PATHOGENIC PROFILE OF GREEN COLOURED VEGETABLES USING DIFFERENT WASHING PROCEDURES

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Abstract
Green Vegetables and their Ready-To-Eat (RTE) salads are an important source of nutrients, but they can also host a large microbial population, particularly bacteria. Fresh green vegetables carry the risk of microbiological contamination due to the poor handling practices, usage of untreated irrigation water or failure during harvesting, handling, processing and packaging anywhere from the farm to the fork. Appropriate washing can mitigate the risk of food borne illness consequent to vegetable consumption by reducing pathogen levels. Therefore, the study analyses the effectiveness of different domestic washing methods (hot water, salt water, tap water, and vinegar) in reducing microbial levels in RTE green vegetable samples. A combination of differential and selective agar mediums were utilized to characterize the microbes present as well as to assess the efficiency of various washing process in minimizing the microbial load. All samples were found to harbour a greater number of gram positive bacteria compared to gram negative bacteria. Results showed that washing with hot water (70°C) in combination with salt (8%) produced the maximum reduction in the number of microorganisms in all the vegetables studied. The Colony Forming Units present on the outer surface of the vegetables reduced to 49±0.4, 44±0.9, 33±0.5, 19±0.6, 29±0.7, and 49±0.9 in Capsicum, Cucumber, Broccoli, Coriander, Parsley and Lettuce respectively compared to 1838±1.8, 1780±0.8, 1885±0.7, 2000±1.6, 1498±1.2 and 1751±1.2 in the untreated samples. This analysis therefore attempts to increase awareness among consumers about health hazards of food as well as effective methods of sanitization which in turn could minimize the associated health risks.

Keywords: Foodborne, Gram positive, microbes, vegetables, washing

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1. INTRODUCTION

Vegetables play an important role in human nutrition. They supply dietary fiber and are important sources of essential vitamins, minerals, and trace elements. Inclusion of vegetables in a balanced diet has been found to reduce incidences of several diseases including cancer, stroke, cardiovascular disease, gastrointestinal disorders and other chronic ailments. (Thunberg et *al.,* 2012). The USDA Dietary Guidelines recommends daily consumption of five to nine servings of fruit and vegetables, although the total amount consumed varies according to age and gender (Bertoia et *al*., 2016). Green coloured vegetables are ideal for weight management as they are typically low in calories. Although less in their fat content, these vegetables are rich sources of fiber, folic acid, Vitamin C, potassium and magnesium. Moreover, they host a range of phytochemicals such as lutein, beta-cryptoxanthin, zeaxanthin, and β carotene (Aldoori et *al*., 2018). Leutin, zeaxanthin and β carotene are important in maintaining eye health and protect against incidences of macular degeneration and cataract. Additionally they have also been portrayed to prevent against cancers of breast, lungs and other tissues. (Christen et *al*., 2015).

Since green vegetables are one of the most commonly consumed crop worldwide, it can be targeted as an easy carrier of pathogens due to inappropriate handling. This in turn questions on their safety aspect. A range of fresh vegetable products including lettuce, cauliflower, sprouts; mustard cress, endive and spinach have been implicated in *Salmonella* infection (Quiroz-Santiago et *al*., 2014). *Escherichia coli* O157:H7 is commonly recovered from the faeces of ruminants. Livestock grazing in orchards may contaminate capsicum and broccoli florets culminating in proliferation of this bacterium in the vegetable tissues (Sagoo et *al*., 2013). Indeed, *E. coli* has been associated with the outer skin of green vegetables (Janisiewicz et *al*., 2017). Moreover, *Campylobacter* infection has been severely linked to consumption of contaminated leafy and non leafy greens. (Wang et *al*., 2014). *Aeromonas sp* has been isolated from a wide range of fresh produce including sprouted seeds, asparagus, broccoli, cauliflower, carrot, celery and cucumber (Farvid et *al*., 2016). Apart from gram negative coliforms, gram positive bacteria have been profoundly associated with green coloured vegetables. *Listeria sp*. is ubiquitous in the environment and can be isolated from soil, water, faecal matter and vegetation irrigated with contaminated water. The potential for environmental *Listeria* to contaminate fresh produce and lead to enteric infection has long been recognized (Heaton et *al*., 2013). *Listeria monocytogenes* grows at refrigeration temperatures and is hence likely to multiply during storage if present on fresh produce. It is capable of growing on lettuce when exposed to processing conditions. (Warriner et *al*., 2016). Other gram positive isolates associated with green vegetables include *Brevibacillus laterosporus*, *Lactobacillus sp*, *Bacillus licheniformis*, *B lentus* and *B cereus* (Weldezgina et *al*., 2016). Additionally, cultures of *Streptococcus faecalis*, *S liquefaciens and Staphylococcus aureus* have been reported in fresh green beans (Pzzuto et *al*., 2016). Therefore direct consumption of raw vegetables without appropriate washing may increases the potential of food borne diseases.

