Standard pour and spread plate technique was used for the enumeration of total viable count (TVC) and TC count of the samples using nutrient agar (NA) and Chromocult coliform agar (CCA) media respectively (Bell *et al.*, 2005). Upon inoculated with samples NA plates were incubated at 37°C for 24 hours for TVC count, and For TC enumeration and presumptive- positive *E. coli* identification CCA plates were incubated at 37°C for 24-48 hours. The coliforms produced Salmon pink to red colonies while *E. coli* produced typical dark blue to violet colonies on CCA medium (González *et al.*, 2003). All samples were duplicated for these counts and expressed as the average colony forming unit (CFU) per centimeter square (cm^2). Calculation was done using following formula (FDA, 2023),

$$N = C/V (n_1 + 0.1n_2) d$$

Where C is the sum of colonies on all plates counted; V is the volume applied to each plate; n_1 is the number of plates counted at first dilution; n_2 is the number of plates counted at second dilution; and d is the dilution from which first count was obtained.

2.5. Biochemical tests for *E. coli*

E. coli colonies of all the four samples were isolated from positive CAA petri-plates with typical colony characteristics, and biochemical tests were performed for the confirmation of fecal *E. coli* presence. The catalase test, methyl red test, citrate utilization test, Indole test, carbohydrate fermentation test (lactose, sucrose and dextrose), hydrogen sulfide (H_2S) production tests were performed according to Cappuccino and Sherman (2014).

2.6. Isolation and identification of bacterial species from HHS samples

From all the positive TC and TVC plates of HHS samples, we identified the presence of different species of bacterium. The Isolation and identification of bacterial species from HHS samples and their biochemical tests were performed on these pure culture isolates according to Cappuccino and Sherman (2014).

2.7. Statistical analysis

Collected data was analyzed using 'JMP Pro 16' statistical software and presented as overall mean, median and quartile values with lowest and highest counts of each sample. The study area and sampling sites map was generated using ArGIS software, version 10.8.1.

3. Results

3.1. Total viable count

Among the four food contact surfaces, TbS had the highest mean TVC value (1.93×10^5 CFU/cm²) (Table 1). TbS also had the highest 3rd quartile value indicating 25% of the study restaurants' tables had a contamination level equal to or above 1.84×10^5 CFU/cm² in contrast to other contact surfaces. Although tables don't come to direct contact of foods, still it was pertinent because it is the surrounding environment of food consumption. The food handlers' hand had the second highest mean TVC count (6.77×10^4 CFU/cm²). Plates comprised of a substantial amount of bacterial contamination with a mean of 5.05×10^4 , while spoon had least count (1.10×10^4 CFU/cm²) among the four (Table 1).

Table 1. Total viable count on nutrient agar plate.

Restaurant ID	Food handlers' hand surface (HHS)	Table surface (TbS)	Plate (PS)	Spoon (SS)
R1	9.50×10^3	3.60×10^4	1.80×10^4	9.00×10^3
R2	6.00×10^3	7.50×10^5	1.25×10^3	5.55×10^3
R3	5.00×10^4	1.67×10^5	5.90×10^3	1.50×10^2
R4	8.50×10^3	7.50×10^5	1.00×10^4	3.50×10^2
R5	2.35×10^4	1.35×10^5	1.55×10^5	2.75×10^3
R6	3.55×10^4	1.95×10^3	1.75×10^3	9.50×10^2
R7	4.20×10^4	3.05×10^4	2.30×10^2	1.40×10^2
R8	4.25×10^5	1.20×10^2	1.65×10^2	4.30×10^4
R9	6.00×10^4	1.55×10^5	2.80×10^2	2.00×10^3
R10	1.35×10^3	2.95×10^4	1.00×10^3	5.00×10^3
R11	2.55×10^4	1.40×10^4	3.50×10^5	2.25×10^2
R12	3.55×10^4	1.05×10^3	3.50×10^4	9.50×10^2
R13	2.15×10^5	2.05×10^3	9.50×10^4	6.00×10^3
R14	8.00×10^4	1.50×10^3	8.00×10^4	4.95×10^4
R15	1.80×10^3	5.05×10^3	2.35×10^5	7.50×10^4
R16	2.40×10^2	1.55×10^4	1.40×10^4	1.15×10^4
R17	6.00×10^5	6.40×10^5	2.10×10^3	3.40×10^3
R18	2.15×10^4	1.90×10^5	2.40×10^2	1.25×10^2
R19	1.10×10^3	1.75×10^2	2.65×10^2	2.20×10^3
R20	9.50×10^4	9.30×10^5	4.15×10^4	1.65×10^3
Lowest count	1.10×10^3	1.20×10^2	1.65×10^2	1.25×10^2
Highest count	6.00×10^5	9.30×10^5	3.50×10^5	7.50×10^4
Mean	6.77×10^4	1.93×10^5	5.05×10^4	1.10×10^4
Median	3.05×10^4	3.0×10^4	5.03×10^3	2.48×10^3
25% Quartile	6.63×10^3	1.975×10^3	4.60×10^2	5.00×10^2
75% Quartile	5.75×10^4	1.84×10^5	6.88×10^4	8.25×10^3
STDEV	1.35×10^5	3.59×10^5	1.93×10^5	2.03×10^4

