

## SHORT COMMUNICATION

# Freshly Eaten Leafy Vegetables: A Source of Food Borne Pathogens?

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## ABSTRACT

The microbiological quality of selected fresh vegetables; lettuce, cabbage, mukunuwenna, gotukola, and tomato were assessed from different market types in the Kandy district. Fifty samples of each vegetable were tested for viable bacterial count (VBC), *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7, before and after washing using tap water. The average VBC in unwashed vegetables was 10<sup>6</sup> cfu/g whereas a 10 fold reduction of VBC was achieved as a result of washing three times. *S. enterica* serotype Enteritidis and *S. enterica* serotype Typhimurium were isolated in 6% of the samples. In addition *L. monocytogenes* was isolated in 2% of the samples investigated. These results reiterate the necessity for awareness among consumers regarding the microbiological quality of fresh vegetables.

**Keywords:** fresh vegetables, food-borne pathogens, viable bacterial count

## INTRODUCTION

Fresh fruits and vegetables provide mankind with an abundance of benefits. These play an important role in health and have the ability to prevent many diseases such as heart disease, cancers and diabetes (Liu, 2003; Pandey and Rizvi, 2009). Green leafy vegetables play a major role in the Sri Lankan diet probably due to its health benefits, low cost availability and active promotion of fresh vegetables as a part of a healthy diet (Chandrika *et al.* 2006).

However, the recent increase in reports of food borne illnesses associated with fresh fruits and vegetables has raised concerns regarding the safety of these products (WHO, 2008). Fresh vegetables are vulnerable to be contaminated by pathogens (Hosein *et al.* 2008; Taban and Halkman, 2011) at several points throughout the pre-harvest and post-harvest systems, *via* soil, water, animals, insects, equipment and human handling (Beuchat, 2002; Hoyle, 2008).

Bacterial pathogens are considered as the most common agents causing food borne diseases and such pathogens involved are *Salmonella* spp., *E. coli* O157:H7, *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Staphylococcus aureus* and *Listeria* spp. (Cetinkaya *et al.* 2008; WHO, 2008; Hosein *et al.* 2008). It is reported that 35% of raw leafy

vegetables samples examined in Malaysia were contaminated with *Salmonella* (Salleh *et al.* 2003). A Study conducted in India on salad vegetables, revealed presence of *E. coli*, *Enterobacter aerogenes*, *Pseudomonas* spp., *S. aureus*, *Salmonella* spp. and *Shigella* spp. (Tambekar and Mundhada, 2006). Further, presence of *Listeria* spp. in vegetables, such as cabbage, lettuce, tomato, watermelon, oranges have been reported in many countries, including Sri Lanka (Gunasena *et al.* 1995; Caggia *et al.* 2009; Ponniah *et al.* 2010). Further, WHO reports that leafy green vegetables are of great concern in terms of microbiological hazards (WHO, 2008).

In developing countries like Sri Lanka, food borne illnesses caused by contaminated vegetables are frequent (Gunasena *et al.* 1994). However, lack of food borne disease investigations and standard diagnostic procedures, cause outbreaks to go undetected and they are hardly reported in scientific literature. Therefore, conducting a scientific research on determining the hygienic condition of fresh vegetables is essential and timely.

The objectives of the current study were to determine the presence of common food borne pathogens including *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 in selected fresh vegetables *viz.* lettuce (*Lactuca sativa*),

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cabbage (*Brassica oleracea*), mukunuwenna (*Alternanthera sessilis*), gotukola (*Centella asiatica*) and tomato (*Lycopersicon esculentum*) purchased from different market types in the Kandy district and to determine the effect of washing of the above vegetables with tap water, on reducing microbial contamination.

## MATERIALS AND METHODS

### Sample collection

The vegetable samples were collected during a period of nine months from July 2009 to March 2010, in the Kandy district, situated in the Central province of Sri Lanka. Ten samples were obtained for each vegetable from road side markets (four samples), retail markets (three samples) and super markets (three samples). A total number of 50 samples of each fresh vegetable, comprising of lettuce, cabbage, 'mukunuwenna', 'gotukola', and tomato were analyzed. All samples were purchased freshly in the morning and transported to the laboratory in separate polyethylene bags (20 cm x 35cm).

