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Sensitivity analysis of *Escherichia coli* and *Staphylococcus aureus* of mixed salad vegetables during washing step at packing house

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ABSTRACT

Fresh cut vegetables are a source of minerals, vitamins, and phytonutrients that are convenient foods for consumers which are following the global trend of inclination toward health food. In terms of food safety, contamination of vegetables with microorganisms can occur at multiple points along the supply chain. This study was conducted to investigate the risk factors of *Escherichia coli* and *Staphylococcus aureus* which contaminate freshly cut vegetables during production (receiving, washing, centrifuging, and packing areas) by using a statistical method for sensitivity analysis and an exposure assessment model complying with the @RiskTM software program. At washing step, the numbers of *S. aureus* found in the vegetables and water were 0.79 ±1.76 Log cfu/g and 0.68±1.52 Log cfu, respectively. For the equipment, the hand, and the table swabbing samples, the numbers of *S. aureus* were 0.48±1.07 Log cfu/25 cm^2, 1.81±1.69 Log cfu/25 cm^2, and 0.54±1.21 Log cfu/25 cm^2, respectively. An amount of *E. coli* of 0.48±1.07 Log cfu/25 cm^2 was found in the table swabbing samples at the packing area. *E.coli* and *S. aureus* were not found in any of the mixed fresh-cut salad samples; therefore, the product samples could be considered safe for consumers. The result of the sensitivity analysis showed that the temperature and pH of water samples were the important factors in the washing process. The suggested interventions included monitoring and maintaining the water temperature at 5°C; also, maintaining the pH of water between 6.5 and 7.5 could help to reduce pathogen contamination of freshly cut vegetables.

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1. Introduction

In recent years, consumers have been increasingly looking for healthy food such as freshly cut vegetables. It causes their nutritive value and convenience. However, freshly cut vegetables are prone to the feasible existence of foodborne pathogenic bacteria which cause illness, intoxication, and, sometimes, the outbreak of fatal diseases (1). Minimally processed vegetables commonly consist of fresh raw vegetables that are washed, peeled, sliced or cut, and kept under refrigeration in sealed packages without dressing (2).

Some of these steps such as peeling, cutting, slicing, washing, and packing increase the feasible of contamination of these products by foodborne microorganisms (3). During harvesting and transportation, raw vegetables may be bruised, resulting in the release of plant nutrients and, thereby, providing substrates for microorganisms present on the surface of the vegetables to grow (4). Some genera of bacteria found in salads include *Aeromonas spp., Bacillus spp., Campylobacter spp., Clostridium spp., Citrobacter spp., Escherichia coli, Listeria, Leuconostoc spp., Pseudomonas spp., Shigella spp., Salmonella spp., Staphylococcus spp., Proteus spp.,* and *Xanthomonas spp.* (5). Because of such exposure to pathogens, vegetables

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have been associated with the outbreak of foodborne diseases in many countries (4). *Staphylococcus aureus* is an important cause of food contamination throughout the world. This bacterium can contaminate several foods which include minimally processed vegetables and processed meat products, and produce several types of enterotoxins (6). *E. coli*, as an enteric pathogen causing hemorrhagic colitis, is becoming increasingly important from public health, particularly the psychotropic strain *E. coli O157: H7* which can grow on fresh-cut vegetables and processed meat products at 4–12°C (7). The existence of pathogens such as *S. aureus* and *E. coli* in ready-to-eat vegetable salads which are eaten without further cooking could be a microbial risk for consumers (3), so enumeration of pathogenic microorganisms, such as was done in this study, is necessary for quantitative risk assessment.

2. Materials and methods

2.1. Freshly cut vegetable and swabbing samples

All samples were collected at Thai Royal Project Packing House, Chiang Mai province, Thailand for measurement of risk of hygienic bacteria indexes such as *E. coli* and *S. aureus*. Mixed freshly cut vegetable samples (red oak leaf, green oak leaf, red loose-leaf lettuce, head lettuce, loose-leaf lettuce, butter head lettuce) at receiving (n=5), washing (n=5), centrifuging (n=5), and packing (n=5); water samples at washing (n=20) were collected at the processing plant. The process flow diagram of the production was shown in Figure 1.

