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Evaluation of different detachment methods for the bacterial recovery from parsley surface

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Abstract

Reliable quantification of microbial growth in food samples has typically been difficult primarily due to the complicacy of food matrices. Therefore, initial sampling and resuspension procedures become critical to the subsequent determination of microbial load. This paper presents a comparison of bacteria recovery methods. Vortexing, homogenization with ultraturrax and stomaching methods were used to remove cells from the parsley surface. For this purpose three types of parsley (the small, curly and large leaf structure) were inoculated with non-pathogenic *Escherichia coli* ATCC 25922 strain by the initial levels of 9.5, 6 and 3 log cfu/ml bacterial culture. Detachment methods were applied and all methods yielded the approximately equal number of bacteria for each inoculation level. Means of recovered bacteria number were found as 8.1, 6.1 and 2.6 log cfu/g, respectively. It was concluded that *E. coli* was recovered in large amounts using all methods. Additionally, total number of aerobic bacteria and total coliform bacteria counts of parsley samples which were purchased from retail markets were determined for sixteen different samples and found as approximately 5.3 cfu/g log level.

Key words: Parsley, *Escherichia coli* ATCC 25922, bacterial recovery, detachment methods.

Introduction

Fresh-cut products were called lightly or minimally processed products and recognized as an important source of nutrients, vitamins and fibre for humans. Consumption of fresh produce has increased over the past two decades. Consumers are more concerned about staying healthy and eating correctly, and in response to this demand a large variety of domestic and imported produce has become available in all seasons [12, 13, 16].

Outbreaks attributed to foods generally consumed raw caused higher hospitalization rates than those attributed to foods generally consumed after being cooked [7]. Since most fresh produce receives minimal processing and is often eaten raw, pathogen contamination can represent a serious risk. Leafy vegetables including parsley were reported as the food products with the highest priority due to their high contamination risk with pathogenic microorganisms[6]. Contaminated fresh parsley is linked to outbreaks of *Shigella sonnei*, enterotoxigenic *Escherichia coli* [11] and verotoxinogenic *Citrobacter freundii* [19]. *Escherichia coli* is among the most commonly isolated pathogens associated with fresh fruits and vegetables. Outbreaks of *Escherichia coli* infections associated with ready-to-eat salads have been occurring with increasing frequency in recent years [20].

The significant increase in the analysis of foodborne pathogens caused by contaminated minimally processed produce in recent years has become of extreme importance [3, 14]. The extensive knowledge of accurate and reliable methods to determine actual quantification of microbial load is crucial for the food industry. Ensuring microbial safety of fresh produce requires efficient recovery, detection and enumeration methods. The choice of the optimal detachment method depends critically on the type of food sample for recovering pathogenic microorganisms. Methods for bacterial recovery need to be carefully chosen such that detachment efficiencies are maximized while cell damage is kept to a minimum [2, 3, 10, 15, 17].

Sanchez *et. al.* [17] focused on the importance of development of a rapid, sensitive, quantitative, and cheap analytical procedures to recover and detect the most relevant foodborne pathogens in fresh vegetables. However, there is a need for the evaluation of different methods for accurate enumeration of pathogens in order to ensure food safety. Kim *et al.* [3] compared the effect of the spindle and stomacher for detaching microorganisms from fresh vegetables. Correlations between the two methods of the spindle and stomacher were very high so that, they suggested that the spindle apparatus can be an alternative device for detaching microorganisms from all fresh vegetable samples for microbiological analysis by the food processing industry.

Kisluk *et al.* [10] previously researched the quantification of low and high levels of *Salmonella enterica* serovar Typhimurium associated with fresh parsley leaves. Recovery of *S.* Typhimurium

from parsley was conducted by mechanical detachment using stomacher, mortar and pestle, vortex, sonicator or homogenizer. So far, in the literature there has been no research on *E. coli* ATCC 25922 recovery on parsley therefore, the aim of this research was to examine the ability of commonly used methods (stomaching, vortexing and homogenizing with ultraturrax) for maximum recovery of *E. coli* ATCC 25922 bacteria inoculated on three types of parsley leaves (curly, small and large flat). Additionally, natural microbial loads of parsley purchased in Mersin, Turkey, were determined.

