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Occurrence and Virulence Characterization of
Aeromonas hydrophila in Salad Vegetables from Punjab, India

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A B S T R A C T

The consumption of fresh and minimally processed vegetables is considered healthy, outbreaks related to the contamination of these products are frequently reported. Present study aimed to evaluate the microbiological quality and the occurrence of *A. hydrophila* in external, internal and macerated part of salad vegetables (cucumber, radish, carrot, tomato, cabbage, long melon and spinach) from the fields of Punjab Agricultural University and local markets of Ludhiana. Epidemiological surveillance conducted showed the occurrence of *A. hydrophila* in 82.5% of total tested samples, cucumber (80%), radish (83%), cabbage (100%), carrot (74%), long melon (85%), spinach (100%) and tomato (68.5%). Total plate count ranged from 4.82 to 6.25 log cfu/g. Aerobic plate count of *A. hydrophila* procured from field and local market ranged between 2.79-3.93 log cfu/g, *A. hydrophila* count from internal, external and macerated part was 2.54, 3.06 and 3.73 log cfu/g. Isolate were molecularly confirmed as *A. hydrophila* by 16s -rDNA specific primer. Virulence was confirmed by gene specific primers, act (232 bp) and ahh (130 bp). Results of the study showed that salad vegetables possess a potential risk for the consumers.

Introduction

Aeromonas hydrophila is quotidian water-borne microorganisms that is often enlaced as a causative agent of clinical infections and has been isolated from animal and plant based food products [1]. It is gram-negative, facultative anaerobe, non-spore forming, rod-shaped motile, catalase, oxidase and positive. The genus is made up of psychrophiles and mesophiles. *A. hydrophila* is frequently known to cause human infections such as septicemia, gastroenteritis and cellulitis, wound sepsis with necrosis, gangrene, pneumonia and traveler's diarrhea resulting from improper handling and consumption of

contaminated food [2]. *Aeromonas* presently is considered as food-borne pathogen of emerging importance and is not listed in the Contaminant Candidate List of food. It has gained attention for potential to grow at refrigeration temperature, association with salad vegetables, assistance of antibiotic resistance and the capability to persist safety treatments in food [3]. Virulence gene detection is important to determine the potential pathogenicity of *Aeromonas* [4] due to the involvement of pathogenic genes and extracellular proteins including enterotoxin, hemolysin, aerolysin, various hydrolytic

enzymes [5]. Despite the nutritional and health benefits of fresh produce, gastroenteritis related outbreaks have increased in recent years [6]. Being sources of high energy and rich in minerals, vitamins, fibers, and phenolics, salad vegetables constitutes an important food group that is linked to maintenance and well-being of individuals and helps to reduce incidence of chronic diseases.

In the farm to table process, there are many sources of contamination of fresh vegetables due to contact with disease causing microorganisms that include on the farm sources and off the farm sources [7]. Enteric bacterial pathogens enters agricultural environment via animal feces. The substantiate routes of crop contamination from feces are water, soil, compost/ seeds. Water can come in direct contact with crops in two ways: the irrigation and the often overlooked, pesticide or fertilizer diluents. The pathogen sources include those animals with freedom to wander into fields. The pathogens associated with these feces can be mobilized during rain or aerosolized by high winds.

Once mobilized in water, these pathogens can flow into surface water commonly used for irrigation and pesticide and fertilizer diluents in some growing regions. Surface water can flow directly into field crops by flooding or percolate through the soil column into groundwater.

Fresh produce following cutting has more water activity and possess easily accessible nutrients on cut surfaces which than intact and supports the growth of food-borne pathogens by serving as the potential organic and inorganic substrates for microorganisms [8]. Preferential niches of plants for these bacteria include wounds, roots, trichomes, stomata and substomatal chamber.

The occurrence of antibiotic resistance in prevailing and pathogenic microbes in vegetables contributes to the horizontal proliferation of resistance within distinct isolates. The resistance gene on transferable elements assist dispersal of resistance and extensive utilization of antimicrobials enables direct or co-selection of resistance [9]. Consequently, the occurrence of antibiotic resistant *Aeromonas* in fresh produce develops a principal interest for the safety of consumers [10]. The objective of this study was to evaluate the prevalence *A. hydrophila* on salad vegetables.