*The calculated values are rounded up at two decimal places; STDEV=Standard deviation

3.2. Total coliform count

We found the presence of total coliform (TC) in all the contact surfaces in most of the restaurants (14 out of 20). Some restaurants had coliform absent on one or two surfaces (<1 CFU/cm²) but present on the rest, showing none were entirely coliform-free on all immediate food contact surfaces. The highest number of TC obtained in HHS and SS samples, 2.88×10^2 and 2.80×10^2 CFU/cm² respectively. The SS samples median TC value was also

the highest compared to other contact surfaces, 3.0×10^2 CFU/cm². PS and TbS samples harbored nearly same amount of coliform 2.01×10^2 and 1.95×10^2 CFU/cm² respectively (Table 2).

Table 2. Total coliform count on CCA agar plate. Result presented as CFU/cm².

Restaurant ID	Food handlers hand surface (HHS)	Table surface (TbS)	Plate (PS)	Spoon (SS)
R1	7.00×10^2	2.50×10^2	3.00×10^2	3.50×10^2
R2	1.50×10^2	2.00×10^2	2.50×10^2	3.00×10^2
R3	6.00×10^2	5.0×10^1	5.00×10^2	1.50×10^2
R4	1.50×10^2	1.00×10^2	3.50×10^2	5.0×10^1
R5	3.50×10^2	1.00×10^2	5.0×10^1	4.00×10^2
R6	2.50×10^2	1.50×10^2	1.20×10^2	3.50×10^2
R7	<1	3.50×10^2	1.50×10^2	<1
R8	5.50×10^2	3.00×10^2	<1	2.00×10^2
R9	1.00×10^2	1.00×10^2	2.50×10^2	5.0×10^1
R10	2.00×10^2	2.50×10^2	2.50×10^2	6.50×10^2
R11	3.00×10^2	4.50×10^2	<1	5.0×10^1
R12	1.15×10^2	5.00×10^2	1.50×10^2	3.00×10^2
R13	6.00×10^2	2.00×10^2	4.00×10^2	5.0×10^1
R14	3.50×10^2	3.00×10^2	1.50×10^2	2.00×10^2
R15	<1	<1	5.0×10^1	4.50×10^2
R16	5.00×10^2	5.0×10^1	2.00×10^2	1.50×10^2
R17	3.00×10^2	<1	1.00×10^2	3.40×10^2
R18	1.50×10^2	2.00×10^2	5.0×10^1	5.55×10^2
R19	<1	1.00×10^2	2.00×10^2	4.00×10^2
R20	4.00×10^2	2.50×10^2	5.00×10^2	6.00×10^2
Lowest count	0.00	0.00	0.00	0.00
Highest count	7.00×10^2	5.00×10^2	5.00×10^2	6.50×10^2
Mean	2.88×10^2	1.95×10^2	2.01×10^2	2.80×10^2
Median	2.75×10^2	2.00×10^2	1.75×10^2	3.00×10^2
25% Quartile	1.24×10^2	1.00×10^2	6.25×10^1	7.50×10^1
75% Quartile	4.75×10^2	2.88×10^2	2.88×10^2	4.00×10^2
STDEV	2.15×10^2	1.39×10^2	1.51×10^2	1.95×10^2

*The calculated values are rounded up at two decimal places; STDEV=Standard deviation

3.3. Percentages of fecal *E. coli* contaminated contact surfaces

We confirmed the presence of fecal indicator organism *E. coli* in the contact surfaces samples. Around 65% of all studied contact surfaces had fecal contamination among which highest contamination occurrence rate was 75% in SS samples. The HHS samples had the second highest (60%) fecal contamination and PS had the lowest (40%) (Table 3).

Table 3. Percentages of fecal *E. coli* contaminated contact surfaces.