Figure1. Process flow diagram of mixed salad production

The swabs were sterile cotton screw-capped plastic tubes ready for use. Buffered peptone water 1% was used as rinsing and diluents fluid. The solution was distributed to small heat resistant screw-capped tubes, each containing 10 ml of rinsing fluid, and then sterilized in the autoclave at 121°C for 15 min. Equipment swabbing by thoroughly swab a standard sample area (10 x 10 cm) (centrifuge and inside the machine) at washing (n=5 at each point), and hand swabbing at packing (n=5) were collected at the processing plant. After the sample collection, the samples were kept in iceboxes and sent to the laboratory within 2 h.

2.2. Microbiological Analysis

2.2.1. Plating media methods for *E. coli* and *S. aureus*

A quantity of 25 g of mixed vegetable samples was weighed, rinsed in a 225 ml bottle containing 225 ml of sterile 0.1% peptone water, thoroughly mixed using sterile homogenizer for 1-1.5 min, and then the suspension diluted from 1:10−2 to 1:10−4. A volume of 0.1 mL of each dilution was spread on Chromocult agar plates (Merck, Germany) and Baird Parker agar plates (BPA) (Merck, Germany) for enumeration of *E. coli* and *S. aureus*, respectively. The plates were incubated at 37°C for 24 h for the isolation of bacteria. The colony-forming units of characteristic *E. coli* and *S. aureus* were counted and reported as cfu per ml of sample dilution by using the FDA BAM, 2002 (Chapter 4) (Feng et al., 2002) and FDA BAM, 2001 (Chapter 12) (Bennett and Lancette, 2002), procedures, respectively.

2.2.2. Isolation and identification of *E. coli* and *S. aureus*

The suspected colonies of *E. coli* and *S. aureus* were identified. Positive colonies of direct plating on BPA and CA (Merck, Germany) were picked up for *E. coli* and *S. aureus* identifications, respectively. Then, the isolates were confirmed for *E. coli* and *S. aureus* by biochemical tests. The morphological typical colonies of *S. aureus* on BPA that are black colonies, shiny with narrow white margins and surrounded by clear zone were picked up. Five colonies from each sample were streaked in tryptone soy agar (TSA) slants. For colonies growing on the CA plates, the total viable counts were determined by counting both red (Coliforms) and blue (*E. coli)* colonies. Twenty presumptive colonies of *S. aureus* (two from each sample) as well as 20 presumptive colonies of *E. coli* (two from each sample) were randomly picked up and identified using the conventional method, which includes colony characteristics on selective media, gram-staining, and biochemical reactions, according to Bergey's Manual of Systematic Bacteriology (10). The biochemical tests used to confirm *S. aureus* were the coagulase test, catalase test, indole production analysis, methyl red test, Voges–Proskauer reaction, urease production test, citrate utilization analysis, and sugar fermentation test. *E. coli* were continued with several biochemical tests such as indole, citrate, and MR-VP (11) All the strains were stored at −40°C in brain heart infusion (BHI) broth (Oxoid Ltd., England) containing 20% glycerol.

2.3. Data Analysis and Model Development

Probabilistic risk assessment as the quantitative model was created to explain the propagation of *E.coli* and *S. aureus* through the various steps of processing of freshly cut vegetables, and stochastic models of *E.coli* and *S. aureus* contamination at each step were developed.

2.4. Probability distributions of the exposure assessment model

The probability distributions of the parameters for the multiple regression models were analyzed using @RISKTM software package 7.0 (student version). First, a regression analysis of the results was carried out to calculate the parameter values. In this study, the probability distributions of the data at each step were evaluated and incorporated into the model by using the @RiskTM software package version 7.0 (student version) (Palisade, Newfield, NY). The exposure assessment model was then simulated to obtain the output, the contamination of pathogen in the process. The Monte Carlo simulation (10,000 iterations) method was used to generate the input and the output values in the exposure assessment model.