Materials and Methods

Sample collection

A total of 205 sample of salad vegetable (cucumber, radish, carrot, cabbage, tomato, long melon and spinach) were collected from the local market, Ludhiana and vegetable farm Punjab Agricultural University (PAU), Ludhiana, Punjab for a period of one and a half year (2014-2016).

Vegetables from the farm were freed from coarse dirt, and samples from the market were randomly purchased at the vegetable counter. All vegetables were sold loose and unprocessed. Samples were individually packaged into plastic bags to avoid cross contamination and stored at 4^oC until further processing within 48 h. 95 sample of salad vegetables from the local market and 110 samples from PAU field was procured during the study (Table 1).

Microbiological analyses

Aerobic mesophilic count and documentation of *A. hydrophila* were quantified using classical methodologies, with the results being expressed as colony forming units (CFU/g).

Enumeration of *A. hyrophila* was done from the external, internal and macerated part of the vegetable. Maceration of the whole vegetable sample was done using sterile scalpel blade. Compacted leaves of cabbage were used as internal and external part, spinach was used as a whole leaf in the study. Dilutions of the surface, internal and macerated portion of sample (25 grams) were enriched in Buffered Peptone water (BPW) (225ml) for 4 hours and suspension was plated in triplicate on *Aeromonas* Isolation HiVeg Medium Base using pour plating technique and incubated at 37°C for 24-48 hours. Colonies were counted per dilution as log cfu/g.

Phenotypic characterization of *A. hydrophila*

Gram's stain was used to examine the isolated bacteria for studying the microscopic properties as initial identification of *A. hydrophila.* Morphological colonies characteristics were recorded on the *Aeromonas* isolation HiVeg Medium Base for primary identification of *A. hydrophila.* Presumptively confirmed by biochemical tests; oxidase, catalase, Indole, Methyl- red, Voges- Proskauer test, motility, Arginine, Lysine, Gas from Glucose, sugar fermentation test, Urease, citrate, and H_2S production. Isolates were further confirmed for virulence by virulence based tests: Crystal violet (CV) binding, Congo-Red binding test, Deoxyribonuclease (DNase) test, Protease production**,** Hemolysin production**,** Pyrazinamidase activity, Siderophore production and molecular method (Table 4).

PCR detection of virulence genes

Template DNA for PCR screening was prepared by processing 5 ml of culture grown for 18 h at 30°C, using an Easy DNA ® Isolation Kit (Invitrogen Inc.). The presence,

concentration, and purity of total DNA in the prepared samples were detected by measuring the absorbance at 260 and 280 nm using TECAN 2000 Nanoquant Plate. PCR analyses were carried out to detect haemolysin gene (ahh) and AHCYTOEN gene (act). The PCR products and the ladder marker were resolved by electrophoresis on 0.8% agarose gel.

Conditions for PCR amplification

Polymerase Chain Reaction Polymerase chain reaction was used to detect 685 bp 16sRNA gene in isolates for confirmation of *A. hydrophila*. Primers specific for act gene: (232 bp product) and ahh: (130 bp) were used as the target genes for PCR amplification. A 30 µl PCR mixture contained 1.5 mM of 25mM $MgCl_2$, 1X Go Taq TM buffer, 10 nmole each 200 µM dNTPs, 0.2 mM of dNTPs (Promega Inc.), 0.5µM of each primers, 2U of GoTaqTM DNA polymerase and 25 ng/µl of the DNA template.

The PCR was run under the following conditions: denaturation at 94°C for 5 min followed by 94°C for 30 sec, primer annealing at 57.5°C for 30 sec (for act gene) 50°C for 40 sec (for ahh gene), extension for 40 sec at 72°C and 7 min final extension at 72°C. Amplicons were examined and visualized by electrophoresis in 0.8% agarose gel in TBE buffer. The gel was stained with EtBr (Sigma) and viewed in Gel Doc System.

