BACTERIOLOGICAL AND MYCOLOGICAL ANALYSIS OF MIXED VEGETABLE SALADS

S. Hameed, A. A. Anjum*, S. Sana and M. I. Najeeb

Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
*Corresponding Author's Email: aftab.anjum@uvas.edu.pk

ABSTRACT: Salads are considered healthy food but poor hygienic practices may destroy the quality of salads. The poor quality salads may cause health issues if consumed. A study was carried out to monitor microbiological quality of salads. Samples (n=90) were collected from road side venders, fast food outlets and family restaurants (30/each type), in different localities in Lahore. Samples were processed to determine aerobic bacterial, coliform and fungal counts. The highest mean bacterial count (9.64±2.43), coliform (9.57±2.79) and fungal counts (2.51±2.62) were observed in salads from road side venders. According to the International Commission on Microbiological Specifications for Foods (ICMSF) 73.33 percent of salad samples by bacterial, 98 from coliform and 26.67 by fungal counts were found unacceptable for human consumption. Among isolated bacteria were identified as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiells*, *Salmonella*, *Enterobacter*, *Aeromonas* and *Bacillus*. The predominant fungal species in salads were *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus flavus*. *A. fumigatus* is an obligate pathogen and Aflatoxin (B_1 and B_2) produced by *A. flavus* cause of mycotoxicosis. It was concluded that the salads from road side venders are source of bacteria and fungi having potential for toxins production.

Key words: Salads, microbial load, bacterial plate count, coliform count, fungal count.

(Received 07.09.2020 Accepted 20.11.2020)

INTRODUCTION

Fruits and vegetables provide important nutrients, micronutrients, vitamins and fibers. Both are part of balanced human diet (Abakari *et al.*, 2018). Salads are type of food prepared by mixing chopped vegetable and/or fruits in raw form (Obaji *et al.*, 2018). The other additional ingredients of salads are mayonnaise and cheese (Nawas *et al*., 2012). Salad provides protein, fats, carbohydrates, vitamins, minerals and antioxidants (Weldezgina and Muleta, 2016). Carrot, cabbage, cucumber and lettuce are vegetable ingredients of salad (Younus *et al.*, 2020).

The raw vegetables are carrier of pathogenic microorganisms. The source of microbial contamination of vegetables are sewage water used for irrigation during production (Iwu and Okoh, 2019), harvesting (if damaged), transportation, packaging, storage and processing, handler, soil and dust. Therefore, vegetables harbor a diverse range of human pathogens and the cut surface of vegetables provide ideal environment for microbial growth. Other than raw material the salads may be contaminated from environment, handling procedures and storage conditions (Obaji *et al.*, 2018).

The salads are enriched media for microorganisms, so higher number of microorganisms can be detected, which may spoil quality of salad (Osamwonyi *et al.*, 2013). Salads are considered as a source of transmission of food borne pathogens to human beings. In most of the cases the microorganisms in salads do not cause spoilage, so salad is considered safe and consumed by the people leading to food borne illnesses (Abaza, 2017). The possible contaminants of vegetable salads are *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, Shigella, *Salmonella*, *Campylobacter*, enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* (Lucero *et al.*, 2013). Among fungi yeast, *Aspergillus*, *Penicillium*, *Cladosporium* and *Rhizopus* are important (Obaji *et al.*, 2018). Some fungi produce toxic metabolites (mycotoxins) which are teratogenic, neurotoxic hemorrhagic, estrogenic, immunotoxic, hepatotoxic, nephrotoxic, dermotoxic and immunosuppressive (Zohri *et al.*, 2014).

Keeping in view, a study was conducted to evaluate bacteriological and mycological quality of mixed vegetable salads collected from different localities in Lahore, Pakistan.

MATERIALS AND METHODS

Sample Collection: Mixed vegetable salad samples (n=90) were collected from different localities in Lahore including road side vendor, fast food outlets and family restaurants (30/each). Samples were collected in sterile plastic bags separately. The bags were placed in cooler containing ice packs and transported to laboratory within shortest possible time not exceeding than three hours for microbiological analysis (Osamwonyi *et al.*, 2013).