Green salad vegetables have now become an attractive choice not only because of their nutritive values but also since most of them can be consumed either raw or with minimal processing. Green vegetables carry the potential risk of microbiological contamination due to the usage of untreated irrigation water, inappropriate organic fertilizers, wildlife or other sources. Faecal material, soil and sewage overflow introduce pathogens directly to watercourses from which irrigation water may be extracted. Pathogens within soil may contaminate crops during heavy rain or irrigation. The present study enlightens the presence of pathogens on the outer skin of green vegetables that can be mostly eaten raw in the form of salads. Maintenance of proper hygiene and sanitation is principal towards preserving good health and limiting the development of diseases. Previous researches have reported chemicals like peracetic acid, percitric acid, sodium bicarbonate and sodium hypochlorite to reduce *Salmonella* and *Listeria* counts. (Valentin-Bon et.*al*., 2018) However these treatments were found to be effective only at high concentrations. This investigation therefore attempts to study the effectiveness of different washing procedures in controlling microbes using reagents available at the domestic scale. Moreover, care was taken to ensure maximum reduction of microbial numbers without compromising the vegetable quality. This study attempts to add on to the limited researches carried out on the safety assessment of green vegetables and provide valuable tools to limit food associated disorders. Maintenance of appropriate hygienic practices including effectively washing vegetables prior to consumption may aid towards improved health and wellbeing of the population at large.

2. MATERIALS AND METHODS

2.1 Sample Preparation

The green coloured vegetable samples used for microbial analysis (Table 1) were dissolved in autoclaved peptone water (Peptone powder 15 gm, and distilled water 150ml). 5g of sample was grinded in a previously sterilized mortar pestle, until a uniform mixture was obtained and mixed in peptone solution followed by filtration through Whatman filter paper to obtain a clear extract. The extract was stored at 4°C until further use.

Table 1: Green coloured vegetable samples analyzed

Green vegetables	Sample Codes	Number of samples
Green Capsicum (*Capsicum annuum*)	A	3
Cucumber (*Cucumis sativus*)	B	3
Brocolli (*Brassica oleracea var. italica*)	C	3
Coriander (*Coriandrum sativum*)	D	3
Parseley (*Petroselinum crispum*)	E	3
Lettuce (*Lactuca sativa*)	F	3
Total		18

2.2 Microbial Analysis

The culture mediums were prepared and sterilized at a temperature of 121°C and a pressure of 15 psi for 15 minutes. A combination of selective and differential agars was selected for evaluating the presence of pathogens on outer surface of vegetables. Nutrient agar, MacConkey agar, Malachite Green agar and Mannitol Salt agar were used to enumerate and characterize microorganisms. While Nutrient agar is a general purpose, non selective media, MacConkey agar and Malachite Green agar was utilized for isolation of gram negative and positive bacteria respectively. Mannitol Salt agar was used to select for gram positive cocci. All samples were pour-plated onto the respective agar medias and incubated for growth at 37°C for 48 hours.