Results and Discussion

Detection of *A. hydrophila*

A. hydrophila was detected in 169 from 206 (82.5%) samples collected during the year 2014-2016. Altogether 25 isolates were obtained for further testing. Salad vegetables *viz.,* carrot, cucumber, radish, tomato, long melon, spinach and cabbage showed significant difference (p<0.05) in the mean

count (log cfu/g) of *A. hydrophila.* Mean count in cucumber was 3.17 log cfu/g, 3.20 log cfu/g in radish, 3.51 log cfu/g in carrot, 3.41 log cfu/g in tomato, 3.19 log cfu/g in spinach, 4.02 log cfu/g in cabbage and 3.55 log cfu/g in long melon.

Total plate count of vegetables was found to be 5.42 log cfu/g in cucumber, 5.34 log cfu/g in radish, 5.68 log cfu/g in carrot, 5.79 log cfu/g in tomato, 6.25 log cfu/g in spinach, 5.2 log cfu/g in cabbage and 5.77 log cfu/g in long melon. It was found that 169/206 (82.5%) sample comprising of 20/25 (80%) of cucumber, 25/30 (83%) of radish, 20/28 (100%) of cabbage, 26/35 (74%) of carrot, 34/40 (85%) of long melon, 20/20 (100%) of spinach and 24/35 (68.5%) of tomato were bacteriologically contaminated (Table 2).

Aerobic mesophilic count from vegetables collected from vegetable farm PAU and local market Ludhiana ranged from a geometric mean of 4.82 to 6.25 log cfu/g (Fig. 1). Mean count in cucumber from market and field was 5.42 and 6 log cfu/g, 5.34 and 5.46 log cfu/g in radish, 5.68 and 5.8 log cfu/g in carrot, 5.79 and 5.56 log cfu/g in tomato, 6.25 and 5.23 log cfu/g in spinach, 5.2 and 5.26 log cfu/g in cabbage and 5.77 and 4.82 log cfu/g in long melon.

The highest level of contamination was observed in spinach from the market samples with the mean count of 6.25 log cfu/g followed by cucumber with the count of 6 log cfu/g from field samples and least was observed in the long melon with mean count 4.82 log cfu/g from field sample.

Results of [11] showed mean total plate count of ready to eat salad to be 6.7 log cfu/g.

Contamination of fresh produce by human pathogens can occur at the pre and post-harvest stage. Pre-harvest application of raw or insufficiently composted animal faeces or sewage as fertilizer, irrigation with contaminated water is possible vehicles for the spread of human pathogens [12]. Post-harvest practices include washing off the vegetables with contaminated water with fecal coliforms, improper packaging, transportation, contamination by food handlers during display. The agricultural practices and hygienic conditions used during harvesting, processing, packaging, transport, and storage in influence the initial microbial population [13].

High level of contamination in cucumber could be due to direct contact of fruit with soil or due to irrigation of crop with contaminated water. Vegetables that are often in contact with soil, insects, and animals during growing and harvesting in the field are more prone to be contamination by bacteria [14].

Cabbage and carrot being having good pH range of 4.9- 6.0, provides very favorable environment for the growth and proliferation of microorganisms. Additional factors like contaminated water, cross contamination, poor handling after harvest increments the survival of bacteria on it. Radish and long melon grows in the close proximity of soil from where it can harbor pathogenic bacteria, their water activity and pH ensures the bacterial survival on them.

The high load of contamination in leafy vegetables is due to surfaces exposure to contaminated environment, extensive utilization of untreated manure and more handling steps during post-harvest. The heavy load in spinach can be accounted to poor water conditions used for washing vegetables and sanitation conditions resulting in higher incidence of food and water borne diseases causing epidemics which then severely threatens the physical well-being of urban population. Pathogens on surface of produce

can contaminate the inner surface during cutting and multiply if held at room temperature [15] due to high humidity, suitable pH, temperatures and nutrients. Saddik *et al.,* (198h5) [16] documented the aerobic count of salads which was more than 10^6 cfu/g as vegetables get contaminated with pathogenic microorganisms in fields or amid harvesting, post-harvesting handling, preparing and dissemination.