2.5. Modeling with multiple regression equations

Regression analysis was employed using the technique of probabilistic sensitivity analysis demonstrated by Iman et al. (1985) and Frey et al. (2003). The distributions were used in the multiple regression models. The outputs demonstrated the contamination levels of *E. coli* and *S. aureus* of the samples. These could also be estimated. It was based upon the values of the input parameters (temperature of the water, residual chlorine in water, pH of water, and temperature of vegetable). Multiple linear regression models were developed by adding probability distributions of input and output data to a Microsoft Excel TM spreadsheet (Microsoft Corp., CA). The results of the microbiological analysis were converted into log 10 units and subjected to simple regression analysis by using the analysis tool program in Microsoft Excel TM. It normally involves fitting a relationship between inputs and outputs such as the following linear one (13). The equation is as follows:

(equation 1): Yi = bo + b1X1, i + b2X2,i + … + bmXm,i + ei ,

whereYi= ith output data point (concentrations of *E. coli* and *S. aureus* found in the samples) for the ith input data point; Xj, i= ith input data point (conditional factors of the process) for the jth input; bj= regression coefficient for the jth input; and ei= error for the ith data point. Each term of the regression model can have a different basis function that can be linear or nonlinear. For a linear model, the regression coefficient, bj, can be explained as the change in the output Yi when the input Xj, i for a given value of j increases by one unit and the values of all other inputs remain fixed (13, 14).

2.6. Sensitivity Analysis

Sensitivity analysis was carried out using the features of rank-order correlation analysis of the @RiskTM software package version 7.0 (student version). The regression sensitivity analysis employed rank order correlations based on Spearman's rank correlation calculations, a non-parametric statistic for quantifying the correlational relationship between two means. The analysis required sampling with Latin Hypercube and running by Monte Carlo simulations in which inputs were allocated probability distributions and assessing the effect of variance in the inputs on the output distribution (13). Tornado graphs could then be generated. Horizontal bars of the tornado graph represented the different input variables, with the length of the bars representing the level of correlation with the mean numbers (Log cfu per ml of sample dilution) of *E. coli* and *S. aureus* found in the samples (output variables).

3. Results

3.1. Microbiological Analysis

E. coli and *S. aureus* were not found in any sample of the mixed salad product which was collected from

Table 1. Summary of microbiological concentrations of contaminated samples at receiving, washing, centrifuging, and packing steps

Step	**Receiving**		**Washing**		**Centrifuging**		**Packing**		**Packing**	
Sample	**vegetable**		**water**		**Centrifuged arm**		**Hand swabbing**		**Table swabbing**	
m.o.	***S. aureus***	***E. coli***	***S. aureus***	***E. coli***	***S. aureus***	***E. coli***	***S. aureus***	***E. coli***	***S. aureus***	***E. coli***
Level of m.o. (Mean Log cfu)	0.79±1.76	N.D.	0.68±1.52	N.D.	0.48±1.07	N.D.	1.81±1.69	N.D.	0.54±1.21	0.48±1.07
Number of samples	5	5	5	5	5	5	5	5	5	5
Positive samples	1	0	1	0	1	0	3	0	1	1

Table 2. Summary of physical and chemical analysis of vegetable and water samples at receiving, washing, centrifuging, and packing steps

Step	Receiving	Washing		Centrifuging	Packing
Factor	**vegetable**	**vegetable**	**water**	**vegetable**	**vegetable**
Temperature (°C)	6.75–10	21.2–22.8	17.3–19.1	19.7–21.5	17.3–19.1
pH	-	-	10.33–10.81	-	-
The residual chlorine (ppm)	-	-	0.23–1.67	-	-
Number of samples	5	5	5	5	5

the processing plant (January–February 2015). As a result, information and data for developing the dose-response model were obtained from the concentration number of *E. coli* and *S. aureus* contamination on the table at the packing step as the representative of the end product. (See Table 1).

From Table 1, the mean concentrations of *S. aureus* recovered from the vegetable samples at the receiving step, water samples at the washing step, centrifuged arm swabbing samples at the centrifuging step, hand swabbing samples at the packing step, and table swabbing samples at the packing step were carried. From the results, it can be seen that the numbers of *S. aureus* found in the vegetable and the water samples were 0.79±1.76 Log cfu/g (0 – 3.93 Log cfu/g) and 0.68 ±1.52 Log cfu/ml (0 – 3.40 Log cfu/ml), respectively. As for the equipment, the hand, and the table swabbing samples, the numbers of *S. aureus* found were 0.48±1.07 Log cfu/25 cm^2 (0 – 2.40 Log cfu/25 cm^2), 1.81±1.69 Log cfu/25 cm^2 (0 – 3.24 Log cfu/25 cm^2), and 0.54±1.21 Log cfu/25 cm^2 (0-2.70 Log cfu/25 cm^2), respectively. An amount of *E. coli* of 0.48±1.07 Log cfu/25 cm^2 (0 – 2.40 Log cfu/25 cm^2) was found in the table swabbing samples at the packing area. As for the mixed salad vegetables, *E. coli* and *S. aureus* were not found in any of the freshly cut vegetable samples at the packing step.