Material and Methods

Materials: Three types of parsley were used as curly leaf, small and large flat leaf parsley and purchased from local markets in Mersin, Turkey. *Escherichia coli* ATCC 25922 strains were loaded on the parsley. *Escherichia coli* ATCC 25922 is non-pathogenic specie of *E.coli* that is usually used as a surrogate of *E.coli* O157:H7 the cause of high pathogenicity [5].

Culture preparation: *E.coli* ATCC 25922 strains were inoculated in 10 ml of Tryptic Soy Broth (TSB) from stock culture and incubated for 18 h at 37°C. After incubation second transfer was done to 10 ml of sterile TSB (second passage) and incubated for 8 h at 37°C. In order to prepare the final culture suspension, activated *E. coli* ATCC 25922 was inoculated in 300 ml of sterile TSB and incubated for 18 h at 37°C. Final concentration of culture suspensions was 10^9 cfu/ml (This culture was also used as active stock culture).

Bacterial inoculation on parsley: Firstly parsley was washed to clean it from coarse dirt with tap water and dipped into 5% acetic acid solution for 30 min for microbial decontamination. Microbiologically free parsley was washed with sterile water and immersed in sterile water for 10 min and left to dry in sterile laminar air flow cabin for 1 h. When the parsley was dried, 10 g of parley was weighed and inoculated by immersion in 300 ml of *E.coli* ATCC 25922 culture suspension for approximately 10 min. Samples were removed and transferred to baskets in sterile laminar air flow cabin for 1 h to dry. To recover the *E.coli* ATCC 25922 from parsleys, three different methods were used.

Stomacher: Ten g of bacteria inoculated parsley was placed into stomacher bag with 90 ml dilution liquid (0.1% peptone +0.1% Tween80). Bags were pummeled in stomacher for 3 min at normal speed and these homogenized liquids were the first dilutions of the samples and serial dilutions were performed. For each dilution, standard spread plate was applied like 0.1 ml of liquid was plated directly on Violet Red Bile (VRB) agar (Merck 1.01406). All agar plates were incubated at 37°C for 24 h. Bacterial count was calculated as colony forming unit per gram of parsley (cfu/g parsley).

Vortex: Ten g of bacteria inoculated parsley was transferred into Erlenmeyer flasks with 90 ml dilution liquid and shacked on Vortex at 1000 rpm for 5 min. These liquids were the first dilution and serially diluted and spread method was applied on VRB agar. The incubations were done at 37°C for 24 h.

Ultraturrax: Ten g of bacteria inoculated parsley was transferred into a sterile glass bottle. After adding 90 ml of dilution liquids to bottles, homogenization was applied by ultraturrax at 9500 rpm for 30 s. The suspensions were filtered by sterile gauzes and the supernatants were serially diluted. Spread method was applied to dilutions and incubated at 37°C for 24 h.

Different bacterial culture and sample concentration: Activated stock culture concentration was determined as 10^9 cfu/ml. To obtain different culture concentrations, activated stock culture was diluted by sterile TSB to 10^6 and 10^3 cfu/ml. Inoculation procedure was carried out for all types of parsley with 10^9, 10^6 and 10^3 cfu/ml culture concentrations as explained in bacterial inoculation on parsley section. Detachment was done by stomaching as it is explained above. Small flat leaf parsley was weighed at 1, 5, 10 and 25 g. Samples were inoculated with 10^9 and 10^6 cfu/ml culture concentrations to analyze the effect of the sample amount. Homogenization was done by stomacher and spread method was applied. All the plates were incubated at 37°C for 24 h.

Determination of total bacterial load of parsleys: Sixteen different parsley samples were purchased randomly from local markets at Mersin, Turkey. A ten gram sample was placed into stomacher bag and homogenized with 90 ml of dilution liquid in stomacher for 30 s then serially diluted. Speared method was applied to all dilutions on Plate Count Agar (PCA, Merck) for Total Aerobic Mesophilic Bacteria (TAMB) count and on VRB agar for coliform group bacteria count. Incubations were done at 37°C for 24 h.

Statistical analysis: Experiments were conducted at least three independent times in duplicates. One-way analysis of variance (ANOVA) was performed with the software IBM SPSS Statistics 20. Statistical analysis was run with a confidence level of 95%. Comparisons between methods were evaluated with the Tukey test.

Results and Discussion

Raw eaten or mildly treated products have potentially high risk to be contaminated with foodborne pathogens including *Salmonella*, *Shigella*, *E. coli* O157:H7, *Listeria monocytogenes* and *Campylobacter* during growth, harvest, transport and further processing and handling. Therefore, the removal of potentially hazardous microorganisms from the vegetables is vital and to determine the actual microbial amount is very important. With this aim, accurate methods were investigated for the recovery of *Escherichia coli* from parsley samples.