2.3 Washing procedures

Several types of washing procedures were employed as follows:

Tap water washing: 5gms of the samples was thoroughly rinsed under running tap water for 2 minutes at room temperature.

Hot water washing: 5 gm of sample was soaked in 100ml of hot water (70°C) for 2 minutes.

Vinegar washing: 5 gm of sample was soaked in vinegar (5%) for 2 minutes.

Salt water washing: 5 gm of sample was soaked for 2 minutes in table salt solution (2%, 4%, 6%, 8% and 10%).

Commercial sanitizer: 5 gm of sample was soaked for 2 minutes in a solution prepared by adding 2 drops of the commercial formula (FSSAI approved; lic. no. 11517034000917) in 100ml of water. This was taken as a positive control.

Statistical analysis

The data was represented as CFU/ml (mean ± SEM) of N≥ 3. P value was evaluated using ANOVA Data analysis pack of Microsoft Excel (version 2007). Data was analyzed at 95% Confidence interval and $P \leq 0.01$ was considered as significant.

3. RESULTS AND DISCUSSION

Microbial content of green coloured vegetables

Raw vegetables harbor huge numbers of microbial colonies which can host a range of pathological outcomes. Food borne pathogens can contaminate raw vegetables at any stage of their production process with a potential for human infection. The culture mediums were prepared followed by inoculation of samples into each agar. Nutrient Agar showed the growth of big circular red colonies in all the raw samples (Figure 1). The samples were inoculated on MacConkey Agar which is specific for the growth of gram negative microbes especially *Escherichia coli* and other lactose fermenting *Coliforms*, revealing a characteristic red colour of the colonies. The crystal violet and bile salts present in it are attributed for their selective action. Malachite Green Agar was utilized for the selective enrichment of gram positive microorganisms especially *Bacillus*, and *Staphylococcus*. A significant number of milky white colonies were observed in raw samples of the leafy and non leafy vegetables indicating improper handling of the vegetables. Cross contamination with the irrigated water and sewage accelerate the deterioration of the food quality whereas unhygienic practices, high moisture content and water activity generate favorable conditions for the growth of bacteria. Moreover all vegetables displayed a higher content of gram positive microorganisms compared to their gram negative counterparts (Figure 1).

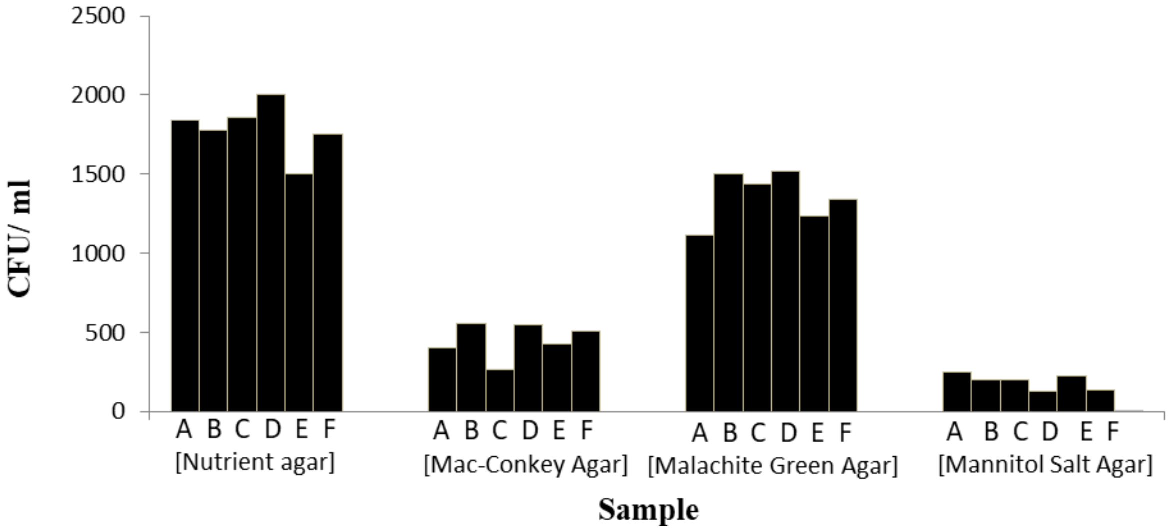

Figure 1: Average CFU/ml of microbes on agar mediums (n≥3)

