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Incidence, Enumeration and Confirmation of *Listeria* and its Species in Ready-to-eat Street Vended Salads Sold at Various Outlets of Faisalabad City, Pakistan

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Abstract

The desire for a healthy lifestyle and faster mode of preparation has supported the consumption of ready to eat fresh salad. Street vended salads are recognized as a source of pathogenic transamination in different parts of the world. The present study was designed to evaluate the safety status of fresh vegetable and Russian salads being sold at various food outlets of Faisalabad. Samples of freshly prepared salads were collected from representative selected different areas of Faisalabad city divided into four different zones (zone 1, zone 2, zone 3 and zone 4). Prevalence and enumeration of *Listeria* was done through microbial testing via the spread plate method. Among samples of vegetable salad, the highest prevalence of *Listeria* was found in the zone 2 (75%) whereas Russian salad samples from zones 1 and 3 exhibited 62% prevalence, the highest among all 4 zones of study. On the whole, the lowest prevalence of *Listeria* was found in zone 4 (50% vegetable salad and 58% Russian salad). Biochemical conformation of Listeria done through different tests for the identification of various *Listeria* species, exhibited that *Listeria monocytogenes* and *Listeria innocua* were highly prevalent in samples from zones 1 and 3 respectively. The results will help to improve safety concerns associated with street vended foods

Keywords: *Listeria*, ready-to-eat, vended salads

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INTRODUCTION

Pathogenic microorganisms significantly exist in street vended foods and cause foodborne illnesses in all parts of world. Food borne illnesses not only effects consumer health also results in negative economic impacts, loss of customers, product recall and potential compensation costs[1]. Contaminated foodstuff is the main cause oftwenty to thirty per cent spreading of *Listeria* infection that causes ninety-five percent cases of food poisoning[2]. Every year, almost one third population is distressed with foodborne infections in industrialized countries whereas the problem is worse in developing countries due to poverty, overcrowding, inadequate sanitary conditions and poor general hygiene[3].

Listeria is a Gram-positive, bacillus, non-spore forming, ubiquitous, motile, facultative anaerobe and one of the most environmental pathogenic microflora of food[4]. *Listeria* can easily deteriorate the raw ready-to-eat foods because of its high salt tolerance and ability to thrive at low preservation temperatures. *Listeria* increases the rate of death up to 25-30% just because of hygienically poor environment and inadequate management of food processing[5]. Due to consumption of contaminated and unhygienic foods nearly 76 million infections, 325,000 hospitalizations and 5000 deaths are caused annually in United States of America[24].

Rapid change in the socioeconomic status and changing life style have greatly increased the consumption of street vended foods[6]. Control of safety of ready to eat street vended foods is difficult and much important because they have significant socioeconomic role in meeting food and nutritional requirements of consumers at affordable prices particularly to the lower and middle income people. As these food products are prepared by small street vendors, cheaper rates of these foods attract the low earners. Ready to eat street vended foods constitutes the primary source of meals for many low and middle income people[7]. Salad, a mixture of vegetables and fruits, is an excellent source of dietary fiber, phytochemicals, antioxidants, minerals, vitamins and phenolic that help to cure many diseases and reduce the risk of developing type 2 diabetes[10].

