Quantification of transfer of Salmonella from citrus fruits to peel, edible portion, and gloved hands during hand peeling

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Placeholder Title: Quantification of transfer of *Salmonella* from citrus fruits to peel, edible portion, and gloved hands during hand peeling

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ABSTRACT (2000 characters allowed)

Although studies have quantified bacterial transfer between hands and various materials, cross-contamination between the surface of fresh citrus fruit and the edible portions during hand peeling has not been reported. This study quantifies transfer of *Salmonella* to the edible portion of citrus fruit from a contaminated peel during hand peeling. Citrus fruits used for this study were *Citrus sinensis* (sweet orange) cultivars ‘Valencia’ and ‘Navel’ and *C. unshiu* (Satsuma mandarins), *C. reticulata* × *C. paradisi* (‘Minneola’ tangelo or ‘Honeybell’) and *C. paradisi* (grapefruit) cultivar ‘Marsh'. An avirulent *Salmonella Typhimurium* LT - 2 (ATCC 700720) resistant to rifampicin was used for all experiments. The inoculum containing ~9 log CFU/ml (50 µl) was spot inoculated onto the equator, stem or styler of each fruit and allowed to dry for 24 h. Six volunteers put on single use latex gloves and peeled inoculated fruit. Peel, edible fruit portion and gloves were collected and enumerated separately. Three replicates of the study were performed where each volunteer peeled 2 inoculated fruit of each variety (n=36 fruit per variety). Cross-contamination from contaminated surface of citrus fruits to edible portion or gloved hands during peeling was affected by inoculation sites. Average *Salmonella* transfer to the edible portion ranged from 0.16 % (Valencia inoculated at the equator) to 5.41 % (Navel inoculated at the stem). Average *Salmonella* transfer to gloved hands ranged from 0.41 % (Grapefruit inoculated at the stem) to 8.97 % (Navel inoculated at the stem). Most *Salmonella* remained on the peel of citrus fruits. The average level of *Salmonella* remaining on the peel ranged from 5.37% (Minneola inoculated at the equator) to 66.3% (Satsuma inoculated at the styler). When grapefruit was inoculated, the *Salmonella* that remained on the peel showed a bimodal pattern where some individuals left almost all *Salmonella* on the peel, while others left substantially less.

Foodborne outbreaks associated with fresh produce have increased worldwide as consumption of fresh produce has also increased (*3*, *11*, *16*, *20*, *27*). While no outbreaks have been directly linked to whole fresh citrus fruit at this time, unpasteurized orange juice has been implicated in several outbreaks caused by *Salmonella*, Enterotoxigenic *E. coli*, *Shigella*, hepatitis A virus, and Norovirus (*6*, *8*, *14*, *18*). The majority of outbreaks involving fruit and fruit juice have been attributed to pathogens contaminating the outer skin or rind, although the peel or rind of many fruits is discarded by the consumer and not eaten (*11*, *12*, *25*).

The FDA Food Safety Modernization Act (FSMA) includes fresh produce safety in its scope and focuses on preventing contamination during the production and harvesting of fresh fruits and vegetables (*10*). However, fresh produce including fresh citrus fruits can become contaminated at numerous points during transport, distribution, retailing and food preparation in the kitchen environment as well as production and harvesting (*5*, *16*, *23*, *27*).

Cross-contamination from the surface of fresh produce to edible portions during cutting, slicing, or peeling can occur if the outer skin or rind of fresh produce is contaminated by pathogens (*12*, *18*, *20*, *25-27*). Bacterial transfer from the skin to the edible flesh has been shown to occur during cutting of tomato and cantaloupes, both of which have implicated as the source of infection in some outbreaks (*4*, *12*, *26*). The surface of fresh citrus fruit has also been a source of pathogens, which can be transmitted to the juice during squeezing or peeling. Martinez-Gonzales et al. (*17*) reported that cross-contamination from inoculated fresh orange skin to utensils used in orange juice squeezing occurred, which subsequently resulted in bacterial transfer from contaminated utensils to squeezed orange juice.

Although studies have been conducted to determine the various factors that influence the bacterial transfer between hands and various surface materials (*7*, *15*, *19*), cross-contamination between the surface of fresh citrus fruit and the edible portions during hand peeling has not yet been reported. While a knife or citrus peeler might be used in commercial setting, this study was undertaken to quantify transfer of *Salmonella* to the edible portion of citrus fruit from a contaminated peel that can occur during hand peeling of a single fruit, as might occur in the home. Transfer rates were determined between various inoculation locations on the citrus fruits to the edible portion and gloved hands.