The thick peptidoglycan layer may impart increased resistance and survivability to the former accounting for the above. Since growth on Malachite Green agar indicated the presence of coagulase-positive species, especially *Staphylococcus sp*., samples were plated onto Mannitol Salt Agar which facilitates the growth of *Staphylococcus* family, a major opportunistic foodborne pathogen. Results revealed the occurrence of *Staphylococcus sp* in the vegetables analyzed.

Tap Water Washing

Appropriate washing can reduce the risk of food borne illness consequent to vegetable consumption by minimizing pathogen levels. The six green vegetable samples were washed with tap water at room temperature for 2 minutes and plated on agar mediums to determine the effectiveness of this method in reducing microbial colonies. Untreated controls were designated as "U" and samples treated with tap water were indicated as "T". The reductions in the bacterial content indicated the detachment of microorganism from the outer skin of the vegetables due to the mechanical force of the running tap water treatment. The attachment of microorganisms to the vegetables seems to be governed by physical factors irrespective of the physiological state of the microorganisms. Thoroughly rinsing fresh vegetables under running water is an effective way to reduce the number of microorganisms. Therefore treatment of samples with running tap water reduced the microbial numbers compared to the untreated ones (Table 2). Samples treated with positive control showed negligible numbers of microbial colonies on these culture mediums (data not shown).

Vinegar Water Washing

Treatments of the vegetable samples with vinegar decreased the bacterial numbers on the vegetables (Table 3).

Table 2: Total Viable Count (TVC) of microbes after Tap Water washing

Sample	Nutrient Agar		Mac-Conkey's Agar		Malachite Green Agar		Mannitol Salt agar	
	U	T	U	T	U	T	U	T
A	1838±1.8***	1246±3.3**	403± 2.5***	149±1.4**	1116±2.5***	500±3.4**	248± 2.3***	102± 1.2**
B	1780±0.8***	1340±0.8***	553± 4.1***	278±2.4**	1500±2.8**	349±2.5**	202± 3.1**	101±4.2**
C	1885±0.7***	1253±2.2***	266± 2.5**	223±4.1**	1401±1.3**	1005±3.7**	200± 2.9**	91± 1.8**
D	2000±1.6***	1050±1.6***	546± 2.8**	174±0.9**	1518±2.1**	920±3.2**	125± 3.7**	97±0 .7**
E	1498±1.2***	886± 2.4***	503± 4.1**	305±2.4**	1233±3.2**	1085±2.1**	220± 4.2**	75± 2.1**
F	1751±1.2***	1235±0.8***	400± 2.6**	150±2.9**	1343± 1.4**	886±2.0**	138± 0.8**	94± 2.6**

Table 3: Total Viable Count (TVC) of microbes after vinegar washing

Sample	Nutrient Agar		Mac-Conkey's Agar		Malachite Green Agar		Mannitol Salt agar	
	U	T	U	T	U	T	U	T
A	1838±1.8***	593± 6.4**	403± 2.5***	81± 0.6**	1116± 2.5***	263± 1.5**	248± 2.3***	93± 0.6**
B	1780±0.8***	515±0.7***	553± 4.1***	161± 1.7***	1500± 2.8**	220± 1.3***	202± 3.1**	72± 0.6***
C	1885±0.7***	565± 0.8**	266± 2.5**	105± 2.2**	1401± 1.3**	901± 2.8**	200± 2.9**	65± 2.1**
D	2000±1.6***	550± 0.8**	546± 2.8**	65± 0.8**	1518± 2.1**	506± 0.7**	125± 3.7**	50± 0.4**
E	1498±1.2***	561± 1.1**	503± 4.1**	91± 0.6**	1233± 3.2**	761±3.6**	220± 4.2**	61± 1.6**
F	1751±1.2***	681± 2.3**	400± 2.6**	141± 2.9**	1343± 1.4**	523±1.8**	138± 0.8**	71± 2.1**