Processing of Sample: For microbiological analysis, stock suspensions of samples were prepared by mixing 25g of each sample in 225 mL phosphate buffer saline, separately. It was homogenized using sterilized tissue homogenizer. Ten-fold serial dilutions were prepared by taking 1mL from homogenized mixture to a test tube contained 9mL sterilized phosphate buffer saline and transferring it to next tube containing 9mL of diluent and so on. Serial dilutions were prepared up to 10^{-15} (Mohammad *et al.*, 2012).

Aerobic Plate Count: One mL from each dilution was properly mixed with sterilized molten Nutrient agar at 45 ºC and poured into sterile Petri plates. It was allowed to solidify and plates were incubated at 37ºC for 24 hours. Plates with countable range of colonies (30-300 CFU/plate) were selected and counts were performed using colony counter (Bukar *et al.*, 2010).

Coliform Count: MacCkonkey's agar was prepared according to the recommendations of manufacturer in universal bottles. Following the sterilization and lowering the temperature of medium at 45ºC, one mL from each dilution was mixed and poured into sterilized Petri plates. After solidification, plates were incubated at 37ºC for 24 hours. Colonies were counted in plates having countable range (Bukar *et al.*, 2010).

Fungal Count: For fungal count Sabouraud Dextrose agar (SDA) plates were prepared and properly labeled for respective tenfold serial dilution. One mL from each dilution was spread over agar surface with the help of sterilized glass spreader. Following the incubation at 25ºC for 48-72 hours fungal colonies were counted (Thomas *et al.*, 2013).

Bacterial Identification: The bacterial isolates were randomly purified on Nutrient agar from aerobic plate count and on MacCkonkey's agar from coliform count and identified by colony morphology, microscopy (Gram's staining) and biochemical profile according to scheme provided in Bergeys Manual of systematic Bacteriology. Biochemical tests used for bacterial identification were catalase production, Coagulase production, Indole production, Methyle red, Voges-proskauer, Citerate utilization, Hydrogen sulphide gas production, Starch hydrolysis and sugar fermentation (manitol and lactose) tests (Harrigan, 1998).

Fungal Identification: The fungal isolates were purified on SDA and identified by colony morphology, pigment production and microscopic features including hyphae, asexual spores and special structures; vesicle and foot cell (Pitt and Hocking, 2009).

Statistical Analysis: Data obtained were analyzed by One Way Analysis of Variance (ANOVA) with five percent level of significance using Statistical Package for Social Sciences (SPSS)**.**

RESULTS

Salads samples for assessed for bacterial and fungal pathogens. Aerobic plate, coliform and fungal counts were performed along with isolation and identification of foodborne pathogen includes *S. aureus*, *E. coli*, *Salmonella and Bacillus.* The fungi isolated were from genus *Aspergillus.*

Bacterial Load: The aerobic plate counts indicated in the fall between range 1.1×10^5 to1.1×10^{15}. The lowest count was found in the salad sample obtained from fast food outlet (1.1×10^5) while highest count was observed in the salads collected from family restaurant (1.1×10^{15}). Statistically, the means of aerobic plate count of road side venders and family restaurants differ non-significantly. On the other hand, the mean of APC from fast food outlets differ significantly from both of mentioned earlier (Table 1and Fig 1).

Coliform Load: The coliform count of salad samples collected from different locations of Lahore city in Pakistan were found in the range of 1.0×10^5 -1.3×10^{14}. This shows that highest coliform count was (1.3×10^{14}) present in salad samples obtained from road side vender and family restaurant. While the lowest count (1.0×10^5) was observed in fast food outlet. While one sample obtained from road side vender did not show any count. Statistically, the means of coliform from road side and family restaurants are different significantly from means of fast food outlets.