Conditional factors of the process are shown in Table 2. The temperatures of the vegetable samples at the receiving, washing, centrifuging, and packing areas were in the ranges of 6.75–10°C, 21.2–22.8°C, 19.7–21.5°C, and 17.3–19.1°C, respectively. At the washing area, the temperature, the residual chlorine concentrations, and the pH of water were in the range ranges of 17.3–19.1°C, 0.23–1.67 ppm, and 10.33–10.81, respectively. Therefore, the only number of *S. aureus* found on the samples and the conditional factors at the washing step was analyzed and developed the probability distributions to create the simulation models.

3.2. Data Analysis and Model Development

3.2.1. Probability distributions of the exposure assessment model

Probability distributions of the parameters used for the regression model were analyzed using the Latin Hypercube method and simulated with the Monte Carlo method by using the @RiskTM software package version 7.0 (student version). The probability values of all the factors used for the sensitivity analysis are shown in Table 3.

From Table 3, the simulated probability distribution values for the temperature of the water, residual chlorine in water, pH of water, the temperature of vegetables, and concentration of *S. aureus* in the water at the washing step were simulated.

3.2.2. Modeling with multiple regression equations

The multiple regression equation was used to develop the sensitivity analysis of *S. aureus* in water used for washing at the washing step, where the equation for the multiple regression of *S. aureus* contamination in water is as follows: Y=0.00+(0.59×Time)−(3.63×Temperature of Water) −(7.78×Residual Chlorine in Water)+(0.00×pH of Water)+(3.86×Temperature of Vegetable). (Equation 2).

Table 3. Summary of simulation model of concentration of *S. aureus* at washing step

Factor	Mean	Standard deviation	Distribution	Probability
Temperature of water (°C)	22.82	1.465	Risk Normal (22.82,1.4653,RiskName("Temp.Water"))	2.02E-33
Residual Chlorine in water (ppm)	0.896	0.45885	Risk Normal (0.48, 1.0733, Risk Name ("S. aureus. Washing-equipment"))	3.70E-18
pH of water	10.572	0.20957	Risk Normal (10.572,0.20957,RiskName("pH"))	5.97E-154
Temperature of vegetables	22.08	0.75631	Risk Normal (22.08,0.75631,RiskName("Temp.Veg"))	9.45E-112
S. aureus	0.68	1.5205	Risk Normal (0.68, 1.5205, Risk Name ("S. aureus. Washing-Water"))	4.64E-03

The input parameters in the model were the temp erature of the water, residual chlorine in water, pH of water, and temperature of vegetable at the washing step. The output of the model was the concentration of *S. aureus* contamination in the water used for washing.

3.3. Sensitivity Analysis

The multiple linear regression model was used to find the factors that impact the number of *S. aureus* in the water used for washing. From the sensitivity analysis results, the risk factors associated with the samples contaminated with *S. aureus* were described. The significant factors included time and water temperature. The critical control points (CCPs) of this packing house were the washing and the packing steps. Therefore, these CCPs could be reconsidered to control the pathogens more effectively. The sensitivity analysis results suggest that the washing water temperature and the processing time should be reduced to enhance the effectiveness of chlorine (see in Figure 2).

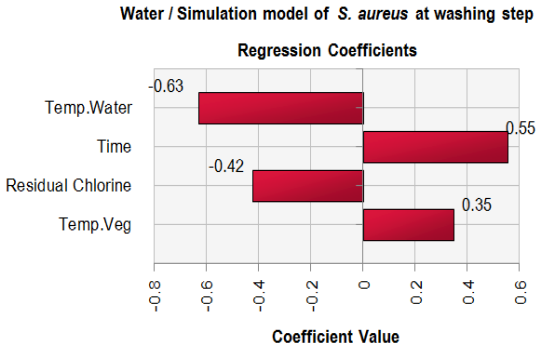

Figure 2. The sensitivity analysis of the risk factors from *S. aureus* in the water used for washing at the washing step.