Contact surfaces	CCA positive plates	No growth on CCA medium	<i>E. coli</i> positive plates	Percentage
HHS	17	03	13 (20)	65%
TbS	18	02	12 (20)	60%
PS	18	02	08 (20)	40%
SS	19	01	15 (20)	75%
Total	72	8	52 (80)	65%

* CCA=Chromocult coliform agar

3.4. Bacterial species isolated from HHS samples

A total of twenty-one bacterial colonies were isolated from NA and CCA media and 10 bacterial species were identified using biochemical tests from the HHS samples (Table 4). These include *K. oxytoca*, *E. aerogenes*, *S. aureus*, *B. cereus*, *S. dysenteriae*, *P. aeruginosa*, *E. coli*, *Lactococcus lactis*, and *S. typhimurium*.

Table 4. Major bacterial isolates from HHS samples.

Organisms	Fermentation test									
	Lactose	Dextrose	Sucrose	Catalase activity	MR	VP	Citrate use	Indole production	Starch hydrolysis	Hydrogen sulfide
<i>E. coli</i>	AG+	AG	A+	+	+	-	-	+	-	-
<i>Salmonella typhimurium</i>	-	AG+	A-	+	+	-	+	-	-	+
<i>Enterobacter aerogenes</i>	AG+	AG	AG+	+	-	+	+	-	-	-
<i>Bacillus cereus</i>	-	-	-	+	+	-	+	-	+	+
<i>Staphylococcus aureus</i>	A	A	A	+	+	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	+	-	-	+	-	-	-
<i>Lactococcus lactis</i>	A	A	A	-	+	-	-	-	-	-
<i>Micrococcus luteus</i>	-	-	-	+	-	-	-	-	-	-
<i>Shigella dysenteriae</i>	-	A	A-	+	-	-	+	-	-	-
<i>Klebsiella oxytoca</i>	A	-	A	-	-	-	+	+	-	-

*AG=Acid and gas producing, A= Acid, '+'- positive, and '-' = negative for respective tests

4. Discussion

This study found the presence of high number mixed microbial contamination on food contact surfaces which were indicated by TVC count. We reported the highest TVC count in HHS and TbS samples with a mean value of 6.77×10^4 and 1.93×10^5 respectively while other contact surfaces also possessed a fair bacterial presence. The occurrences of TC were frequent in the samples, 14 out of 20 samples, where HHS had the highest mean count of 2.88×10^2 followed by SS 2.80×10^2 CFU/cm² (Table 2). Alarming scenarios were reported in terms of *E. coli* presence in the samples. 65% of all samples were *E. coli* positive among which 75% of SS and 65% of HHS were contaminated by this fecal route microbe (Table 3). Both gram-positive and gram-negative bacteria with potential to cause foodborne illnesses were reported in this study in the HHS samples, among them notably identified species were *S. typhimurium*, *Shigella* spp., *S. dysenteriae*, *B. cereus* and *K. oxytoca*. In a microbiological survey of food contact surface's hygiene in public sector cafeterias serving RTE foods in a university of Pakistan reported 7.9×10^7 CFU/cm² (7.9 log) APC count on hands samples, which was much higher than our count. This study also reported higher TVC count in spoon and plate samples, 5.7 log and 5.1 log CFU/cm² respectively, but surprisingly got lower count in serving counters (tables) swabs (3.1 log CFU/cm²) (Giwa *et al.*, 2021). A study in Oman in Salalah state on food safety in restaurants found 1.6×10^5 CFU/cm² TPC count in food handlers' hand (Abdalla *et al.*, 2015). Similar high counts were reported in restaurants provide RTE foods in Cairo, Egypt in hand and plate swab (Fahim *et al.*, 2022), and in Addis Ababa, Ethiopia in plate, glass and food serving tray (Tenna *et al.*, 2023). Our TVC values were somewhat lower compared to these studies but still high enough to introduce substantial contamination of the foods of these restaurants.

Results from previous studies widely vary in microbiological counts (TVC and TC) of food contact surfaces, from countries to countries and places to places, which is expected as food culture and regulatory approaches and their application, and many other integral factors influence these counts. It also varies with the level of food preparation or types of food handled, for example meat and fish food handlers' associated food contact surfaces ought to be heavily contaminated than processed foods handlers. Differences were also observed in using terminology and reporting results. We reported TVC as total viable count which has been reported as aerobic plate count (APC), total plate count (TPC) and standard plate count (SPC) in different studies while the units used as log CFU/cm², log₁₀ CFU/cm² and exponential factor (as we reported). A substantial amount TC count was reported in respect to all the study contact surfaces by Giwa *et al.* (2021). They reported the highest number of TC presence in knife followed by hand, cleaning cloth and spoon and plate. Similar findings were reported in Johannesburg, South Africa (Christison *et al.*, 2008) where they found around 3.5 log or 3.1×10^3 CFU/cm² in hand samples and around 1.7 log in spoon and utensils samples. Align to these results, we got the highest mean TC value in HHS and SS samples (2.88×10^2 and 2.80×10^2 CFU/cm²) (Table 2). In our observation, the high TC value in SS samples is attributed to the fact that we collected the whole spoon swab, and the customers in these restaurants are people from all sorts of work for example, raw vegetables, poultry and fish sellers, rickshaw and motor vehicle drivers and so on, all take their breakfast and evening snacks and due to their uncleaned hands, they use spoons. We presume these along with improper cleaning after use of these spoons may attribute to the