Fungal Load: The highest yeast and mold count (5×10^6) found in fast food outlet, while the lowest count (1.0×10^3) was found in sample obtained from fast food outlet. While 62 samples did not show any mold count. The means of fast food outlets and family restaurants are insignificantly different, while both differ significantly from mean of road side vender as mentioned in Table 1. Fig. 1 shows comparison of bacterial, coliform and fungal counts of salads.

Salads Quality: International Commission on Microbiological Specifications for Foods (ICMSF) for bacterial and coliform counts and DOD FOOD SAFETY & QA ACTION LEVELS for fungal counts provided criterion for determining the quality of raw material for human consumption (Table 2). According to these 73.33 percent of salad samples by aerobic bacterial, 98 from coliform and 26.67 by fungal counts were found unacceptable for human consumption.

Bacterial and Fungal Contaminants: A total of 494 bacteria were isolated from salad samples (n=90). Out of these 177 bacteria were recovered from road side venders, 152 from fast food outlets and 165 from family restaurants. The bacteria identified based on colony morphology, Gram's staining and biochemical profile

was *S. aureus* and *E. coli*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Aeromonas* and *Bacillus.* A total of 50 mold species were isolated from road side venders (23), fast food outlets (12) and family restaurants (15). Fungi isolated belonged to genus *Aspergillus* were *A. fumigatus*, *A. flavus* and *A. niger* (Table 3). Representative isolates are shown in Fig. 2.

Table-1: Microbiological Counts in mixed vegetable salads.

Category of Salads	Microbiological Counts (Mean Log CFU ± SD)		
	Aerobic Plate	Coliform	Fungal
Road Side Vendors	9.64 ± 2.43^{b}	9.57 ± 2.79^{b}	2.51 ± 2.62^{b}
Fast Food Restaurants	6.62 ± 1.35^{a}	6.81 ± 0.99^{a}	1.1 ± 2.09^{a}
Family Restaurants	8.68 ± 2.46^{b}	8.55 ± 2.11^{b}	1.07 ± 2.23^{a}

Values designated with same superscripts are non-significantly different ($p \geq 0.05$)

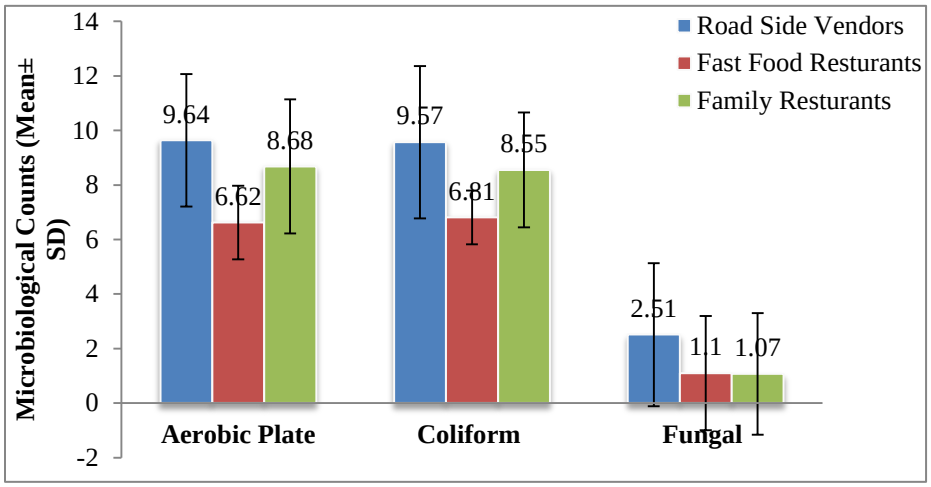

Figure-1: Microbiological counts in mixed vegetables salads

Table-2: Quality of salads based upon bacterial, coliform and fungal counts.

