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Assessment and Reduction of Bacterial Contamination of Fresh Farm Produce

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Assessment and Reduction of Bacterial Contamination of Fresh Farm Produce

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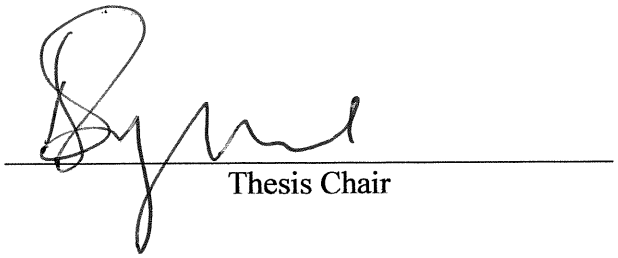

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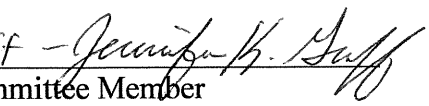
Jennifer Graff - 

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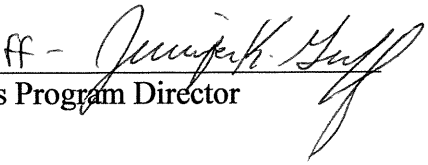
Jennifer Graff - 

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Introduction

Fruits and vegetables provide vital nutrients, vitamins, and enzymes that are essential for everyday function and a healthy lifestyle (Lassale, et al., 2016; "Getting vitamins from food (not pills)", 2011; Rodriguez-Casado, 2016). Long-term benefits of consuming fresh farm produce include protection from many chronic medical conditions and illnesses, such as diabetes, cardiovascular diseases, and cancer due to their antioxidant effects (Oyebode, et al., 2014; Rodriguez-Casado, 2016). Additionally, fruit and vegetable consumption has been shown to benefit psychological health by improving overall happiness and well-being and decreasing depression and anxiety (Conner, et al., 2017). However, these physical and psychological benefits do not come without a risk.

More than nine million foodborne illnesses are reported annually in the United States, and a startling 46% are estimated to be caused by fresh produce (Painter, et al., 2013). Multiple studies have found bacterial contamination can come from any number of places as produce is grown, harvested, stored, transported to grocery stores, displayed, and further handled in grocery stores (Heredia, et al., 2016; Rajkowski & Xuetong, 2008). For example, a study in Mexico looked into the four steps of the production process to examine bacterial contamination of fresh produce (Heredia, et al., 2016). Many studies have shown that produce harbors potentially pathogenic bacterial contamination on the surface, such as *Salmonella* and *Listeria*. *Escherichia coli* is often considered a sign of fecal contamination and a sign that pathogens may be present

(Callejón, et al., 2015; Denis, et al., 2016; Korir, et al., 2016; Painter, et al., 2013). For example, a study in the U.S. and the European Union addressed the rate of foodborne illnesses associated with fresh produce to determine the pathogens involved and the mechanism of contamination (Callejón, et al., 2015). The study found that *Salmonella* was mainly indicated as the cause of multiple outbreaks of gastroenteritis across different states in the U.S. and the patterns of outbreaks differed in the US and the European Union (Callejón, et al., 2015). Contaminated produce also increases the transmission of antibiotic resistant bacteria which pose a significant threat to public health, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Kadariya, et al., 2014; Oyebode, et al., 2014).

General household cleaning methods that have been used are a water wash which involves the mechanical removal of potentially pathogenic microbes, household bleach dilution since its active ingredient sodium hypochlorite denatures proteins, and a vinegar dilution because of its active ingredient acetic acid which also denatures proteins in pathogens. The overarching aim of this study was to develop a method to reliably assess bacterial contamination of fresh produce and evaluate the effectiveness of common household methods in reducing that bacterial contamination.