Salads are economical, convenient in preparation and have delicious taste but the hygiene and safety levels are big challenges for the food administrators. Freshly consumed produce got contaminated on farm or point of sale in market. Reported sources of contamination include soil, handling during pre and post-harvest handling, water and dust[11]. Manures used to promote the growth and cultivation of these produce harbor the large number of pathogenic microorganisms such as *Salmonella, Mycobacterium, Listeria monocytogenes, Klebsiella and Clostridium perfringens*[9].Globally, about 325,000 people are hospitalized along with death of 5,000 children each year due to food borne illnesses[14]. Therefore, despite the freshness and nutritional benefits of fruits and vegetables, there have been an increase in incidence of food borne illnesses thus bacteriologically safe fruit and vegetable products are required to maximize the health benefits promised by their consumption[8]. Ingredients of salads are contaminated with soil, dust, irrigation water which are proved to be associated with pathogenicity of *Listeria*. Findings have reported the highest prevalence of *Listeria monocytogenes* in ready to eat salad samples followed by chicken products, beverages, egg based products, beef and seafood products[13]. In salads, *Listeria* has been reported to be isolated from fresh produce such as parsley, cucumber, cabbage, sprouts and water cress and resulted in morbidity and mortality in various parts of world[12]. The average shelf life of ready to eats ranges from 5-7 days and after opening of package product can be stored for two days at refrigeration conditions. Highly perishable, favorable pH (6.0-7.0), poor abidance to decontamination procedures and temperature abuse can increase the risk associated with these products[27]. In Pakistan street vendors to large restaurants are responsible for sale of ready to eat salads. Most common salads on retail sale include vegetable salad and Russian salad. Common vegetables used in vegetable salads include onion, cucumber, tomatoes, lettuce green pepper, radish, salt and spices commonly stored at ambient temperature during retail conditions.. While ingredients in Russian salads include fresh or boiled vegetables, mayonnaise, cream and

spices stored at refrigeration conditions. This study provides the prevalence data of *Listeria monocytogenes* for Vegetable salad and Russian salad. The objectives of this study were to assess microbiological quality of freshly prepared street vended salads and to get information regarding the major sources of contamination.

MATERIALS AND METHODS

Sample of Vegetable and Russian salads were collected from four different zones of the Faisalabad city (Table 1). A total of 192 samples were picked during five weeks. Samples were kept in strict hygienic conditions in sterile zipper polythene bags, stored in an icebox under controlled temperature and brought to the Food Microbiology and Microbiology Laboratory, National Institute of Food Science and Technology, University of Agriculture, Faisalabad within an hour of its collection. *Listeria* selective agar was used for microbial analysis. Samples were homogenized in a peristaltic blender (Seward Europe) at 200 rpm for 2 minutes. For this purpose, 25g salad along with 25 mL of peptone water was taken in specialized stomacher bags[15]. Six serial dilutions were made by transferring 1 mL liquid sample from stomacher bag to the test tubes serial wise and transferred to the prepared Petri plates containing solidified *Listeria* Selective Agar Base (Oxoid). Spreading of the poured volume (0.1mL) was done using a sterile spreader and plates were allowed to dry for 15 minutes (Fig. 1)[16]. After drying, incubation was done for 24 hours at 37°C. After incubation, dark brown or blackish colored colonies grew over *Listeria* selective agar as depicted in Fig. 2. Colonies of *Listeria* were counted using the colony counter (galaxy 230-USA) and documented. Colony forming units per gram (CFU/g) were calculated using the following formula.

Table 1. Sampling plan for vegetable and Russian salads collection

Zone 1 locations for collection of samples			Zone 2 locations for collection of samples		
No.	Vegetable salad	Russian Salad	No.	Vegetable salad	Russian Salad
1	Yong Wala	Ghulam Muhammadabad road	1	Canal road	Sargodha road
2	Kotwali road	Tata bazar	2	Abdullahpur overpass	Millat road
3	Narwala road	Sadar bazar	3	Tahir road	Fsd.-Sanghla hill road
4	Jail road	Tariq road	4	Khaleeq road	Kashmir road
5	Ganda nala road	Gulberg road	5	Noorpur road	Iqbal stadium
6	Bakar mandi road	Makki road	6	Chibban road	Lhr.-Fsd. Shk. road
7	Chiniot bazar	Kachari bazar	7	Shadman road	College road
8	Oil depot road	Imam bargha road	8	Ittehad road	Wapda colony road
Zone 3 locations for collection of samples			Zone 4 locations for collection of samples		
No.	Vegetable salad	Russian Salad	No.	Vegetable salad	Russian Salad
1	Rasool park	Susan road	1	Liaqat abad	Samundari road
2	Tufail shaheed road	Tezab mill road	2	Mulyanwala road	D-type colony road
3	Younas road	Jaranwala road	3	Naimatabad road	Dijkot road
4	Satyana road	D-ground	4	Sanni road	Samanabad road
5	Maqbool road	Lower canal road	5	Main road	Railway road
6	Usman road	Khawaja islam road	6	Saifabad road	Jhang road
7	Allama Iqbal road	Illahi abad road	7	Talianwala road	AARI colony road
8	Risalewala road	Warispura road	8	Bilal road	Fazal elahi road