In study conducted by McMohan *et al.,* (2001) [17], 34% of organic vegetables tested in study was contaminated with *Aeromonas* spp. Callister and Agger (1987) [18] detected *A. hydrophila* on vegetables and inferred that vegetable produce from retail could be an important source of *A. hydrophila* gastroenteritis. Saad *et al.,* (1995) [19] reported occurrence of *Aeromonas* in 47.8% of vegetables. It has also been detected in lettuce from restaurant [20], pre-made salads [21] and commercial vegetable salads [22].

Minimally processed vegetables have a physical structure which is susceptible to microbiological invasion. So, both microbiological and physiological activities could play a role in quality degradation during storage. Besides the direct effect of microbiological activity on flavor quality, interaction with physiological and microbiological mechanisms during storage of commodities susceptible to microbiological invasion can occur [23].

Enumeration of *A. hydrophila* from local market and vegetable farm of PAU, Ludhiana

A. hydrophila detected from the local market, Ludhiana and PAU field showed the significant difference (p< 0.05) in the mean log cfu/g count. The mean value of *A. hydrophila* isolated from local market samples (3.93 log cfu/g) was significantly higher by 24.4% than salad vegetable samples collected from PAU field (2.78 log cfu/g) (Fig. 1). In a study conducted by [7] from retail shops in Italy showed 100 percent prevalence of *A. hydrophila* in chicory, mix salad and carrot.

Intense use of contaminated water for washing, the collapse of a biological structure due to poor handling, cut surfaces or abrasions, poor facilities and conditions for transportation and storage with the high risk of contamination can introduce pathogens directly to the produce at the market place.

Post-harvesting practices can damage the surfaces of leafy greens. Injured lettuce and spinach have been shown to provide favourable conditions for the growth and dissemination of *E. coli O157:H7* and *S. enterica.*

The fecal-oral route of transmission of pathogens broadens to include workers handling of fruits and vegetables from the point of removal from the plant throughout all further stages of handling, including preparation at the retail and food service levels.

The possible reason for lower contamination in field samples can be attributed to the less exposed to flies, insects, animals, prudent use of fully composted farm yard manure, safe irrigation water and hygienic practices.

Ibenyassine *et al.,* (2006) [24] reported the contaminated irrigation water and surface runoff water as a major source of contaminant in fields. River water contaminated with human and animal waste poses a serious health risk [25]. A food-borne outbreak of *A. hydrophila* in a college of Xingyi City, Guizhou, China, was reported in 2012 which was caused by salad ingredients washed in contaminated tank water [26].

Table.1 Sample collection

Salad Vegetable	Collection site		Total number
	Local Market	**PAU field**	
Cucumber	10	15	25
Radish	15	15	30
Carrot	15	20	35
Cabbage	10	10	20
Spinach	10	10	20
Long Melon	20	20	40
Tomato	15	20	35

Table.2 Primers used for targeting species specific virulent genes of *A. hydrophila*

Gene	Primer	Sequence (5'→3')	Amplicon size(bp)
16s	F	GAAAGGTTGATGCCTAATACGTA	685
	R	CGTGCTGGCAACAAAGGACAG	
act	*F*	AGAAGGTGACCACCAAGAACA	232
	R	AACTGACATCGGCCTTGAACTC	
ahh	F	GCCGAGCGCCCAGAAGGTGAGTT	130
	R	GAGCGGCTGGATGCGGTTGT	

Table.3 Percentage contamination in salad vegetables

Vegetables	Bacteriologically Unsafe		Total number of unsafe sample	Total percentage (%) contamination
	Location			
	Market	**Field**		
Cucumber	12/15	8/10	20/25	80
Radish	14/15	11/15	25/30	83
Carrot	15/20	10/15	26/35	74
Cabbage	10/10	10/10	20/20	100
Spinach	10/10	10/10	20/20	100
Long Melon	19/20	15/20	34/40	85
Tomato	15/20	9/15	24/35	68.5
Bacteriologically Unsafe	95/110	73/95	169/205	-
Percent contamination	86.3	76.8	82.5	

Table.4 Biochemical characteristics of *A. hydrophila* (%)