Parameters	CFU/g	Status	Road side venders n=30	Fast food outlets n=30	Family restaurants N=30	Total n=90
Aerobic plate count	$<10^5$	Acceptable	0	0	0	0
	$\geq10^5$-$\leq10^6$	Marginally acceptable	3	16	4	23
	$>10^6$	Unacceptable	27	14	26	67
Coliform count	$<10^2$	Acceptable	1	0	0	1
	$\geq10^2$-$\leq10^3$	Marginally acceptable	0	0	0	0
	$>10^3$	Unacceptable	29	30	30	89
Yeast/mold count	$\leq10^3$	Acceptable	17	24	25	66
	$>10^3$	Unacceptable	13	6	5	24

Table-3: Distribution of pathogens in mixed vegetable salads.

Pathogen	Isolate	Road side venders (%)	Fast food outlets (%)	Family restaurants (%)
Bacteria	*S.aureus*	16.9	19.1	18.2
	Bacillus	5.7	5.9	5.5
	Aeromonas	12.9	7.8	9.1
	E.coli	16.4	19.1	18.2
	Salmonella	15.3	7.2	12.7
	Klebsiells	16.4	19.1	18.2
	Enterobacter	16.4	19.1	18.2

Fungi	*A.fumigatus*	26.1	75	40
	A.flavus	21.7	25	26.7
	A.niger	52.1	0	33.3

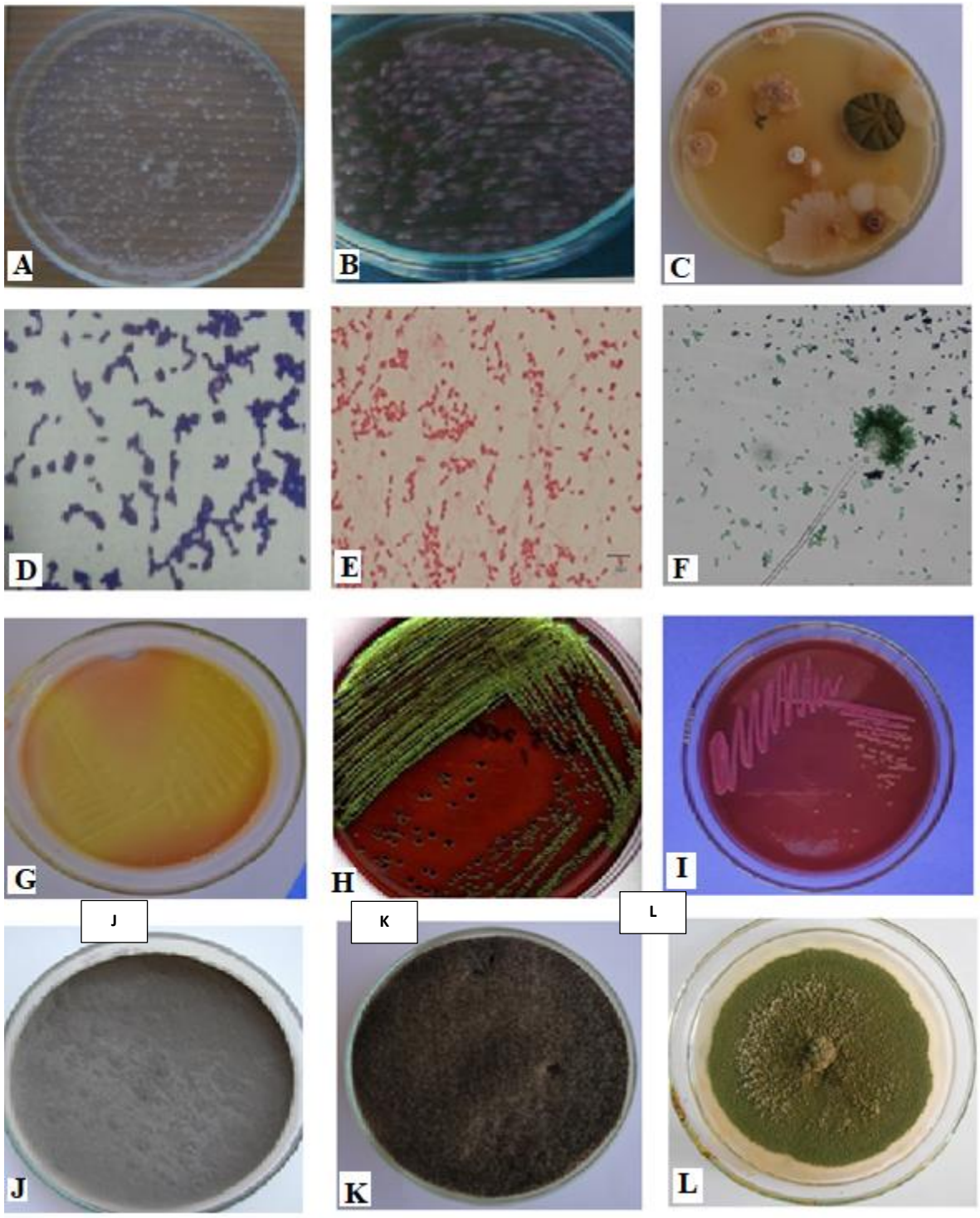

Figure -2: Bacteriological and Mycological analysis of mixed vegetable salads
A: Bacterial count, **B:** Coliform count, **C:** Fungal count, **D:** Gram positive cocci, **E:** Gram negative coccobacilli, **F:** Microscopic view of *Aspergillus*, **G:** *S. aureus* on manitol salt agar, **H:** *E. coli* on EMB agar, **I:** *Klebsiells* on MacCkonkey's agar, **J:** *A. fumigatus*, **K:** *A. niger*, **L:** *A. flavus.*

DISCUSSION

Salads are made from nutritionally enriched raw material providing a good growth medium for microorganism. Bacterial and fungal pathogens contaminate salads by several ways leading to food borne illnesses to end consumer. The unhygienic preparation may increase number of microorganism. So, microbiological evaluation of salads is important to ensure public health.