### Sample preparation

Damaged and diseased particles were removed from vegetable samples. All equipment necessary for the pathogen culture, *viz*: chopping board, knife, vegetable trays, were wiped with 70% ethanol. Two sets of 25 g of each vegetable type were weighed, one set was washed three times separately in tap water and the other set was unwashed. Both sets of vegetables were cut into approximately 1 cm<sup>2</sup> pieces and each set transferred into 10 flasks containing 225 ml of sterile nutrient broth, LAB-M. Tenfold serial dilutions (10<sup>0</sup>- 10<sup>-4</sup>) were prepared from each sample and 0.1 ml of selected dilutions were spread plated on nutrient agar (LAB-M) and incubated at 37 °C for 24 hrs. This procedure was repeated 3 times for each vegetable type. The average viable bacterial count (VBC) was taken and the results were statistically analyzed using the GLM procedure with SAS statistical software (SAS -Windows 6.1). Data were ArcSine transformed prior to statistical analysis at 95% confidence level.

### Isolation and detection of pathogenic bacteria from vegetable samples

#### *Salmonella* spp.

Five grams of each vegetable (washed and unwashed) which were cut into pieces (1 cm<sup>3</sup>), were transferred into buffered peptone water (Oxoid) for pre enrichment of *Salmonella* spp. and incubated overnight at 37 °C. One milliliter

aliquots of each pre enriched sample was subsequently transferred into selenite broth (Oxoid) and incubated at 37 °C for 24 hrs for selective enrichment. A loop full of broth from the enriched samples were streaked on Xylose lysine deoxycholate (XLD) agar (Oxoid) and incubated 37 °C for 24 hrs. *Salmonella* species showing characteristic pink colour colonies with black centers on XLD plates were selected, sub-cultured and stained with Gram's stain prior to biochemical testing. Oxidase, catalase, urease (Oxoid), citrate (Oxoid) and triple sugar iron agar (Oxoid) tests were performed for the conformation of *Salmonella* spp. Cultures maintained on nutrient agar were further subjected to serological tests using polyvalent 'O' and 'H' antisera (Difco) according to the Laboratory Protocol of WHO, 2003.

#### *Listeria monocytogenes*

Vegetables (25 g) were separately (washed and unwashed) immersed in 200 ml of nutrient broth and subjected to cold enrichment at 4 °C for 1-8 weeks. Loop full of the broth was streaked weekly on blood agar and incubated over night at 37 °C. Small, white, round colonies consisting of narrow zones of haemolysis were suspected as colonies of *L. monocytogenes* and subjected to biochemical tests, *viz*. oxidase, catalase, urease (Oxoid), citrate (Oxoid) and triple sugar iron agar (Oxoid).

#### *Escherichia coli* O157:H7

Vegetables (25 g) were separately (washed and unwashed) immersed in 200ml of nutrient broth (LAB-M) and incubated at 37 °C overnight. Subsequently, a loop full of broth from each vegetable (washed and unwashed) was streaked on Sorbitol MacConkey agar (SMAC, Oxoid) and on Violet Red Bile agar (VRBA, Oxoid) separately for selective isolation of *E. coli* O157. Sorbitol non fermenting *E. coli* colonies (colorless/white) were selected and sub cultured on MacConkey agar (MAC, Oxoid) plates. Colonies consisting of a pink colour, shiny surfaces with entire margins on MacConkey agar medium were suspected as *E. coli* O157.

Following incubation of VRBA agar plates at 37 °C for 24 hrs, pink colour colonies showing blue florescence under UV light (670 nm) were selected and subjected to Gram's staining and biochemical tests [oxidase, catalase, urease (Oxoid), citrate (Oxoid), triple sugar iron agar (Oxoid) and Indole tests] and confirmed by latex agglutination test (Oxoid).