The acetic acid chemically interacts with proteins and other important biomolecules inside the bacterial cells causing them to lose their native structure resulting in increased control with respect to tap water washing. However, this treatment can only control the neutrophilic microorganism having little effect on the acidophilic ones. Moreover, the effectiveness of the washing method also depends on the growth phase of the cells, where logarithmic growth phase cells were more sensitive and easily killed than stationary phase cells.

Hot Water Washing

Washing with hot water (70°C for 2 minutes) lead to a significant reduction of the food borne pathogens from the outer skin of the vegetable samples analyzed. Moreover, the efficacy of this method was found to be higher in terms of decreasing the microbial load compared to tap water and vinegar washing. Since majority of the bacteria present on the surface of vegetables are mesophilc, the temperature of this treatment diminishes the rate of their multiplication and therefore survival of these organisms. The increased temperature of hot water may be causing inactivation of enzymes, degradation and as well denaturation of proteins and other cellular macromolecules accounting for a lower yield (Table 4). Moreover, the time for treatment was standardized such as to obtain maximum microbial reduction without compromising the quality of the samples. Hot water washing displayed a marked reduction in all the agar mediums studied showing its efficacy against both gram positive and negative bacteria (Table 4).

Salt Water Washing

Salt water washing (4%) showed the best result with respect to reduction of microbial numbers compared to all other washing methods employed (Table 5). The salts may be responsible for control of microorganism through the process of dehydration and osmosis. High concentration of salt leads to loss of moisture from the bacterial cells resulting in loss of cell structure and alteration in cell physiology and metabolism which may eventually lead to cell death. Salt water washing showed the maximum decrease in gram negative and gram positive microbes including *Staphylococcus sp* as tabulated in Table 5.

Hot water combined with salt washing

Since the above data clearly implicated hot water and salt water treatment to perform best in terms of reducing the microbial load, it was further evaluated whether combining the above could lead to a better control compared to the individual methods. Additionally, the efficacy of this treatment procedure was also checked across a range of salt concentrations in order to analyze the best combination bringing about maximum control. It was indeed observed that washing the vegetables with hot water combined with salt resulted in an improved reduction of microorganism compared to either of the individual methods (Table 6). An increase in salt concentration increased the control efficiency of the above treatment. However, a further increase in salt concentration beyond 8% caused no further significant decrease in the microbial numbers (Table 6). Therefore, a salt concentration of 8% accompanied with hot water was found to bring about maximum control of bacterial numbers.

Table 4: Total Viable Count (TVC) of microbes after Hot Water washing

Sample	Nutrient Agar		Mac-Conkey's Agar		Malachite Green Agar		Mannitol Salt agar	
	U	T	U	T	U	T	U	T
A	1838±1.8***	113± 1.7**	403± 2.5***	24± 0.8 **	1116± 2.5***	155± 1.2**	248± 2.3***	50± 0.6**
B	1780±0.8***	1340± 0.8***	553± 4.1***	50± 0.7**	1500± 2.8**	133± 1.7**	202± 3.1**	52± 0.8**
C	1885±0.7***	1253± 2.2***	266± 2.5**	83± 2.51**	1401± 1.3**	184± 0.8**	200± 2.9**	59± 2.6**
D	2000±1.6***	1050± 1.6***	546± 2.8**	44± 0.9**	1518± 2.1**	385± 1.4**	125± 3.7**	42± 0.4**
E	1498±1.2***	886± 2.4***	503± 4.1**	60± 0.8**	1233± 3.2**	213± 2.8**	220± 4.2**	42± 0.4**
F	1751±1.2***	1235± 0.8***	400± 2.6**	53± 1.7**	1343± 1.4**	214±1.05**	138± 0.8**	48± 1.2**