Rajvansh in Jaipur found 0.320 to 1.38×10^4 CFU/g of bacterial count in salad samples collected from Jaipur city (Rajvanshi, 2010). In a study carried out in Iran, it was observed the aerobic plate count in salads was in range of 10^4-10^8 (Mohammad *et al.,* 2012). Nawas *et al.* in Chittagong revealed that the viable count was in range from 1.60×10^4 to 4.38×10^5 CFU/g in salad samples (Nawas *et al.,* 2012). In another study conducted by Osamwonyi on Bacteriological Quality of Vegetable Salads Sold at Restaurants within Okada Town, Edo State, Nigeria, resulted in the count 10^4 (Osamwonyi *et al.,* 2013). The above mentioned data are contradictory to present findings of bacterial counts (10^5- 10^{15} CFU/g). According to ICMSF (International Commission on Microbiological Specifications for Foods), no sample was in acceptable limit and 26.66 percent salad samples were found marginally acceptable. On the other hand, 73.33 percent samples were unacceptable. The highest no. of aerobic plate count can be attributed to the unhygienic conditions that were adopted during preparation (Eni *et al.,* 2010), handling, and storage of salads samples. It can be linked with the health status of workers over there. This may be due to the exposure with soil and dust.

Coliform also harbor salads. Mohammad in Iran, coliform was detected in salad samples in range from 10^3 to 10^8 CFU/g (Mohammad *et al.,* 2012). In a study conducted by Rehman and Noor, in Bangladesh, coliform was detected in salad sample (Rahman and Noor, 2012). The range of CFU/g was from 10^4 to 10^6 CFU/g. These studies have higher level of coliform count. According to the standards of ICMSF, 98.8 percent samples were unacceptable and only 1.11 percent samples were fallen in the category of acceptable limit. Presence of coliform indicates the fecal contamination (Abdullahi and Abdulkareem, 2010). The sources of fecal contamination may be water used for washing of vegetables and equipment. If the water is contaminated with sewage water it will definitely transfer coliform to human through uncooked food. Presence of higher no. of coliform can cause diarrhea, abdominal pain, abdominal cramps and nausea.