MATERIALS AND METHODS

Preparation of samples. Citrus fruits selected for this study were *Citrus sinensis* (sweet orange) cultivars 'Valencia' and 'Navel' and *C. unshiu* (Satsuma mandarins) grown in California, *C. reticulata* × *C. paradisi* ('Minneola' tangelo or 'Honeybell') grown in Florida and *C. paradisi* (grapefruit) cultivar 'Marsh' grown in Texas. Satsuma mandarins were shipped from a California packing facility; all other varieties were purchased from a local supermarket (Winter Haven, FL). The citrus fruits were stored at 4 °C until use, and left overnight at ambient temperature (ca. 20 °C) prior to inoculation.

Preparation of cultures. An avirulent *Salmonella* Typhimurium LT - 2 (ATCC 700720) isolate was used for all experiments to minimize risk to study participants. *Salmonella* was made resistant to 200 µg/ml rifampicin (Thermo Fisher Scientific, Waltham, MA) by serial exposure to increasing (1:2) concentrations of rifampicin in tryptic soy broth (TSB; Difco Becton Dickinson & CO., Sparks, MD) incubated at 37±2°C for 24 ± 2 h (*21*).

A frozen culture of *Salmonella* Typhimurium LT-2 was streaked onto tryptic soy agar supplemented with 100 µg/ml rifampicin (TSAR; Difco Becton Dickinson & CO), and incubated at 37±2°C for 24 h prior to each experiment. An isolated colony was transferred to 10 ml of TSB supplemented with 100 µg/ml rifampicin (TSBR), and incubated at 37 ± 2°C for 24 h. The overnight culture was sub-cultured twice by transferring 0.1 ml to 10 ml fresh TSBR and incubated at 37±2°C for 24 h. The culture was centrifuged at 2,095 × g for 10 min (Allegra X-12, Beckman Coulter, Fullerton, CA). Cells were washed twice by removing the supernatant and suspending the cell pellet in 10 ml of 0.1% peptone (Difco, Beckton, Dickinson & CO.). Washed cells were suspended in half the original volume of 0.1% peptone and diluted to achieve ~ 9 log CFU/ml.

Inoculation of samples. The citrus fruits were held stable by 4 cm diameter plastic rings in a biosafety cabinet. The inoculum (50 µl) was spot inoculated onto either the equator, stem or styler of each fruit and allowed to dry for 24 h with the biosafety cabinet fan running. This inoculation procedure resulted in ~6 log/CFU per fruit after drying. Although unlikely to occur frequently in real world situations, these high initial concentrations are needed to ensure detectable colony counts after cross-contamination.

Cross-contamination during peeling. Six staff members, three women and three men, from the Citrus Research and Education Center put on single use, disposable latex gloves (Thermo Fisher Scientific) and were instructed to peel the inoculated fruit in the same manner as they would at home. Although an avirulent *Salmonella* strain was used (see above), gloves were used to further reduce risk to study participants. Each participant was informed as to the general peeling and sample collecting procedures prior to experiments. Each volunteer was given two inoculated fruit, the first was placed whole into a sterile 532 ml Whirl-pak bag (Nasco, Fort Atkinson, WI) and served as a positive control. Gloves used to handle the whole fruit were removed and discarded. Fresh gloves were donned and a second fruit was given to the volunteer to peel. The peel, edible fruit portion and gloves from the second peeling were each collected into separate sterile 532 ml Whirl-pak bags. For 'Navel' oranges, the navel portion of the fruit was collected and enumerated separately. This procedure was also repeated for uninoculated fruit to serve as negative controls. Three replicates of the study were performed; each volunteer peeled 2 inoculated fruit of each variety (n=36 fruit per variety).

***Salmonella* Recovery.** Fifty milliliters of Dey-Engley (DE) neutralizing buffer (Remel Products, Thermo Fisher Scientific, Lenexa, KS) were added to each bag containing peel, edible portion, or gloves to enhance recovery of cells and to ensure that any antimicrobials present were neutralized. Samples were massaged by hand for 30 s. Samples were serially diluted in 0.1 % peptone and 0.1 ml of appropriate dilutions were spread plated onto TSAR. Plates were incubated for 24 h at 37±2°C, bacterial colonies were counted and log CFU/fruit were calculated.