Methods and Materials

The produce selected for assessment included cucumbers, pre-packaged peaches, and pre-packaged whole mushrooms that were bought from a local commercial grocery store. Produce types were chosen based on their different surface

textures and growing conditions due to the fact that they are commonly eaten raw and are not generally peeled before consumption. Pre-packaged in this context assumes some pre-cleaning of produce. Cucumbers were chosen because of their bumpy, rugged surface that could potentially harbor more bacteria in their ridges, while also being grown low to the ground. Mushrooms were chosen due to their growth on the ground and for their smooth, delicate surface. Lastly, peaches were chosen for their fuzzy surface and their growth completely off the ground.

Three different cleaning treatments were selected based on their common household application, simplicity, and cost-effectiveness. These treatments included a water wash, diluted bleach, and diluted vinegar. For each type of produce, there was an unwashed control sample that served for comparison with the other three cleaning methods in assessing bacterial reduction. As per FDA recommendation, the water wash included holding the produce under running water for thirty seconds while gently rubbing it with gloved hands (Center for Food Safety and Applied Nutrition, 2015). The bleach dilution was made from 200 ppm of household bleach (McGlynn, 2004). Produce samples were immersed in the bleach dilution for sixty seconds and rinsed afterwards with tap water for an additional thirty seconds with gentle rubbing. Vinegar dilutions are commonly used as a household cleaning method. This was made with a 1:3 white vinegar-to-water dilution with distilled water. Produce samples were immersed in the vinegar and allowed to soak for thirty seconds. They were then rinsed under running tap water while gently hand rubbing them, for an additional thirty seconds.

For each produce type to receive each treatment equally, samples were assorted and split apart in different ways (Figure 1). Three cucumbers were used, C1, C2, and C3, and then each cucumber was cut into four equal parts. One section was used as the control while the other three sections of the sample received each of the three cleaning treatments. Three packages of peaches were referred to as P1, P2, and P3. Four peaches were picked from each bag, with one of them being held as the control and the remaining receiving treatment. There were three packages of whole mushroom that were labeled M1, M2, and M3. Four mushrooms were selected from each package with one mushroom from package acting as the control and the remaining ones receiving a different cleaning treatment. Each trial was repeated three times per produce type.

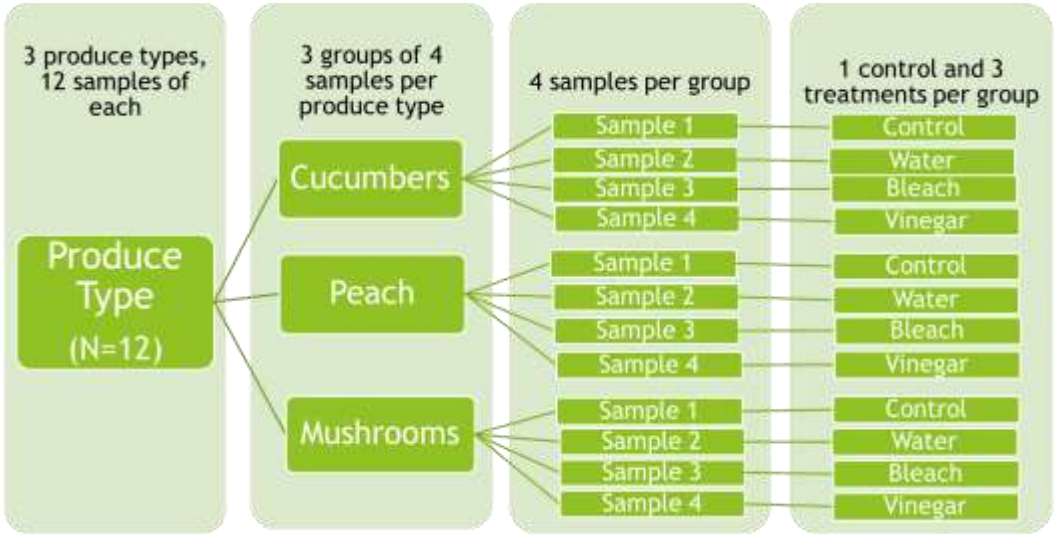

Figure 1. Each produce type was split into three groups of 4 samples per group. Each sample in each group for each produce type received either a treatment or acted as the control.