Eblen *et al.* [5] studied the growth and survival characteristics of 15 nonpathogenic generic *Escherichia coli* strains and one nonpathogenic *E. coli* O157:H43 strain in five different growth media. These strains' growth and survival characteristics were reported to be similar to the pathogenic *Salmonella* and *E. coli* O157:H7 strains. Among these strains of *E. coli, E. coli* ATCC 25922 had the most similar thermal-kinetic properties to *Salmonella* and *E. coli* O157:H7 [5]. Kim and Harrison [21] also studied with non-pathogenic *E. coli* and pathogenic *E. coli* O157:H7 strains based on cryotolerance, cell surface characteristics (hydrophobicity, zeta potential, and morphology) and connection to the lettuce. The results of this study indicated that *E. coli* ATCC 25922 was a useful surrogate for *E. coli* O157:H7 for surface penetration studies. Hence, *E. coli* ATCC 25922 strain (instead of pathogenic

coliforms) was chosen through the culture preparation and parsley surface inoculation stages.

Recovery of bacteria from parsley leaves: Enumeration of bacteria adhered to food surfaces accurately requires using a proper procedure for detachment from the food matrix. This research focused on commonly used methods (vortexing, stomaching and homogenizing with ultraturrax) and compared their ability to detach and recover *E. coli* from parsley leaves. Large, curly and small leaved samples were inoculated with *E.coli* ranging between 10^3 to 10^9 cfu/ml. Recovered bacteria were plate counted after the detachment process. Table 1 shows that, *E. coli* could be recovered with all methods substantially. Although, there was no significant difference statistically between the detachment methods and types of leaf ($p<0.05$), *E.coli* ATCC 25922 recovery from curly leaf parsley with vortexing was slightly but not significantly higher than the other recovery methods for 9 log bacterial inoculum.

Moreover, there was no significant difference in the number of microorganisms recovered by the three methods statistically; stomaching could be recommended as the most practical method for recovery of microorganisms. This is due to the inoculated sample that can be collected directly into sterile processing bag. This method is relatively timesaving and disposal of the bagged samples is simple [4]. Both the vortexing and homogenization with ultraturrax require sterilization and decontamination of containers. Additionally vortexing does have the disadvantage of contacting of all sample surface with the dilution liquid due to non flexible structure of Erlenmeyer flasks which may be inappropriate for large volume samples. Difficulties of ultraturrax homogenization include roughing filtration of homogenized sample. Low bacterial recovery may be due to bacterial injury resulting from the high speed of homogenization with ultraturrax, occasionally [18].

Kisluk *et al.* [10] researched on quantification of low levels of pathogens. Recovery of *S.* Typhimurium from parsley by mechanical detachment using stomacher, mortar and pestle, vortex, sonicator or homogenizer followed by plating resulted in underestimation with less than 1% recovery when leaves were inoculated with 3.5-6.5 log CFU/g. Lower levels were undetectable by most assayed methods. Direct plating following detachment with mortar and pestle gave more repeatable results. However, this was reported as a time consuming method and cannot be used for large numbers of samples. On the contrary, in our study bacterial recovery was very high and there was no significant difference between detachment methods.

Effect of different bacterial culture and sample concentration: Decontaminated parsleys were inoculated with 9.5 - 6 and 3 log cfu/ml *E.coli* bacterial cultures then 8.1, 6.1 and 2.6 log cfu/g bacteria were recovered from parsley leaves, respectively. (The reason of high recovered level for 6 log inoculums could be plate counting).

Although detection of the low level of microorganisms in food matrices is difficult, high proportion of recovery was obtained for low level culture concentration. When the samples were contaminated by 6 and 3 log cfu/ml bacterial suspensions, detached levels were 6.1 and 2.6 log cfu/g, respectively. On the contrary, at high culture concentration (9.5 log), recovery was lower (8.1 log). Based on these results, it can be assumed that the surface of samples could have reached a saturation point to attach the microorganisms. The number of attached cells increased with culture concentration, until the attachment surface approached saturation.

The effect of sample amount on number of recovered bacteria was investigated. For this purpose small flat leaved parsley was weighed as 1, 5, 10 and 25 g and inoculated with 10 and 6 log bacteria, bacterial recovery was evaluated and the results are shown in Fig 1.