Samples were collected in triplicate from each location.

CFU/g = (Average No.of Colonies x Dilution Number) / (Dilution Factor x Volume plated)

(Formula A)

DF = (Volume of sample) / (Sample Volume + Diluent Volume)

(Formula B)

Biochemical Testing

Two species of *Listeria* (*Listeria monocytogenes* and *Listeria innocua*) were identified and biochemically confirmed by through Gram staining, catalase, citrate, hydrogen sulfide, indole, methyl red and oxidase tests as described by the Laboratory Manual of Food Microbiology[16]. Statistical analysis was performed for to determine the significance of test results[17] by using the completely randomized design with the help of Statistix 8.1 software.

RESULTS AND DISCUSSION

Vegetable Salad

Prevalence of *Listeria* in street vended vegetable salad samples gathered from zone 1, zone 2, zone 3 and zone 4 are shown in Table 2. Total of 24 samples of vegetable salad were collected separately from all four zones and the occurrence of *Listeria* was documented in percentage. Among 24 samples of vegetable salad 15 samples of vegetable salad illustrated positive results for the pathogen and the total prevalence of *Listeria* was recorded 62%. The highest count of $1.59x10^8$ CFU/g was recorded from Ganda Nala Road and the lowest count of $4.47x10^6$ CFU/g was recorded from oil depot road (Table 3). Highest prevalence was found in the area of Yong wala and Narwala road whereas *Listeria* was not detected from the samples of Chiniot bazar. The following results are in accordance with the findings of

Table 2. Prevalence of *Listeria* in salad samples from different zones

Locations	Total prevalence in vegetable salad	Total prevalence in Russian salad
Zone 1	15/24 (62%)	15/24 (62%)
Zone 2	18/24 (75%)	11/24 (45%)
Zone 3	13/24 (55%)	15/24 (62%)
Zone 4	12/24 (50%)	14/24 (58%)

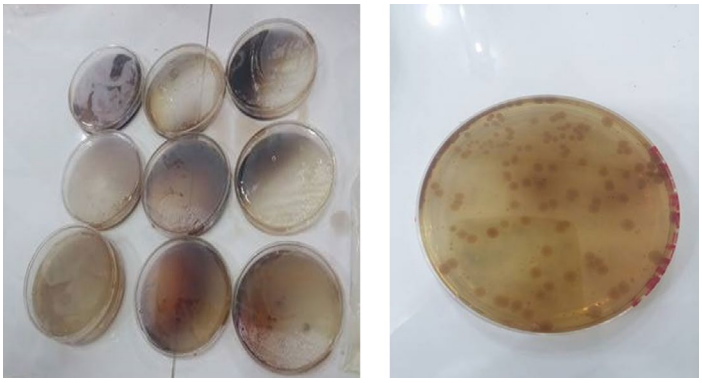

Fig. 1. Plates poured with *isteria selective agar*

Fig. 2. Brown colored colonies of *Listeria* grown on Plates.