Each produce sample that was partitioned accordingly, was swabbed three times around the circumference with a cotton applicator that had been dipped in nutrient

broth. Once the samples were swabbed, the cotton applicator was set into 1 ml of nutrient broth for five minutes. Each 1 ml Eppendorf tube of nutrient broth suspension was then vortexed at 50 rpm for thirty seconds and these tubes were considered the stock bacterial suspensions. All of the stock suspensions were then diluted with a 1:100 serial dilution (Ben-David & Davidson, 2014). Three 0.1 ml aliquots of each suspension was then deposited on to three nutrient agar plates, inoculated with the spread plate technique, and then incubated for 24 hours (Lagier, et al., 2015; Sanders, 2012). Distinct colony forming units (CFU's) were counted, described and isolated after the incubation period (Figure 2). Each treatment for every produce type had a sample size of nine since each treatment was done three times per produce type.

Colony forming units per milliliter (CFU/ml) were recorded for each treatment. The effectiveness of the cleaning treatments was evaluated based on CFU/mL and compared to the control with a one-way ANOVA test using SPSS (Ben-David & Davidson, 2014; Sanders, 2012). To compare the effectiveness amongst the cleaning treatment, a Tamhane's T2 post hoc analysis was used again with SPSS (Figure 2).

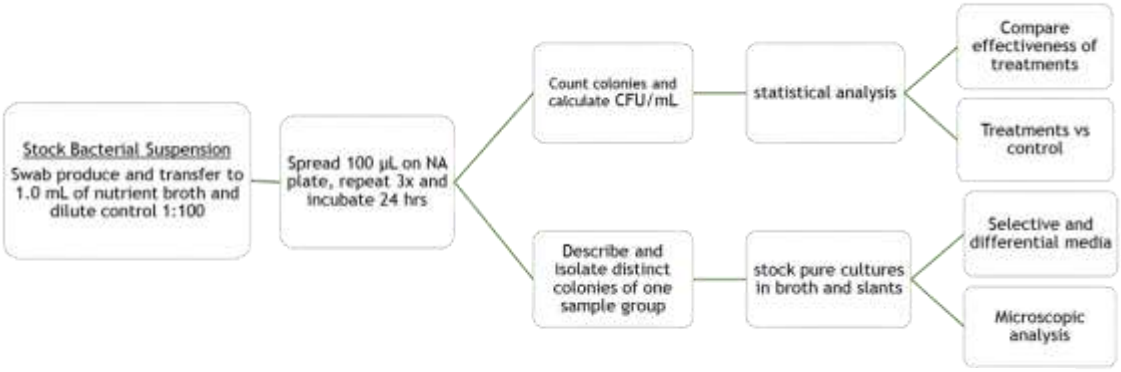

Figure 2. Produce was swabbed and placed in a nutrient broth to create the stock solution and later the dilutions. Each dilution was then plated and incubated. Colonia counts were established and statistical analysis was done to compare the effectiveness of each treatment. Distinct colonies were isolated for purity and the assessed with selective and differential media and microscopic analysis for further identification.

Distinct and different colonies that were observed were taken as representative samples from the spread plates to be isolated to test for purity (Figure 2). These colonies were described by their shape, color, elevation, and size (Bautista-Trujillo, et al., 2012; Lagier, et al., 2015). These isolates were taken from each control and treatment sample from all produce types. All isolates were incubated for 24 hours to assess purity by visual and microscopic analysis that included gram staining (Bautista-Trujillo, et al., 2012; Johnson & Case, 2015). Pure isolates were then moved using disposable sterile loops to nutrient agar slants and broths and stored in the refrigerator at 4° C to be used later on in qualitative analysis (Lagier, et al., 2015).

Isolates were qualitatively analyzed to identify the bacterial species, establish pathogenic risk, and evaluate the effectiveness of the cleaning methods in the reduction of pathogens. Different selective and differential media were used for initial characterization. Each medium was inoculated with a single line streak with a loopful of bacteria from the stored nutrient agar containing the pure colonies (Lagier, et al., 2015).