Sensitivity analysis was carried out by using the @RiskTM software. The horizontal bars of the tornado graph were plotted for each input variable, with the length of the bars representing the level of correlation with the output variable.

4. Discussion

Overall, the microbial qualities of mixed salad samples were excellent. There were no any pathogens found in the samples. These results are similar to those presented in the U.S. Food and Drug Administration's survey of domestic production of the United States of America, which reported no *E. coli O157: H7* and low prevalence (,1%) of Salmonella among leafy greens by USDA, (2003). In Thailand, the Department of Health, Ministry of Public Health collected 148 samples of fresh fruits and vegetables from the fresh market in Bangkok province. All samples were contaminated with *E. coli* (13.8%), *Salmonella spp.*, (2.6%), and *S. aureus* (0.9%) as defined by Thai Department of Health (2016).

Our results show the existence of *E. coli* in the samples investigated could be as a result of fecal contamination. The bacterium is present in sewage, feces, soil, and water, and typically comes in contact with vegetables as a result of the water used for growing of vegetables (15). *S. aureus* is found in the samples of vegetables, water equipment, hand swabbing, and table swabbing could be from pre-harvest and post-harvest handling. *S. aureus* is an opportunistic pathogen found in the nasopharynx and skin of up to 50% of normal people (16). It can contaminate raw-mixed vegetable salads as a result of poor hygiene practices of farmers, retailers, and food vendors. S. aureus can spread by direct contact during harvesting, processing, packing, and contaminate of vegetables during the selling process (17). The sensitivity analysis results (see Figure 2) indicate that the conditional parameters affecting the concentration of S. aureus at the washing step were the temperature of the water, time, residual chlorine concentration, and temperature of vegetables. Only a few studies have characterized the change in microbial levels throughout the production and packaging of fresh produce. In the study of Johnston et al., 2005, A total of 398 produce samples (leafy greens, herbs, and cantaloupe) were collected through production and the packing shed and assayed by enumerative tests for total aerobic bacteria, total coliforms, total Enterococcus, and *E. coli*. The study demonstrates that each step from production to consumption may affect the microbial load of produce and reinforces government recommendations for ensuring a high-quality product. The significant intervention steps that contribute toward controlling the effect on freshly cut vegetables were water temperature and pH of water (FAO, 2004). The main decontamination method available to the fresh produce industry has been washing with potable water, often chlorinated. This washing method is routinely applied to leafy green vegetables (salad vegetables) like lettuce, especially for ready-to-eat salads, but its effectiveness is limited (5). The suggested interventions include monitoring and maintaining the water temperature at 5°C (18) and also maintaining the pH of water between 6.5 and 7.5 as these steps could help in reducing pathogen contamination of freshly cut vegetables. Additionally,

cross-contamination during production could occur due to the following reasons: using the same knife or chopping board to cut raw meat and ready-to-eat foods (salad, cooked quiche, etc.), defrosting food or placing dirty utensils and equipment in the hand wash basin, storing raw food above ready-to-eat food, etc. Washing with chlorinated water is routinely applied to leafy green vegetables (salad vegetables) like lettuce, especially for pre-packed ready-to-eat salads, but its effectiveness is limited (5). Measures to minimize the risk of microbial contamination at all points, from the field to the table, thorough good agricultural practices, good manufacturing practices, etc. would be the most effective strategy to assure that salad vegetables eaten raw are safe for human consumption.

5. Conclusion

E. coli and *S. aureus* were not found in any of mixtheed freshly cut salad samples, which is appropriate when considering the Thai Health Certification standard (less than 10 cfu/g) for fresh-cut produce (Plant Standard and Certification Division, 2013); therefore, the mixed salad product would be safe for consumers. As for sensitivity analysis, the results showed that the significant factors in the washing process included pH of water and water temperature. This finding suggests that control of pH and water temperature during the washing process is a useful practice for reducing potential contamination. However, if the quality of water is not good enough, pathogenic microorganisms can contaminate the produce.

Conflict of interest

The authors have no conflict of interest.

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