Galactose	66
Xylose	100
Fructose	100
Arabinose	83
Sucrose	83
Dextrose	100
Cellobiose	66
Lactose	33
Maltose	100
Mannitol	83
Methyl Red (MR) test	100
Voges-Proskauer's (VP) test	96
Oxidase test	98
Catalase test	100
Indole test	100
Citrate utilization	94
Triple Sugar Iron(TSI) test	92
Esculin hydrolysis	100
Phenylalanine deaminase agar test	100
Urease test	0
Nitrate test	61
Lysine decarboxylase	100
Ornithine decarboxylase	0
Arginine dihydrolase	100
Gelatinase production	100

Fig.1 Enumeration of *A. hydrophila* from local market and vegetable farm of PAU, Ludhiana
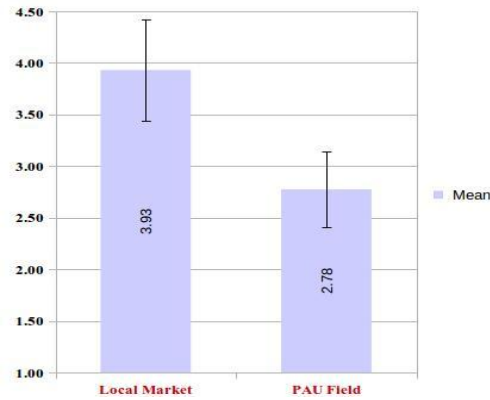

Fig.2 Enumeration of *A. hydrophila* from external, internal and macerated
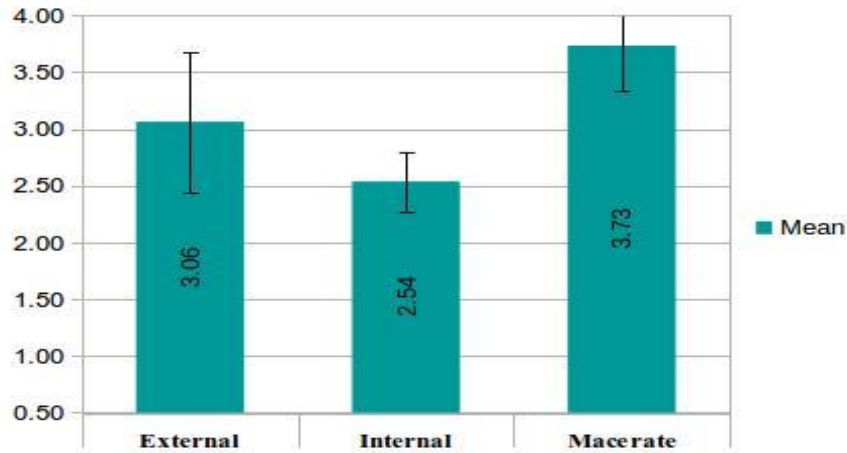portion of salad vegetables