Table 3. Prevalence and enumeration of *Listeria* in vegetable salad

Zones	Location	Satisfactory Samples % ($<10^1$ CFU/g)	Un satisfactory Samples % ($>10^2$ CFU/g)	Colony forming unit (CFU/g)
1	Yong Wala	-	100	$1.30x10^7$
	Kotwali road	33	66	$6.19x10^6$
	Narwala road	-	100	$8.42x10^6$
	Jail road	33	66	$1.12x10^8$
	Ganda nala road	33	66	$1.59x10^8$
	Bakar mandi road	33	66	$9.4x10^5$
	Chiniot bazar	100	-	ND*
	Oil depot road	66	33	$4.47x10^6$
2	Canal road	66	33	ND*
	Abdullah pur overpass	33	66	$5.61x10^6$
	Tahir road	-	100	$1.80x10^7$
	Khaleeq road	33	66	$7.06x10^7$
	Noorpur road	-	100	$3.86x10^6$
	Chibban road	33	66	$8.73x10^7$
	Shadman road	-	100	$1.40x10^8$
	Ittehad road	33	66	$6.20x10^6$
3	Rasool park	33	66	$4.60x10^6$
	Tufail shaheed road	66	33	$2.80x10^7$
	Younas road	33	66	$6.39x10^6$
	Satyana road	66	33	$4.86x10^5$
	Maqbool road	-	100	$2.47x10^7$
	Usman road	33	66	$1.06x10^8$
	Allama iqbal road	33	66	$3.56x10^6$
	Risalewala road	33	-	ND*
4	Liaqat abad	66	33	$6.73x10^6$
	Mulyanwala road	33	66	$8.46x10^5$
	Naimatabad road	33	66	$3.73x10^5$
	Sanni road	66	33	$4.93x10^5$
	Main road	100	-	ND*
	Saifabad road	-	100	$2.99x10^7$
	Talianwala road	33	66	$6.28x10^6$
	Bilal road	66	33	$1x10^7$

Christson et al[18] who reported the presence of *Listeria monocytogenes* in street vended salad which may cause health hazards.

In zone 2, prevalence of the pathogen was recorded as 75% in vegetable salad. Total 18 samples were positive for *Listeria* as shown in Table 3. Tahir road, Noorpur road and Shadman road have shown highest prevalence while no pathogen was detected in samples taken from Canal road with the non-significant difference (P> 0.05). Enumeration results indicated highest count of $1.40x10^8$ in samples from Shadman road while the lowest count of $3.86x10^6$ CFU/g was recorded in samples collected from Noorpur road. The following results are in agreement with the study reported by El-Shenawy et al[19] according to which *Listeria* is present everywhere in our environment and its various species like *Listeria monocytogenes* is responsible for many outbursts.

In zone 3, overall 55 % prevalence was observed in vegetable salads. None of the sample was found acceptable for the consumption. Highest count of $1.06x10^8$ CFU/g was found in the area of Usman road whereas lowest count was shown in the area of Satyana road ($4.86x10^5$ CFU/g) with no detection in the area of Risalewala road as presented in the Table 3. The following results are in accordance with the study of Lianou, & Sofos[20] who stated that inefficient methods of handling of street vended salads can support the growth of pathogenic micro