The plates were either divided in two, three, or four quadrants where each quadrant was streaked with a different isolate. Each of the isolates were tested on each medium three times to ensure greater confidence in the purity of isolates. For this study, specific bacteria of interest included *Salmonella*, and *S. aureus* because they are known for their occurrence as pathogenic contaminants of fresh produce. *E. coli* was another bacterium of interest because of its ability to indicate fecal contamination (Callejón, et al., 2015; Heredia et al., 2016; Kadariya, et al., 2014; Martin, et al., 2016; Painter, et al., 2013; Reddy, et al., 2016; Sargeant, et al., 2004).

The differential and selective media used included: Mannitol salt agar (MSA), MacConkey agar (MAC), and Eosin Methylene Blue agar (EMB). MSA was used for the identification of gram positive bacteria and to differentiate between *S. aureus* and *S. epidermis* based on mannitol fermentation (Bautista-Trujillo, et al., 2012; Johnson & Case, 2015). MAC was used for the identification of gram negative bacteria and to differentiate between *Salmonella* and coliforms based on the ability to ferment lactose (Johnson & Case, 2015; Martin, et al., 2016; Reddy, et al., 2016). EMB was also used to identify lactose fermenting gram negative bacteria and further evaluate if there was a presence of *E. coli* if a green sheen color change was observed (Johnson & Case, 2015; Martin, et al., 2016; Reddy, et al., 2016).

Results

Mean abundance of the CFU/mL for the unwashed cucumber, peach, and mushroom samples were analyzed and compared to the CFU/mL of the treated samples for each produce type. A one-way ANOVA analysis indicated that the treated sample's bacterial contamination was significantly lower than the control samples for all produce types at the critical level of p = 0.05 (Table 1).

PRODUCE TYPE	CONTROL	WATER	BLEACH	VINEGAR	ONE-WAY ANOVA
CUCUMBER	32,000	232	12	51	F=14.86 P<0.05*
PEACH	2,732	19	4	21	F=15.10 P<0.05*
MUSHROOM	92,222	13,289	272	208	F=11.29 P<0.05*

Table 1. Summary statistic of mean CFU/ml of produce types and one-way ANOVA analysis, * means significance difference between two or more cleaning methods and control of each produce type at a critical level of 0.05, n=9 for each produce type and cleaning method.

Tamhane's T2 post-hoc analysis was used to compare the bacterial loads between each treatment type within each produce type. The post-hoc analysis for the cucumber sample group indicated that the bacterial load of the cucumber control was significantly higher compared the bacterial loads found on the cucumber after each water, bleach dilution, and vinegar dilution treatment (Figure 3a). The bacterial load for the bleach treated cucumber samples was significantly lower than the water treated cucumber, but neither of these treatments were significantly different when compared to the vinegar dilution treated cucumber samples (Figure 3a). The peach control CFU/mL was significantly higher compared to the other peach treated samples consisting of the

water, bleach-dilution, and vinegar dilution treatments (Figure 3b). When each peach cleaning treatment was compared to one another, there was no significant difference found (Figure 3b). The bleach and vinegar dilutions of the mushroom samples were significantly different from each other (Figure 3c). The mushroom sample treated with water was not significantly different from the control, bleach dilution treatment, or the vinegar dilution treatment mushroom samples (Figure 3c).