higher value of TC in SS samples which also explains the highest-level of *E. coli* presence in the SS samples. Total Coliform is considered as an important hygiene indicator (Jackson *et al.*, 2007) while the presence of less than 10^6 TVC load is an indicative of mixed microflora presence. The presence of high TC in these food contact surfaces does suggest that contamination is coming from numerous sources involving soil, raw vegetables and other raw food items, water and so on. Our observation suggests the workers prepare raw salads, handle eggs for RTE foods and often help in the cooking area from where they come in contact to variety of raw and unprocessed foods, thus may harbor coliform bacteria in hands and subsequently contaminate other contact surfaces.

Giwa *et al.* (2021), found *E. coli* presence in above 40% of spoon, 30% of plate and less than 20% of hand samples. In contrast, we got highest in SS followed by HHS and lowest in PS samples (Table 3). The PS samples had the lowest *E. coli* count in respect to other contact surfaces and was much of a surprise. This may be attributed to the fact that the plates go through a kind of washing in these restaurants. Although, they first dip it in water containing accumulated food leftovers from plates and spoons, and then wash it minimally with detergent and finally rinse it. This practice of washing certainly not enough but still lowers the *E. coli* contamination in PS samples.

Numerous studies have isolated different species of bacteria from food contact surfaces. Abdel-Salam *et al.* (2021), studied the influence of food handlers' hand and food contact surfaces hygiene in RTE foods, they reported *S. aureus* and *E. coli* was most abundantly present in these contact surfaces (Fahim *et al.*, 2022). *S. aureus* is commonly found on skin, nose of human and environment thus can easily contaminate food handlers' hands. Our study also reported their presence in HHS samples which was also reported by others along with *E. coli* (Christison *et al.*, 2008; Muhammad *et al.*, 2016). Giwa *et al.* (2021) isolated *E. coli*, *Shigella* spp., *Klebsiella* spp., *Pseudomonas* spp., *S. aureus* and hemolytic *Streptococci* in the hand samples. *Salmonella*, *Bacillus*, *Listeria* spp., were found in food and hand swab samples in restaurants in Salah city, Oman, by Ali *et al.* (2023). These studies are consistent with our findings where the current study found 10 bacteria species from the HHS samples including common pathogenic *Salmonella*, *Shigella*, *Klebsiella* spp, and *B. cereus* which have been implicated in may foodborne illness outbreaks (Bintsis, 2017). In USA, *Salmonella* spp., is the second leading cause of foodborne illness and first for foodborne illness related hospitalization and 28% of death (Scallan *et al.*, 2011).

Frequent handling of raw eggs, commonly used in popular breakfast and evening snacks, along with inadequate hand washing by food handlers, may contribute to *S. typhimurium* contamination. Cross-contamination from other food items like beef, vegetables, and chicken could also play a role (CDC, 2023). The current study highlights restaurants with high TVC, TC count, and *E. coli* presence on food contact surfaces, indicating poor hygiene practices and raising significant public health concerns. These findings also justifies the frequent occurrences of diarrheal incidences and other foodborne illnesses among the consumers (60%) taking foods in these restaurants, reported in a study in the same area (Mali *et al.*, 2020).

5. Conclusions

The study found high contamination levels on contact surfaces of RTE foods in local restaurants, including fecal coliform and pathogenic bacteria. Hands were identified as a significant source of cross-contamination, with spoons also posing a risk. *E. coli* was found on all surfaces, indicating widespread cross-contamination. This raises public health concerns, prompting the need for action to ensure food safety. Further research is recommended to understand the routes and extent of contamination, with a focus on implementing practical solutions applicable to local restaurants.

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Data availability

All relevant data are presented within the manuscript.

Conflict of interest

None to declare.

Authors' contribution

Md. Abu Tareq: participated in planning, coordinating, prepared the methodology, reviewed, and edited the article; Md. Touheduzzaman Rifat: carried out the lab work, formal analysis and wrote the original draft; Md. Shajadul Islam: wrote, reviewed, and edited the article; Prosenjit Mondol and Md Shafiqul Islam Khan: supervised the study. All authors have read and approved the final manuscript.

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