## RESULTS

### Viable bacterial counts in fresh vegetables

VBC in unwashed vegetables was in the range of  $1 \times 10^6$  cfu/g, with the exception in tomato obtained from the retail market, which was  $1 \times 10^4$  cfu/g. When considering all the vegetables subjected to investigation, the VBC in washed vegetables was significantly lower in unwashed vegetables (F value = 18.54, P value= 0.0001) indicating washing 3 times in tap water effectively reduced bacterial content. When considering each vegetable separately, a significant reduction of VBC was achieved as a result of washing with respect to tomato and gotukola while there was no significant effect of washing on cabbage, mukunuwenna and lettuce (Table 1).

When comparing washed vegetable samples, from the three different market types, the highest average number of total VBC ( $1.38 \times 10^6$ ) was recorded in vegetables purchased from the road side market, where as the VBC in vegetable purchased from retail and super market were  $7.84 \times 10^5$  and  $8.43 \times 10^5$  respectively, which were less than that of road side market. Though a statistical difference was observed between VBC of vegetables in road side market and retail market (F= 3.78, P= 0.0285) the VBC in the road side market was not statistically different from the supermarket. Average VBC of different vegetables purchased from different markets are shown in Table 1.

### Bacterial pathogen types present in fresh vegetables

*Salmonella* spp., *L. monocytogenes* and suspected colony of *E. coli* O157 were isolated from different vegetable samples. *Salmonella* spp. were the most common (in 6% of samples tested) among the three pathogens and the species present included *Salmonella enterica*, serotypes

Enteritidis and Typhimurium. Table 2 presents the different *Salmonella* serotypes isolated from different vegetables and the market type from which the vegetables were obtained. *L. monocytogenes* and *E. coli* O157 were observed in 2% of the samples investigated (Table 2). In addition colonies of *Pseudomonas* spp., *Staphylococcus aureus*, *E. coli*, *Proteus* sp. and *Klebsiella* spp. which are of a health concern were isolated during the current study. However, further investigations are needed to confirm this result.

## DISCUSSION

This study provides clear evidence of contamination of fresh vegetable from common food-borne pathogens including *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7. Importantly, the results confirmed that the level of contamination was equally high in most of vegetables studied that were bought from different market types. It was also noted that thorough washing was not sufficient to reduce pathogen levels to safe limits in leafy vegetable types studied.

Viable bacterial count (VBC) is an important indication of bacteriological quality of food products and high bacterial load in foods could pose a health risk to consumers (Aycicek *et al.* 2006 and Hosein *et al.* 2008). The maximum safe count to consume for aerobic mesophilic microorganisms in vegetables in general was established as  $10^5$  cfu/g (Silva *et al.* 2007). Majority of the vegetable samples investigated in the current study accounted for bacterial counts higher than the  $10^5$  cfu/g, which indicates a high percent of contamination. Washing of vegetables in tap water for three times results only a 10-fold reduction of VBCs and stresses the importance of using a disinfecting substance (e.g. salt, warm water).

**Table 1.** Average viable bacterial counts in vegetables before and after washing

Vegetable	Average viable bacterial count (cfu/g)					
	Roadside market		Retail market		Super market	
	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed
Tomato	$2.6 \times 10^4$ <sup>a</sup>	$2.7 \times 10^6$ <sup>b</sup>	$3.0 \times 10^3$ <sup>a</sup>	$1.2 \times 10^4$ <sup>b</sup>	$1.5 \times 10^4$ <sup>a</sup>	$1.1 \times 10^6$ <sup>b</sup>
Cabbage	$2.1 \times 10^6$ <sup>a</sup>	$3.1 \times 10^6$ <sup>a</sup>	$1.2 \times 10^6$ <sup>a</sup>			
Mukunuwenna	$2.9 \times 10^6$ <sup>ab</sup>	$4.3 \times 10^6$ <sup>a</sup>	$8.6 \times 10^5$ <sup>b</sup>	$1.4 \times 10^6$ <sup>ab</sup>	$1.6 \times 10^6$ <sup>ab</sup>	$3.4 \times 10^6$ <sup>a</sup>
Gotukola	$5.1 \times 10^5$ <sup>c</sup>	$1.7 \times 10^6$ <sup>ab</sup>	$1.1 \times 10^6$ <sup>bc</sup>	$3.4 \times 10^6$ <sup>a</sup>	$9.1 \times 10^5$ <sup>bc</sup>	$3.4 \times 10^6$ <sup>a</sup>
Lettuce	$1.3 \times 10^6$ <sup>ab</sup>	$2.9 \times 10^6$ <sup>a</sup>	$7.6 \times 10^5$ <sup>ab</sup>	$1.8 \times 10^6$ <sup>ab</sup>	$4.3 \times 10^5$ <sup>b</sup>	$1.8 \times 10^6$ <sup>a</sup>