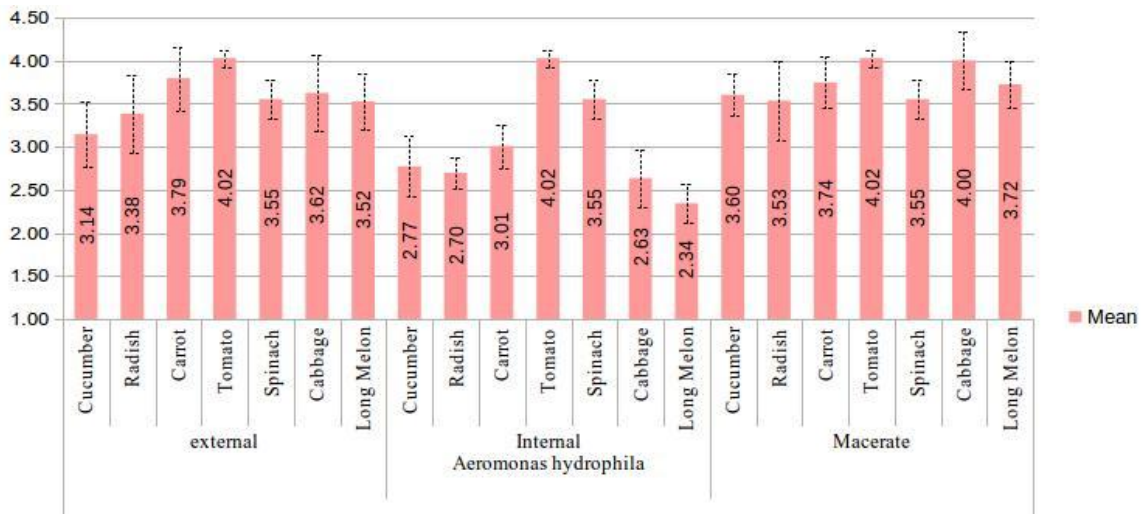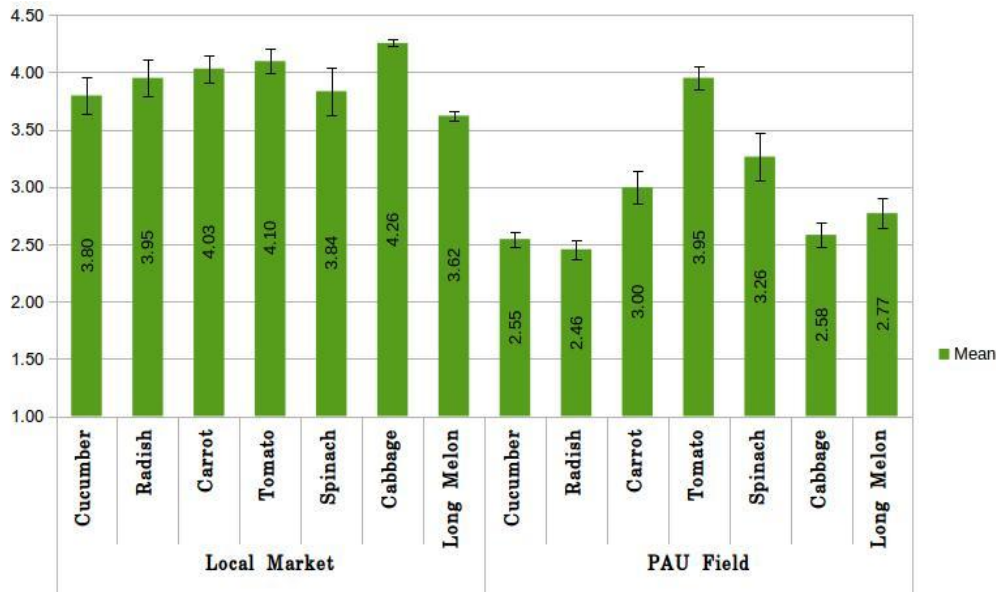**Fig.3** Comparative mean count of *A. hydrophila* in vegetable and vegetable part

Fig.4 Comparative mean count of *A. hydrophila* with source of isolation and vegetable

Enumeration of *A. hydrophila* from external, internal and macerated portion of salad vegetables

The internal tissue of leafy foods are generally considered as aseptic [27] but bacteria could be present there in low number but by up-taking the contaminated water through irrigation and washing processes, bacteria can be introduced [28]. *A. hydrophila* was isolated from internal, external and macerated part of salad vegetables and it showed the significant difference (p <0.05). The mean value (log cfu/g) for macerated portion was significantly higher (3.73 log cfu/g) than the external portion (3.06 log cfu/g) followed by the internal portion (2.54 log cfu/g) of the vegetables (Fig. 2).

The presence of pathogens in the internal parts of fresh produce is generally linked to the irrigation water used, contaminated soil could pose a serious risk factor to users because internalised pathogens are ineffective by normal washing with disinfectants. Donkor *et al.,* (2010) [29] assessed the internalisation of microorganism in vegetables using

standard microbiological methods, and the overall mean count was found to be 3.9×10^3 cfu/g; 8.3×10^2 cfu/g; 2.1×10^2 cfu/g; 3.4×10^2 cfu/g for total plate count, coliform, faecal coliform counts and yeast counts respectively.