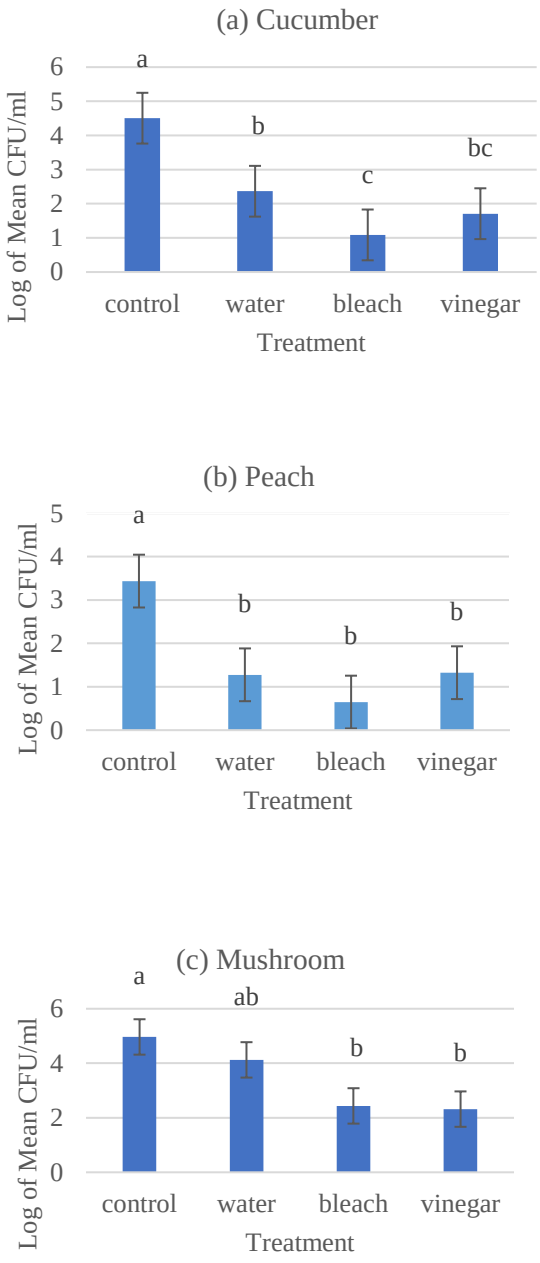

Figure 3. Comparison of cleaning methods in reducing CFU/ml of cucumber (a), peach (b), and mushroom (c) samples with Tamhane's T2 post-hoc analysis. Bars with the same letter were not significantly different at a critical level of 0.05.

From every produce sample, bacterial colonies were able to be successfully cultured and isolated. From the cucumber samples, seven individual colonies were isolated and identified as being *Staphylococcus epidermidis* or *S. aureus* by using selective and differential media, visual appearance, and gram staining techniques (Table 2). From the peach samples, nine bacterial colonies were isolated analyzed with selective and differential media and microscopic analysis (Table 3). Further identification of the peach isolates continues, but the pathogens *Salmonella, S. aureus,* and non- *E. coli* coliforms have been able to be identified (Table 3). From the mushroom samples, fourteen bacterial colonies were isolated and characterized with selective and differential media and microscopy (Table 4). Further identification of mushroom isolates continues, but so far M1 and M14 have been identified as *Bacillus cereus*. M3 and M11 are most likely to be *Salmonella* (Table 4).

CUCUMBER ISOLATES

ISOLATE IDENTIFICATION			MICROSCOPIC ANALYSIS			MSA		MAC		EMB	
ID	**Treatment Received**	**Identity**	**Gram stain**	**Arrangement**	**Motile**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**
C1	Control	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
C2	Control	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
C3	Water	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
C4	Water	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
C5	Bleach	*S. epidermidis*	+	Staphylococcus	-	+	-	-	-	-	-
C6	Vinegar	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
C7	Vinegar	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-

Table 2. Summary of cucumber qualitative results.

PEACH ISOLATES

ISOLATE IDENTIFICATION			MICROSCOPIC ANALYSIS			MSA		MAC		EMB	
ID	**Treatment Received**	**Identity**	**Gram stain**	**Arrangement**	**Motile**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**
P1	Control	*Salmonella*	-	Single bacilli	+	-	-	+	-	+	-
P2	Control		+			-	-	+	-	+	-
P3	Water	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
P4	Water		+	Streptobacillus		+	+	-	-	-	-
P5	Bleach	*S. epidermidis*	+	Staphylococcus	-	+	-	-	-	-	-
P6	Bleach	Non- *E. coli* coliform	-	Single bacilli		-	-	+	+	+	-
P7	Vinegar	*S. aureus*	+	Staphylococcus	-	+	+	-	-	-	-
P8	Vinegar		+	Bacillus	+	+	+	+	-	+	-
P9	Vinegar	*S. aureus*	+	Cocci	-	+	+	-	-	-	-

Table 3. Summary of peach qualitative results.