<sup>a,b,c</sup> values followed by the same letter within each line are not significantly different (p = 0.05)

**Table 2.** Pathogens isolated from different vegetables obtained from different market types (RSM - roadside market, RM- retail market, SM- super market)

Pathogen	Market type	no. of times the pathogens were isolated and the leafy vegetable	Percentage occurrence of pathogens (n=50)
<i>Salmonella</i> spp.	RM	1 (mukunuwenna) - (before washing)	6%
<i>Salmonella</i> Typhymurium	RSM	1 (gotukola) - (after washing)	
<i>Salmonella</i> Enteritidis	SM	1 (lettuce) - (before washing)	
<i>L. monocytogenes</i>	SM	1 (mukunuwenna)	2%
Suspected <i>E.coli</i> 0157:H7	RM	1 (mukunuwenna)	2%

During the current study the presence of pathogenic bacteria and a high VBC in mukunuwenna, gotukola and salad leaves were observed from all three market places. According to investigations, *Salmonella* spp. have been recorded as one of the most common pathogens responsible for food borne illnesses worldwide in which *Salmonella* Enteritidis and *Salmonella* Typhymurium are common serovars recovered (Salleh *et al.* 2003; WHO, 2008; CDC, 2012). Similarly, results of the current study showed high numbers of *Salmonella* spp. (serotype Typhymurium and Enteritidis) when compared to *L. monocytogenes* and *E. coli*.

It was a noteworthy observation that, *L. monocytogenes* a common food born pathogen in vegetables was found in mukunuwanna obtained from super markets. *L. monocytogenes* has a special ability to survive in cold temperatures (Becker *et al.* 2000; Sergelidis and Abraham, 2009). Therefore the cold temperatures prevailing in the super market may have favored the growth of *Listeria* sp. According to a previous study conducted in Sri Lanka, *L. monocytogenes* has been isolated in 38% of food samples tested, and was the highest among green leafy vegetables (49%) (Gunasena *et al.* 1995).

Leafy vegetables (Mukunuwenna leaves, Gotukola leaves, and Salad leaves) resulted in the highest contamination of pathogens and highest number of VBC in comparison with tomato and cabbage. However, when considering tomatoes, as the pericarp was taken, the surface area is lower than leafy vegetables consisting of the same weight. This indicates extra caution is necessary in processing of leafy vegetables. Although the highest contamination of VBC in vegetables was recorded from the road side markets, results obtained in the study did not support the view that vegetables sold in super markets have less

pathogens or superior quality. Similar observations were made in a study conducted in Thailand (Minami *et al.* 2009). Thus, hygienic condition of vegetables purchased in different markets cannot be discriminated.

Most of the leafy vegetables are easily contaminated by soil and they provide increased surface area for bacterial colonization. Infiltration of pathogens into cracks, wounds, crevices, stomatal cavities and intercellular spaces of fruits and vegetables has been demonstrated by several researchers (Harapas *et al.* 2010; Taban and Halkman, 2011; Saldaña *et al.* 2011). Therefore even though thorough washing can be effective more vigorous processing is needed to reduce the pathogen number and make vegetable safe for consumption. These results emphasize the necessity for awareness among consumers regarding the microbiological quality of fresh vegetables and the potential health hazard of pathogenic bacteria present in vegetables should not be underestimated.

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