Table 4. Prevalence and enumeration of *Listeria* in Russian salad

Zones	Location	Satisfactory Samples % (<101 CFU/g)	Un satisfactory Samples % (>102 CFU/g)	Colony forming unit (CFU/g)
1	G.M Abad road	66	33	$3.86x10^5$
	Tata bazar	100	-	ND*
	Sadar bazar	33	66	$1.26x10^7$
	Tariq road	33	66	$1.16x10^8$
	Gulberg road	-	100	$2.62x10^6$
	Makki road	33	66	$1.30x10^8$
	Kachari bazar	33	66	$3.73x10^6$
	Imam bargha road	66	33	$4.13x10^6$
2	Sargodha road	100	-	ND*
	Millat road	-	100	$1.84x10^7$
	Fsd.-Sanghla hill road	33	66	$7.66x10^5$
	Kashmir road	66	33	$7.40x10^6$
	Iqbal stadium	100	-	ND*
	Lhr Fsd Shk road	66	33	$5.13x10^7$
	College road	-	100	$2.99x10^8$
	Wapda colony road	66	33	$4.33x10^6$
3	Susan road	100	-	ND*
	Tezab mill road	-	100	$2.63x10^6$
	Jaranwala road	33	66	$8.87x10^6$
	D-ground	33	66	$2.33x10^8$
	Lower canal road	33	66	$4.44x10^7$
	Khawaja islam road	33	66	$2x10^6$
	Illahi abad road	33	66	$5.75x10^7$
	Warispura road	33	66	$9.40x10^7$
4	Samundari road	-	100	$1.67x10^7$
	D-type colony road	100	-	ND*
	Dijkot road	33	66	$7.14x10^6$
	Samanabad road	33	66	$7.01x10^6$
	Railway road	33	66	$1.21x10^7$
	Jhang road	33	66	$4.33x10^5$
	AARI colony road	66	33	$3.33x10^5$
	Fazal elahi road	33	66	$1.06x10^8$

flora in street vended foods. Most of the vendors openly store leftover food in an open place for the next day use[21]. Some of them also use stagnant water for washing which in result can cause serious health hazards.

In zone 4, vegetable salads have shown the total prevalence of 50%. Highest count was found in the area of Saifabad road that is $2.99x10^7$ CFU/g whereas lowest count was observed in the samples taken from Naimatabad road ($3.73x10^5$ CFU/g). No pathogen was detected in the Main road samples. The results are in agreement with the work of Gelfand and colleague[22] who describes the sources for the high prevalence of *Listeria* which may include the dusty unygenic premises and poor sanitary conditions.

Russian Salad

Result of the study (Table 2) presented the significant difference in samples of Russian salad in zone 1 and zone 3 with 62% unsatisfactory results. None of the samples was found to be in the acceptable limits. In zone 1, highest count of $1.30x10^8$ CFU/g from Makki roadand the least count of $3.86x10^5$ CFU/g were observed in the area of Ghulam Muhammadabad road whereas no *Listeria* was detected in the samples taken from

Table 5. Biochemical testing of *Listeria* spp.

Zones	Types of Salad	*Listeria monocytogenes*	*Listeria innocua*
Zone 1	Vegetable Salad	+(15/24)	+ (3/24)
	Russian Salad	+(11/24)	+(2/24)
Zone 2	Vegetable Salad	+(14/24)	+(4/24)
	Russian Salad	+(10/24)	+(2/24)
Zone 3	Vegetable Salad	+(9/24)	+(5/24)
	Russian Salad	+(12/24)	+(1/24)
Zone 4	Vegetable Salad	+(10/24)	+(4/24)
	Russian Salad	+(11/24)	+(2/24)

Tata bazar with the overall count ranging from 105-10^8 CFU/g. These results are closely related to the previous work done[23] which reports that fresh and mixed salads sold by road merchants harbor numerous pathogenic micro flora.

In zone 2, the percentage of total prevalence in Russian salad samples was 45% as documented in the Table 2. Enumeration of *Listeria* in Russian salad samples collected from zone 2 shown the significant results and the highest count was found in the area of College road with 2.99x10^8 CFU/g while the lowest count of *Listeria* was recorded in samples taken from Faisalabad-Sanghla hill road (Fsd.-Sanghla hill road) as 7.66x10^5 CFU/g. No *Listeria* was detected in the samples gathered from the areas of Sargodha road and Iqbal Stadium as described in Table 4.These results are in accordance with the results of research in which shows that by adopting good hygiene practices like use of hygienic tops, aprons, utensils and good sanitary conditions can reduce the risk of *Listeria* by half.