Mohammad recovered 3.85 to 6.7 log/g yeast and molds from salad samples (Mohammad *et al.,* 2012). The presence of yeast and mold is of less important. However it is reported that presence of some fungi may cause allergic reactions if they survive, grow and produce a large no. of conidia in salads consumed by the people. According to the standards established by DOD FOOD SAFETY & QA ACTION LEVELS, 73.33 percent salad samples were acceptable while remaining 24 were unacceptable. The results of all above studies are in accordance to our study. In another study 86 salad samples out of 224 were declared unacceptable (Berrada *et al.,* 2016). The above mentioned studied indicates the presence of large no. of microorganisms in salads. *S. aureus* and *E. coli* contaminants of vegetable salads in Egypt (El-Hadedy and El-Nour, 2012) are in accordance to present study. Isolation of *E. coli*, *Enterococcus* (Campos *et al.,* 2013), *Salmonella*, *E. coli* and *L. monocytogenes* in Turkey (Gurler *et al.,* 2015), *S. aureus*, *Enterobacter*, *Enterococcus* and *Klebsiella* (Hannan *et al.,* 2014), *E. coli*, *L. monocytogenes* and *Salmonella* from salads in Brazil (Oliveira *et al.,* 2011) may be correlated with present study results. Abakari *et al.* (2018) isolated *Bacillus*, *E.coli*, *Salmonella* and *Shigells spp*. from salad samples in Ghana. *Aspergillus* is frequently isolated genus from vegetable salad in agreement to present study (Iu *et al.,* 2015). The presence of pathogens in salads can be linked with poor hygienic practices during salad preparation.

Conclusion: It was concluded that salad samples sold in various places of Lahore are highly contaminated with microorganisms. This is because; safety guidelines are not being fulfilled. Government should take action in order to force the authorities over there in order to follow hygienic practices during all the process of salad preparation. This could help to lower the contamination level in salads.

REFERENCES

Abakari, G., S. J. Cobbina and E. Yeleliere (2018). Microbial quality of ready-to-eat vegetable salads vended in the central business district of Tamale, Ghana. Int. J. Food. Contam. 5(1): 3.

Abaza, A. (2017). Bacteriological assessment of some vegetables and ready-to-eat salads in Alexandria, Egypt. J. Egypt. Public. Health. Assoc. 92(3): 177-187.

Abdullahi, I.O. and S. Abdulkareem (2010). Bacteriological quality of some ready to eat vegetables as retailed and consumed in sabon-gari, Zaria, Nigeria, Bayero. J. Pure. Appl. Sci. 3: 173-175.

Avazpour, M., M.R. Nejad, F. Seifipour and J. Abdi (2013). Assessment of the microbiological safety of salad vegetables from different restaurants in Ilam. J. Paramed. Sci. 4: 111-115.

Berrada, L., A. El Ouali Lalami, B. Salame, M. Aabouch and L. Bennani (2016). Assessment of the Microbiological Quality of Salads in the North

Central Region of Morocco. Int. J. Innov. Sci. Res. 25: 510-516.

Bukar, A., A. Uba and T. I. Oyeyi (2010) . Occurrence of some enteropathogenic bacteria in some minimally and fully processed ready - to - eat foods in kano metropolis, Nigeria. Afri. J. Food. Sci. 4: 032-036.

Campos, J., J. Mourao, N. Pestana, , L. Peixe, C. Novais and P. Antunes (2013). Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. Int. J. Food. Microbiol. 166: 464-470.

De Oliveira, M. A., V. M. De Souza, A. M. M. Bergamini and E. C. P. De Martinis (2011). Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. Food. Control. 22: 1400-1403.

El-Hadedy, D. and S. A. El-Nour (2012). Identification of Staphylococcus aureus and Escherichia coli isolated from Egyptian food by conventional and molecular methods. J. Genet. Eng. Biotechnol. 10: 129-135.

Eni, A.O., I. A. Oluwawemitan and O. U. Solomon (2010). Microbial quality of fruits and vegetables sold in sango ota, Nigeria. Afri. Food. Sci. 4: xxxx-xxxx,

Gurler, Z., S. Pamuk, Y. Yildirim and N. Ertas (2015). The microbiological quality of ready-to-eat salads in Turkey: A focus on Salmonella spp. and Listeria monocytogenes. Int. J. Food. Microbiol. 196: 79-83.