Data analysis. Individual transfer rates for each experiment were calculated using the following equation:

$$\text{Transfer rate (\%)} = \left(\frac{\text{CFU measured on peel, edible portion, or gloved hands}}{\text{CFU measured on inoculated whole fruit}}\right) \times 100$$

Transfer rates between samples were logarithmically transformed as recommended by Schaffner (*24*) and then frequency histograms were created using Excel (Microsoft, Redmond, WA) and SigmaPlot (Systat Software Inc., San Jose, CA). Standard deviations, median, maximum, minimum and ranges were also calculated.

Average log percent transfer rates were analyzed using Tukey's HSD test (SAS University Edition; SAS Institute Inc., Cary, NC, USA) to determine statistically significant differences between citrus varieties and *Salmonella* transfer to edible and gloved hands of the individual doing the peeling. Note that because of the effect that log transformation has on calculation of the means, average relative transfer rates may not show the same relationship after transformation.

RESULTS

Transfer patterns (log percent transfer) of *Salmonella* from the stem, equator and styler inoculation sites on the peel of citrus fruit varieties to the edible portions and gloved hands of the individual doing the peeling are shown in Figures 1-5. Transfer rates have also been characterized using six different statistical parameters (mean, standard deviation, median, maximum, minimum and range) as presented in Table 1 to provide a more detailed summary of the results.

During the peeling of 'Valencia' fruits, the majority of *Salmonella* remained on the peel (Figure 1) from all inoculation locations. Note that in the Valencia experiments, participants separated the edible portions into two halves, but the differences were not statistically significant. We combined these data and report 72 edible portion observations in Fig 1. Log percent transfer of *Salmonella* inoculated onto the stem or styler to edible portion of the fruit was significantly higher than from the equator to the edible portion ($P<0.0001$). Whether inoculated at the stem, equator, or styler, the log percent transfer of *Salmonella* to gloved hands were not significantly different ($P=0.077$). When the styler was the source of the contamination, the level of *Salmonella* that remained on the peel was more variable, as evidenced by the flattening of the distributions shown in Figure 1C. The highest percentage of *Salmonella* remaining on the peel portion was observed when the stem was the source of contamination (Table 1).

When 'Navel' oranges were peeled (Figure 2) as with Valencia oranges shown in Figure 1, relatively higher level of *Salmonella* remained on the peel of Navel compared to Valencia oranges. When inoculated at the stem, equator, and styler on Navel, the log percent transfer of *Salmonella* to the gloved hands and the edible portions were not significantly different. Table 1 shows the percent transfer from the previously defined inoculation locations to the navel portion. The mean and standard deviations shown in Table 1 for Navel oranges support the observation from Figure 2C, that when the styler was inoculated on Navel orange, a significant portion of the contamination remains with the navel portion (P<0.0001).

Salmonella transfer from inoculated 'Satsuma mandarin' fruit to the edible portions and the gloved hands of person peeling fruit is shown in Figure 3. When the stem, equator, and styler were the source of contamination, the log percent transfer to the edible portion was not significantly different, while transfer to the gloved hands did showed a significant difference (P<0.0001). Higher bacterial transfer to the gloved hands occurred when inoculated at the equator or the styler than at the stem portion. The levels of *Salmonella* that remained on the peel of Satsuma mandarin was highest among all citrus varieties during hand peeling no matter whether the fruit was inoculated on the stem, equator, or styler. This may be due to the easy-to-peel nature of Satsuma mandarins we observed compared to the other citrus varieties.

When 'Minneola' tangelo was inoculated (Figure 4), the highest log percent transfer of *Salmonella* to the edible portion was from the styler, followed by the stem, equator portion (P<0.0001). Log percent transfer to the gloved hands was also significantly high in the styler (P<0.0001). The *Salmonella* transfer distributions for the Minneola experiments are characterized by rather broad distributions with wide variability and low frequency peaks (Figure 4). The *Salmonella* that remained on the peel portion was highest when the stem was inoculated, followed by when the styler was inoculated and when the equator was inoculated, showing similar pattern to the other citrus varieties except Satsuma mandarins.