Logarithmic number of detached bacteria was increased by increasing sample weight. Measured bacterial numbers per unit weight were equal for all sample amounts and as a consequence

Table 1. Recovery of *E. coli* ATCC 25922 from parsley surface by different detachment methods.

Approximate inoculation level (log cfu/ml)		9.5	6	3
		Recovery level (log cfu/g)		
Large flat leaf parsley	Vortex	8.2±0.06	5.4±0.08	3.1±0.10
	Stomacher	7.8±0.46	6.6±0.20	2.8±0.14
	Ultra turrax	7.7±0.23	6.3±0.07	2.5±0.28
Curly leaf parsley	Vortex	8.5±0.15	5.7±0.07	2.7±0.34
	Stomacher	8.4±0.12	6.3±0.20	2.8±0.10
	Ultra turrax	8.3±0.35	6.4±0.07	2.0±0.00
Small flat leaf parsley	Vortex	8±0.40	5.6±0.08	2.7±0.00
	Stomacher	8±0.36	6.4±0.03	2.3±0.00
	Ultra turrax	8±0.25	6.4±0.02	2.5±0.11

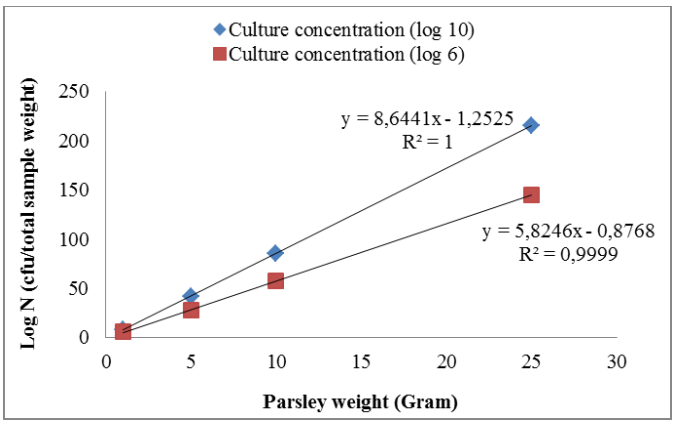

Figure 1. Effect of culture and sample concentration on bacteral recovery.

of that logarithmic number of total detached bacteria was increased linearly with increasing sample weight for both two culture concentrations.

Total bacterial load of parsleys: Natural microflora of samples was evaluated in terms of TAMB and total coliform bacteria (TCB) counts which is the indicator of hygiene. TAMB and TCB counts were observed as 5.3±0.6 and 5.3±0.7 log cfu/g, respectively. About 82% and 50% of the parsley had TAMB and TCB counts of 5 - 6 log cfu/g, respectively. The number of aerobic bacteria in parsleys was greater than 6 log cfu/g in 12.5% of the samples. In general, our data provides consistent results with previous studies conducted by Aycicek *et al.* [1]. They examined microbial levels on parsley and stated the TAMB and TCB counts as 5.7 and 5.8 log cfu/g. respectively. on parsley. Johnston *et al.* [9] reported the level of TAMB as 5.6 log cfu/g on parsley, similarly.

The natural microbial load of parsley was between the ranges of our *E.coli* inoculation level, and the recovery of *E. coli* was high (almost all was recovered) at this inoculation level. Therefore, it can be concluded that, the natural bacterial load on parsley is estimated accurately.

Conclusions

Parsley is a topsoil crop, but due to open leaves, it could be in contact with soil and irrigation water and may contain pathogenic bacteria and therefore could represent a risk to the consumers. Therefore, efficient detachment of bacterial cells is crucial for assessing bacterial abundance. However, there is no agreement on which procedure gives the best results. We tested the effect of three detachment methods on the release of bacteria associated with three different leaf types. Stomaching, vortexing and ultraturrax homogenization methods were applied experimentally. Neither the detachment methods nor the leaf structure affected the number of bacteria recovered. However, stomacher-type blender was preferable due to practicality. The choice of the appropriate detachment device depends critically on the type of food sample. Additionally, *E coli* ATCC 25922 could be inoculated at low and high levels and almost completely recovered. At this point by using proper washing methods a large amount of bacteria could be removed from food surface easily.

References

[1]Aycicek, H., Oguz, U. and Karci, K. 2006. Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. Int. J. Hyg. Environ. Health **209**:197-201.