Comparative mean count of *A. hydrophila* in vegetable and vegetable part

The present study showed a significant difference (p<0.05) in the presence of *A. hydrophila* between the salad vegetable (cucumber, radish, carrot, tomato, spinach, cabbage and long melon) and the portion of salad vegetable (external, internal and macerated). According to results, the mean value (log cfu/g) is highest 4.02 log cfu/g in macerated portion of cabbage and in internal and external parts of tomato. The mean log cfu/g in external part for all vegetable parts ranges from 3.14 log cfu/g to 4.02 log cfu/g, in internal part it ranges from 2.77 log cfu/g to 4.02 log cfu/g and in macerated part it ranged from 3.53 log cfu/g to 4.02 log cfu/g.

Overall, macerated portion of all vegetable showed more percent of *A. hydrophila*

contamination by 16.3% followed by external (8.8%) and then internal part (6.6%) (Fig. 3).

A. hydrophila could have contaminated tomatoes and cabbage due to the poor quality of water used in irrigation and washing purposes. The agricultural practices and hygienic conditions used during harvesting, processing, packaging, transport, and storage influences the initial microbial population [30]. Fruits and vegetables are cleaned, cooled, and conveyed in water and are treated with aqueous formulations of chemicals after harvesting to remove foreign matter and microorganisms [31]. The degree to which washing reduces the microbial load depends on the produce and the procedure used. Recycling of the wash water, a common practice in washing and hydrocooling, can lead to cross- contamination [32]. Microorganisms in the water may contaminate fruit through lenticels [33], stomata, and injuries incurred during harvesting [34]. Warmed tomatoes immersed in cool water containing bacteria were found to absorb water and bacteria. When the tomatoes immersed in water were at the same temperature as the water, they did not absorb water, but their surfaces were contaminated with bacteria [35].

Cabbage and carrot being having good pH range of 4.9- 6.0, provides a very favorable environment for the growth and proliferation of microorganisms. Additional factors like contaminated water, cross contamination, poor handling after harvest increments the survival of bacteria on it. Radish and long melon grow in the close proximity of soil from where it can harbor pathogenic bacteria, their water activity and pH ensures the bacterial survival on them.

The frequent utilization of polluted irrigation water, market handling and provision for sanitary standards (water for crop irrigation and washing) is lacking. Faecal coliform in irrigation water ranged between 498×10^3 and 2.7×10^6 100 ml^{-1} [36] which are exceeding the WHO recommended level of 1×10^3 100 ml^{-1} for unrestricted irrigation assessment of personal hygiene in food vendors.

Comparative mean count of *A. hydrophila* with source of isolation and vegetable

Present investigation yields significant difference ($p<0.05$) in the presence of *A. hydrophila* in salad vegetable (cucumber, radish, carrot, tomato, spinach, cabbage and long melon) with respect to the source of sample collection (local market and PAU, field).

Cabbage collected from local market showed the highest mean count of 4.26 log cfu/g followed by tomato 4.10 log cfu/g, carrot, radish, cucumber (4.10 and 4.08 log cfu/g) from the same source. From field highest mean log cfu/g was observed in tomato 3.95 log cfu/g followed by spinach (3.26 log cfu/g), carrot (3.00 log cfu/g), long melon (2.77 log cfu/g), cabbage (2.58 log cfu/g), cucumber (2.55 log cfu/g) and radish (2.46 log cfu/g).

Briefly, salad vegetables collected from the local market had 22.3 percent high contamination level of *Aeromonas hydrophila* than salad vegetables collected from the PAU field (Fig. 4).

Vegetables contaminated with pathogenic microorganisms are capable of causing human diseases while still on the plant in the farm, or during harvesting, transport, processing, distribution and marketing, or in the home [37]. Cabbage, tomato, carrot and radish samples procured from local markets and retail stores were found to have higher $\log_{10}$ cfu/ml value in comparison to samples procured from university field. It has been

suggested that a favourable environment allows pathogenic bacteria grow to detectable levels in these vegetables [38].

The issue of fresh produce spoilage is of high importance in the efforts to combat hunger, raise income and improve food security. Food spoilage by bacteria has an impact on food security for poor people, on food quality, safety and on the economic development. Roughly one-third of food produced for human consumption is lost or wasted globally, which amounts to about 1.3 billion tonnes per year [39]. Food losses take place at production, post-harvest and processing stages in the food supply chain [40].