Data for transfer of *Salmonella* from the different inoculation locations on 'Marsh' grapefruit to the edible portion and gloved hands during hand peeling is presented in Figure 5. There is no significant difference in bacterial log percent transfer to the edible portion no matter where the grapefruit is inoculated. However, when the equator was the source of contamination, the highest transfer to the gloved hands was observed, followed by the styler, and stem portions, and the differences were significant (P<0.001). No matter where the grapefruit was inoculated, the *Salmonella* that remained on the peel showed an interesting bimodal pattern, where on some occasions almost all the *Salmonella* (100% or 2 log percent) remained on the peel as shown in Figure 5. The reason for this bimodal pattern is not clear, but it may be due to the large size of the grapefruit, and may represent different peeling strategies of the volunteers observed.

The highest log percent transfer of *Salmonella* to the edible portion was found from the styler of all citrus fruits, followed by the stem and the equator portion (P=0.0001). As noted above, the navel portion (present in Navel oranges only) also had the highest log percent transfer when the styler was inoculated. The log percent transfer of *Salmonella* to the edible portion on Satsuma mandarin (Figure 4) was noticeably higher than in the other citrus varieties (P<0.0001). The significantly higher log percent transfer of *Salmonella* to the gloved hands was observed in Minneola tangelo compared to the other citrus fruits (P<0.0001).

DISCUSSION

Cross-contamination from outer skin or rind of fresh produce to edible portion, hands or utensils can occur during food preparation such as cutting, extracting or peeling if the surface of fresh produce is contaminated as our results show the bacterial transfer from inoculated citrus fruits to edible portion and gloved hands during hand peeling. Castillo et al. (*6*) isolated four different types of *Salmonella enterica* serotypes on fresh oranges and orange juices collected from public markets in Mexico and hypothesized that *Salmonella* found in orange juices was due to the contaminated oranges or improper sanitation during orange juice extraction or serving process. Martinez-Gonzales et al. (*17*) demonstrated transfer of *Salmonella*, *E. coli* O157:H7, and *L.*

monocytogenes from contaminated oranges to squeezed orange juice, hands and utensils such as juice extractor during extraction. Their study also indicated that contaminated utensils or hands caused another cross-contamination to orange juice extracted from uninoculated fresh orange. A study conducted by Vadlamudi et al. (*27*) showed that preparation procedure can affect bacterial transfer from contaminated cantaloupe skin to the edible portion. In their study, cutting cantaloupe after removing the contaminated rind effectively reduced the *Salmonella* transfer to edible portion compared to cutting cantaloupe prior to removing the rind. As citrus fruits are sliced or peeled before consumption, bacterial transfer rate from the surface of citrus fruits to edible portion could vary depending on preparation procedures like peeling or slicing. Previous studies reported that surface characteristics of fresh produce influence the microbial transfer from contaminated outer skin to edible portion or utensils (*22*, *28*). Our data clearly show the different transfer pattern of *Salmonella* inoculated onto the stem, equator, or styler of Minneola tangelo to the edible portion and the gloved hands compared to the other citrus fruit varieties, which might be due to the relatively thin and smooth skin that tends to adhere to edible flesh portion of fruit (*13*). The leathery skin of Satsuma mandarin tends to easily separate from edible portion as fruit matures (*2*). With this surface characteristic, Satsuma mandarin peels can be removed quickly, easily and in very few pieces when compared to the other citrus varieties, requiring much less contact between the hand and the fruit. It is also possible that physical characteristic of fresh citrus fruits such as resistance to rupture, puncture, and shearing stress may influence transfer of *Salmonella* from peel to edible portion when they are peeled, although the relationship between bacterial transfer during hand peeling and susceptibility to damage is not clear. Further research is needed to determine the effect of physical characteristics of citrus fruits on bacterial transfer to edible portion during peeling.

Cross-contamination from contaminated surface of citrus fruits to edible portion or gloved hands during peeling can be affected by inoculation sites as this study shows that the highest *Salmonella* transfer to the edible portion and gloved hands were found from the styler and equator of all citrus varieties except Minneola tangelo, respectively. When the stem portion was the source of contamination, the highest level of *Salmonella* remained on the peel of all citrus fruit types tested except Satsuma mandarin. In a previous study conducted on bacterial transfer from fresh orange to squeezed orange juice, a significantly higher level of pathogenic bacteria on stem portion was observed than on smooth skin and styler of fresh oranges (*18*). Moreover under natural conditions, the stem portion may be more likely contaminated during harvesting because it is susceptible to damage by force needed to pick (*1*, *9*).