[2]Bower, C. K., McGuire, J. and Daeschel, M. A. 1996. The adhesion and detachment of bacteria and spores on food-contact surfaces. Trends in Food Science & Technology **7**(5):152-157.

[3]Kim, D.K., Kim, S.J. and Kang, D.H. 2015. Comparison of a four-section spindle and stomacher for efficacy of detaching microorganisms from fresh vegetables. Journal of Food Protection **78**(7):1380-1386.

[4]Donegan, K., Matyac, C., Seidler, R. and Porteous, A. 1991. Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. Appl. Environ. Microbiol. **57**(1):51–56.

[5]Eblen, D. R., Annous, B. A. and Sapers, G. M. 2005. Studies to select appropriate nonpathogenic surrogate *Escherichia coli* strains for potential use in place of *Escherichia coli* O157:H7 and *Salmonella* in pilot plant studies. Journal of Food Protection **68**:282-291.

[6]FAO 2008. Microbiological Hazards in Fresh Leafy Vegetables and Herbs: Meeting Report. Microbiological Risk Assessment Series No. 14, FAO, Rome.

[7]Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M. and Gould, L. H. 2015. *Escherichia coli* O157 outbreaks in the United States, 2003–2012. Emerging Infectious Diseases **21**:8.

[9]Johnston, L. M., Jaykus, L. A., Moll, D., Martinez, M. C., Anciso, J., Mora, B. and Moe, C. L. 2005. A field study of the microbiological quality of fresh produce. Journal of Food Protection **68**(9):1840-1847.

[10]Kisluk, G., Hoover, D. G., Kneil, K. E. and Yaron, S. 2012. Quantification of low and high levels of *Salmonella enterica* serovar Typhimurium on leaves. LWT - Food Science and Technology **45**:36-42.

[11]Naimi, T. S., Wicklund, J. H., Olsen, S. J., Krause, G., Wells, J. G., Bartkus, J. M., Boxrud, D. J., Sullivan, M., Kassenborg, H., Besser, J. M., Mintz, E. D., Osterholm, M. T. and Hedberg, C. W. 2003. Concurrent outbreaks of *Shigella sonnei* and enterotoxigenic *Escherichia coli* infections associated with parsley: Implications for surveillance and control of foodborne illness. Journal of Food Prot. **66**(4):535-541.

[12]Nastou, A., Rhoades, J., Smirniotis, P., Makri, I., Kontominas, M. and Likotrafiti, E., 2012. Efficacy of household washing treatments for the control of *Listeria monocytogenes* on salad vegetables. International Journal of Food Microbiology **159**:247-253.

[13]Olaimat, A. N. and Holley, R. A. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiology **32**:1-19.

[14]Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P and Silva, C.L.M. 2013. Fresh fruits and vegetables - An overview on applied methodologies to improve its quality and safety. Innovative Food Science & Emerging Technologies **20**:1-15.

[15]Rodrigues-Szulc, U. M., Ventoura, G., Mackey, B. M. and Payne, M. J. 1996. Rapid physicochemical detachment, separation and concentration of bacteria from beef surfaces. Journal of Applied Bacteriology **80**(6):673-681.

[16]Rosa, C., Sapata, M. and Guerra, M. M. 2007. Chemical and sensory characteristics and microbiological safety of fresh finely chopped parsley packed in modified atmosphere. Food Control **18**:1008–1012.

[17]Sanchez, G., Elizaquivel, P. and Aznar, R. 2012. A single method for recovery and concentration of enteric viruses and bacteria from fresh-cut vegetables. Int. J. Food Microbiol. **152**(1-2):9-13.

[18]Sharpe, A. N. and Jackson, A. K.1972. Stomaching: A new concept in bacteriological sample preparation. Appl. Microbiol. **24**:175–178.

[19]Tschape, H., Prager, R., Streckel, W., Fruth, A., Tietze, E. and Böhme, G. 1995. Verotoxinogenic *Citrobacter freundii* associated with severe gastroenteritis and cases of haemolytic uraemic syndrome in a nursery school: Green butter as the infection source. Epidemiol. Infect. **114**(3): 441–450.

[20]CDC 2013. Outbreak of *E. coli* O157:H7 linked to ready-to-eat salads. Centers for Disease Control and Prevention, October 2013.

[21]Kim, J. K. and Harrison, M. A. 2009. Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to Romaine lettuce. Journal of Food Protection **72**(7):1385-1391.