The causes of food spoilage in low-income countries like India are mainly connected to financial, managerial and technical limitations in harvesting techniques, storage and cooling facilities in difficult climatic conditions, infrastructure, packaging and marketing systems. Given that many small holder farmers in developing countries live on the margins of food insecurity, a reduction in food losses could have an immediate and significant impact on their livelihoods.

Phenotypic characterization

The colonies of *A. hydrophila* were light brown colored colonies on HiVeg media base, with diameter ranged from (2-3) mm. *A. hydrophila,* a gram negative bacillus, straight shape, singly or pairs and rarely as short chains, and not spore forming.

The results of biochemical tests were adopted as a complementary characteristic of the initial diagnosis of *A. hydrophila*, where the results indicated that isolates belong to *A. hydrophila*, all isolates were positive result for oxidase test. *A. hydrophila* isolates are characterized by their ability to ferment glucose, it produces (alkaline) red color top

and bottom (acidic) yellow color with gas formed but not H_2S on Triple Sugar Iron agar; it gives a positive result to catalase, indole, Simmon's citrate tests.

Detection of virulence genes

For the validation of *A. hydrophila* identification at molecular level, chosen strains were identified by 16S rDNA PCR analysis. Amplified products were checked on 0.8% agarose gel, electrophoresis generated 685 bp size fragment.

PCR amplification of DNA from *A. hydrophila* isolates using specific primers for AHCYTOEN (Act) genes resulted in the expected PCR products of 232 bp sizes. Amplification result obtained through the present study shows the 130 bp of haemolysin gene (ahh)**,** which is reported earlier as a potent virulence gene of *Aeromonas*.

The band appearance of amplified virulent genes of samples was obtained through the 0.8% agarose gel electrophoresis and viewed in gel documentation system with EtBr stain and analyzed.

The pathogenicity and virulence characteristics of *A. hydrophila* are associated with presence of different exotoxins e.g., haemolysin, enterotoxins and cytotoxins and exoenzymes e.g., proteases and lipases [41].

Enterotoxin Act is the most cytotoxic virulence factor of and a core gene within *A. hydrophila.* An aerolysin related cytotoxic enterotoxin (Act) of *Aeromonas hydrophila* possesses multiple biological activities, which include its ability to lyse red blood cells, destroy tissue culture cell lines, evoke a fluid secretory response in ligated intestinal loop models, and induce lethality in mice. The role of Act in the virulence of the organism has been demonstrated [42].

Similar results were found by [43], where PCR amplification of DNA from pathogenic *A. hydrophila* isolates using specific primers for act genes resulted in the expected PCR products of 232 bp sizes. Isolates have been classified as pathogenic due to presence of 232 bp gene in 0.8% agarose gel.

Screening of 232 bp act gene has been reported to be the most effective way of diagnosis of *Aeromonas* virulence factors [44]. Previous clinical studies [45] reported that the expression of multiple biological activities, as in the case of the AHCYTOEN gene, is necessary for the expression of microbial pathogenicity. This cytolytic enterotoxin gene is considered as a characteristic virulence trait in *Aeromonas* [46].

Hemolysin (ahh1) has also been detected by [47] from the clinical isolates. The production of hemolytic cytotoxins has been regarded as strong evidence of pathogenic potential in *Aeromonas* [48].

Minimally processed vegetables have a physical structure which is susceptible to microbiological invasion. So, both microbiological and physiological activities could play a role in quality degradation. Results obtained in this study indicate the need for implementing food safety assurance programmes in the production chain of salads to improve their microbiological quality and need for food processors and consumers to adopt hygienic practices to minimize risks of transmission of foodborne pathogens through this kind of foods. This study revealed the potential hazard of ready-to-eat salads and emphasizes the need of surveillance studies for redressal of public health. Though microorganisms are part of the epiphytic flora of salads, their persistence and further proliferation contaminates the ready to eat foods which are dressed with fresh salad vegetables. There are an increasing number of outbreaks of human pathogens related to fresh produce. Thus, the growth of human pathogens on plants should be explored.

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