Similar to zone 1, percentage of total prevalence of *Listeria* in Russian salad samples was 62 % with the highest count in the area of D-groundas 2.33x10^8 CFU/g while the least count was recorded in the samples taken from Khawaja Islam road (2x10^6 CFU/g) and no *Listeria* was detected in the area of Susan road (Table 4).These results are in accordance with the work reported by Razzaq and colleagues[21] that declared that most of the vendors openly store their leftover food in an open place for the next day use which is the significant reason of prevalence of *Listeria*. Results for the Russian salad samples collected from zone 4 depicted 58% total prevalence of *Listeria* with the highest count in the area of Fazal Elahi roadwith 1.06x10^8 CFU/g count while the least count was recorded in the samples taken from AARI colony road (3.3x10^5 CFU/g). No *Listeria* was detected in the area of D-type colony road (Table 4). The results are in agreement with the work of Gelfand and coworkers[23] that describes the sources for the high prevalence of *Listeria*. Similarly in another study conducted in Malaysia commercially available minimally processed vegetable salads were analyzed microbiologically and *Listeria monocytogens* and *Listeria* spp. were detected in 22.5% and 33.3% samples respectively[26] .

Biochemical Testing

Biochemical conformation for the identification of various *Listeria* species (resulted shown in Table 5) indicated that the percentage of *Listeria monocytogenes* in vegetable salad and Russian salad was 62% and 45% respectively in the samples collected from zone 1, while the occurrence of *Listeria innocua* in the samples of vegetable salad and Russian salad was 16% and 8%. In the zone 2, the prevalence of *Listeria monocytogenes* in vegetable and Russian salad samples was 58% and 41% while that of *Listeria innocua* was 16% and 8% in vegetable and Russian salad samples respectively. In zone 3, the total percentage of *Listeria monocytogenes* in vegetable and Russian salad was 37% and 45%, whereas the percentage of *Listeria innocua* was 16% and 12% respectively. Similarly, in the zone 4, the occurrence of *Listeria monocytogenes* 41% and 45% while in case of *Listeria innocua* the prevalence was recorded as 16% and 8% in vegetable and Russian salad samples respectively. A study conducted in Brazil on 162 minimally processed vegetable salad samples sold locally also showed similar results containing *Listeria monocytogenes, Salmonella* and *Escherichia coli* at levels above (World Health Organization) WHO recommended limits[25]. In present study high *Listeria* count indicates increased consumer's exposure to microorganisms as it is assumed that the pathogen level were not reduced from post salad production to the utilization point because salads were served as ready to eat form and were stored at room temperature for sale. Possibility is that rework salad from last day was also mixed in new lot to utilize it risking the overall quality of product. Therefore holding temperature

and Thermal processing have strong impact on microbiological safety. The quality and safety of vegetables used for ready to eat salad preparation also depend upon use of clean irrigation water and appropriate washing to remove residues of soil and organic fertilizers. Thus for the prevention of increased bacterial load application of good hygiene practices, effective control on storage condition, decontamination and selection of appropriate packaging material is necessary.

CONCLUSION

The study revealed that the microbiological quality of salads sold at various markets of Faisalabad were unwholesome for human consumption. On overall basis, *Listeria monocytogenes* was the most prevalent specie of *Listeria* that was found in most of the samples of salads collected from all zones. On the other hand, the prevalence of *Listeria innocua* was very low as compared to *Listeria monocytogenes.*

The contamination could be attributed to the productuion source of vegetables, use of contaminated water for washing, improper handling, use of dirty processing utensils, knives and trays, poor personal hygiene and improper storage conditions generally along environmental conditions and cross contamination from rotten vegetables in value chain Simple washing of vegetables before use can possibly reduce the risk of microbiological hazards. The need is to provide basic food hygiene training to food handlers and to create consumer awareness to consume properly processed food products. Further it is recommended that research should be carried out on salads to isolate bacterial pathogenic strains identified in present study and antimicobial susceptibility of identified bacteria.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All data set generated or analyzed during the study are included in the manuscript.

ETHICS STATEMENT

This research does not contain any studies with human participants or animals performed by any of the authors.

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