Hannan, A., R. Rehman, S. Saleem, M.U. Khan, M.U. Qamar and H. Azhar (2014). Microbiological analysis of ready-to-eat salads available at different outlets in Lahore, Pakistan. Int. J. Food. Res. 21.

Harrigan, W.F. (1998). Laboratory Methods in Food Microbiology, 3rd Ed. Academic Press; San Diego. 198p.

Iu, N., E. Vc, O. Ce and F. Ju (2015). Evaluation of the degree of contamination of salad vegetables sold in Umuahia main market. Am. J. Microbiol. Res. 3: 41-44.

Iwu, C.D. and A.I. Okoh (2019). Preharvest Transmission Routes of Fresh Produce Associated Bacterial Pathogens with Outbreak Potentials: A Review. Int. J. Environ. Res. Public. Health. 16(22): 4407.

Lucero, E.C.S., L.E. Alcaráz, S.E. Satorres, E. Manfredi and L.D.C. Velázquez (2013). Presence of enterotoxigenic Staphylococcus aureus in artisan fruit salads in the city of San Luis, Argentina. Braz. J. Microbiol. 1155-1161.

Mohammad, B., H. Najafi and M. Bahreini (2012). Microbiological quality of mixed fresh-cut vegetable salads and mixed ready- to-eat fresh herbs in Mashhad, Iran. I.C.N.F.S. 39: 62-66.

Nawas, T., R. M. Mazumdar, S. Das, M. N. Nipa, S. Islam, H. R. Bhuiyan and I. Ahmad (2012). Microbiological quality and antibiogram of *E. Coli*, *Salmonella* and *Vibrio* of salad and water from restaurants of chittagong. J. Environ. Sci. Nat. Resour. 5: 159-166.

Obaji, M., A. N. Oli, I. Enweani, I. Udigwe, J. N. Okoyeh and I. M. Ekejindu (2018). Public Health challenges associated with street-vended foods and medicines in a developing country: A mini-review. J. Med. Res. 4(6): 283-287.

Osamwonyi, O.U., O. N. Obayagbona, W. Aborishade, F. Olisaka, E. Uwadiae and O.N. Igiehon (2013). Bacteriological quality of vegetable salads sold at restaurants within okada town, Edo state, Nigeria.Afri. J. Basic. Appl. Sci. 5: 37-41.

Pitt, J. and A. Hocking (2009). Fungi and Food Spoilage, 3rd Ed. Springer ; Berlin (Germany).

Rahman, F. and N. Rashed (2012). Prevalence of pathogenic bacteria in common salad vegetables of Dhaka metropolis. Bangladesh. J. Bot. 41: 159-162.

Rajvanshi, A. (2010) .Bacterial load on street vended salads in Jaipur city, India. Inter. J. Food. Saf. 12: 136-139.

Thomas, B.T., R. Kolawole, O. M. Adebayo and S.O. Makanjuola (2013). Relationship between total viable bacterial counts, fungal counts and the intrinsic parameters of garri. Nat. Sci. 11: 47-50.

Weldezgina, D. and D. Muleta (2016). Bacteriological contaminants of some fresh vegetables irrigated with Awetu River in Jimma Town, Southwestern Ethiopia. Adv. Biol. 2016.

Younus, M.I., A.A.M. Sabuj, Z.F. Haque, S.M. Sayem, S. Majumder, M.S. Parvin and S. Saha (2020). Microbial risk assessment of ready-to-eat mixed vegetable salads from different restaurants of Bangladesh Agricultural University campus. J. Adv. Vet. Anim. Res. 7(1): 34.

Zohri, A.A., S.M. Saber, M.S. Youssef and M.M. Abdel-Kareem (2014). Isolation and identification of toxigenic fungi from foodstuffs at Sohag Governorate. Egypt. Environ. Stud. 13: 1-12.