MUSHROOM ISOLATES

ISOLATE IDENTIFICATION			MICROSCOPIC ANALYSIS			MSA		MAC		EMB	
ID	**Treatment Received**	**Identity**	**Gram stain**	**Arrangement**	**Motile**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**	**Growth**	**Ability to Ferment**
M1	Control	*B. cereus*	+	Bacillus	+	+	-	-	-	-	-
M2	Control		+	Diplobacilli	+						
M3	Control	*Salmonella*	-	Streptobacillus	+	-	-	+	-	+	-
M4	Control		+	Streptobacillus	+	+	+	-	-	-	-
M5	Control		+	Streptococcus	-	+	+	-	-	-	-
M6	Control		+	Diplobacilli	+	+	+	-	-	-	-
M7	Control		+	Streptobacillus							
M8	Control		+	Streptococcus	-						
M9	Control		+	Diplobacilli							
M10	Water		-	Streptobacillus							
M11	Bleach	*Salmonella*	+	Diplobacilli	+	-	-	+	-	+	-
M12	Bleach		+	Streptobacillus	+	+	+	-	-	-	-
M13	Vinegar		+	Streptobacillus	+						
M14	Vinegar	*B. cereus*	+	Bacillus	+	+	-	-	-	-	-

Table 4. Summary of mushroom qualitative results.

Discussion

Even though reports of foodborne illness in developed countries have been declining, foodborne illnesses due to contaminated fresh farm produce that is consumed raw has been steadily increasing (Eylen, et al., 2007; Lassale, et al., 2016; Painter, et al., 2013; Rodriguez-Casado, 2016). Bacterial contamination of fruits and vegetables can come from any of the many stages between the field and the fork (Heredia, et al., 2016; Rajkowski & Xuetong, 2008). Potentially pathogenic bacteria on each produce type were identified and cultured from all samples. The bleach-dilution cleaning method was the most effective at disinfecting produce, followed by the vinegar dilution, with the water wash being the least effective.

When comparing microbial loads, the mushroom samples had the highest CFU/mL and the peach samples has the lowest CFU/mL. Surface texture and growing conditions of produce may have contributed to bacterial contamination. The effectiveness of each cleaning treatment varied by produce type. This indicates that the surface texture and initial microbial load impacts bacterial removal. All three cleaning treatments did effectively reduce bacterial contamination in regards to the cucumber and peach samples. For the cucumber samples, the bleach dilution treatment was the most effective and the water treatment was the least effective, with the vinegar dilution being of intermediate effectiveness. The cleaning treatments for the peach samples were equally effective, suggesting that no distinction was made between the treatments because of the low initial microbial load. For the mushroom samples, the bleach and vinegar dilution

treatments were equally effective in microbial load reduction, but the water treatment was not effective as it did not significantly differ from the control. The water treatment was not as effective due to the mushrooms fragility which did not allow effective mechanical removal of bacteria.

Pathogenic bacteria were identified and cultured from all produce samples. No notable trends were observed between the cleaning treatments and the bacteria classified, but future trials may disclose possible trends. *S. aureus, Salmonella, and B. cereus* were the pathogenic bacteria characterized and are common causative agents of foodborne illnesses (Callejón, et al., 2015; Heredia et al., 2016; Painter, et al., 2013; Reddy, et al., 2016). Fecal contamination is evident on the peach samples, due to the presence of coliforms (Martin, et al., 2016; Sargeant, et al., 2004). *S. epidermidis* is non-pathogenic and part of the regular flora of humans and was identified on the peach and cucumber samples (Davis, 1996), suggesting human handling as a possible source of contamination.

Reducing pathogenic contamination of fresh produce is crucial and effectively accomplished with cost-effective cleaning methods as the trends and findings of this study has shown. This study was limited in its findings due to unidentified bacteria in our mushroom samples. Future work would involve complete specific strain identification via molecular methods such as the polymerase chain reaction, and expanded testing and cleaning to include other fresh farm produce that is normally consumed raw such as tomatoes, lettuce, and grapes.

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