Table 5: Total Viable Count (TVC) of microbes after salt water washing

Sample	Nutrient Agar		Mac-Conkey's Agar		Malachite Green Agar		Mannitol Salt agar	
	U	T	U	T	U	T	U	T
A	1838±1.8***	113± 1.2***	403± 2.5***	14± 0.7***	1116± 2.5***	102± 0.7***	248± 2.3***	40± 0.5***
B	1780±0.8***	150± 0.5**	553± 4.1***	25± 0.8**	1500± 2.8**	129± 0.8**	202± 3.1**	47± 0.9**
C	1885±0.7***	189± 1.1***	266± 2.5**	66± 1.2***	1401± 1.3**	161±1.2***	200± 2.9**	42± 1.2***
D	2000±1.6***	170± 0.8**	546± 2.8**	25± 0.9**	1518± 2.1**	372± 1.9**	125± 3.7**	33± 1.9**
E	1498±1.2***	190± 0.7**	503± 4.1**	39± 1.2**	1233± 3.2**	202± 2.3**	220± 4.2**	39± 1.3**
F	1751±1.2***	248± 0.8**	400± 2.6**	71± 1.2**	1343± 1.4**	195± 2.2**	138± 0.8**	35± 1.4**

Table 6: Total Viable Count (TVC) of microbes after hot water combined with salt water washing

Sample	Untreated	Hot water (70°C)	Salt water (4%)	Hot water + Salt water (2%)	Hot water + Salt water (4%)	Hot water + Salt water (6%)	Hot water + Salt water (8%)	Hot water + Salt water (10%)
A	1838±1.8***	113± 1.2***	102± 1.2***	98±1.9*	81±1.7*	72±0.9**	49±0.4**	47±0.5**
B	1780±0.8***	150± 0.5**	127± 0.5***	120±1.5**	80±0.7**	67±2.5**	44±0.9**	40±1.4**
C	1885±0.7***	189± 1.1***	101± 1.1***	96±0.8**	87±0.5**	75±0.8**	33±0.5**	33±0.3**
D	2000±1.6***	170± 0.8**	123± 0.8**	94±0.9**	60±0.8**	49±0.9**	19±0.6*	18±0.7*
E	1498±1.2***	190± 0.7**	139± 0.7**	101±1.8**	74±1.2**	61±0.8**	29±0.7**	26±0.8*
F	1751±1.2***	248± 0.8**	205± 0.8**	167±2.1**	100±1.8*	93±0.5**	49±0.9**	47±0.6**

4. CONCLUSION

Recent food borne illness outbreaks have revealed links between pathogens and some green vegetables such as lettuce, coriander and cucumber and their RTE salads since fresh produce carry the potential risk of microbiological contamination due to failure during harvesting, handling, processing and packaging. Considering this, common salad vegetables were chosen and analyzed in the laboratory in defined manner which revealed the abundant growth of gram positive bacteria compared to their gram negative counterparts. Appropriate washing can limit the risk of food associated disorders consequent to vegetable consumption by reducing pathogen levels. In this present work, four different washing methods, (Hot water, salt water, tap water, vinegar) were evaluated for their efficiency in reducing pathogenic levels. Results showed that washing with Hot water (70ºC) and salt water (8%) was the most effective method in achieving the above. This highlights the importance of safe washing practices to circumvent vegetable contamination by food borne microorganisms. This manuscript summarizes current observations concerning contaminated green vegetables and their RTE salads as important vehicles for the transmission of foodborne pathogens in humans and highlights the importance of appropriate washing in controlling the same.

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