The results of our study demonstrate that pathogens present on the surface of fresh citrus fruits can be transferred to the edible portion of the fruit, or to the hands of the person doing the peeling. Although most *Salmonella* remained on the peel of citrus fruits, the cross-contamination from the surface of citrus fruit varieties to the edible portion and gloved hands can occur during hand peeling. The average level of *Salmonella* remaining on the peel ranged from 5.37 % (Minneola inoculated at the equator) to 33.3 % (Satsuma inoculated at the styler). Average *Salmonella* transfer to the edible portion ranged from 0.16 % (Valencia inoculated at the equator) to 5.41 % (Navel inoculated at the stem). Average *Salmonella* transfer to gloved hands ranged from 0.41 % (Grapefruit inoculated at the stem) to 8.97 % (Navel inoculated at the stem). Our study also shows that bacterial transfer rate varies depending on the citrus varieties and inoculation sites on the citrus fruits. Different surface characteristic of different citrus varieties may affect the transfer rate of *Salmonella* for reasons that are as yet unclear.

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Table 1. Percent transfer of *Salmonella* from inoculated citrus fruit to five citrus varieties during hand peeling

Fruit	Inoculation location	Enumeration location	Mean	Standard Deviation	Median	Max*	Min	Range
Valencia	Stem	Peel	27.06	36.50	15.28	185.71	0.15	185.57
		Edible	1.89	9.99	0.10	83.02	0.02	83.00
		Gloved hands	0.91	1.84	0.14	9.58	0.03	9.55
	Equator	Peel	9.05	10.29	5.67	43.36	0.33	43.04
		Edible	0.16	0.18	0.09	0.87	0.01	0.85
		Gloved hands	0.85	1.39	0.43	7.69	0.03	7.66
	Styler	Peel	23.76	35.33	6.30	122.40	0.04	122.37
		Edible	1.27	3.36	0.26	25.61	0.02	25.59
		Gloved hands	0.53	0.91	0.10	4.03	0.02	4.01
Navel	Stem	Peel	47.85	40.68	29.72	185.00	4.81	180.19
		Edible	5.41	28.31	0.19	170.45	0.04	170.41
		Navel	0.27	0.79	0.03	3.80	0.01	3.79
		Gloved hands	8.97	47.20	0.31	284.09	0.04	284.05
	Equator	Peel	12.08	14.25	7.87	50.00	0.19	49.81
		Edible	1.54	2.20	0.48	10.00	0.02	9.98
		Navel	0.04	0.09	0.02	0.51	0.00	0.51
		Gloved hands	0.79	1.33	0.36	7.00	0.04	6.96
	Styler	Peel	18.25	41.73	9.23	229.57	0.03	229.53
		Edible	1.74	3.30	0.25	13.98	0.03	13.96
		Navel	14.11	13.12	11.90	82.76	1.17	81.59
		Gloved hands	1.56	2.08	0.57	6.43	0.02	6.41
Satsuma	Stem	Peel	61.02	37.22	62.53	131.82	1.70	130.12
		Edible	1.70	2.52	0.29	8.27	0.04	8.23
		Gloved hands	0.63	0.92	0.25	3.72	0.04	3.68
	Equator	Peel	53.76	51.78	40.43	266.67	0.63	266.04
		Edible	1.63	2.80	0.65	16.25	0.04	16.21
		Gloved hands	4.14	5.12	2.10	17.80	0.04	17.76
	Styler	Peel	66.30	37.90	73.10	119.49	0.43	119.06
		Edible	2.46	3.64	0.77	13.83	0.02	13.81

		Gloved hands	3.42	6.33	0.58	23.60	0.04	23.56
Minneola	Stem	Peel	30.33	41.12	12.20	141.67	0.32	141.35
		Edible	1.12	4.28	0.24	25.83	0.02	25.81
		Gloved hands	3.05	3.83	1.14	14.13	0.02	14.11
	Equator	Peel	5.37	10.31	0.79	44.00	0.00	44.00
		Edible	0.57	1.24	0.09	5.00	0.00	5.00
		Gloved hands	2.68	5.86	0.37	25.00	0.04	24.96
	Styler	Peel	17.53	25.90	4.29	101.25	0.15	101.10
		Edible	1.84	2.22	0.91	8.39	0.05	8.35
		Gloved hands	3.53	5.43	1.96	30.16	0.05	30.11
Grapefruit	Stem	Peel	36.20	36.46	36.12	196.43	1.33	195.10
		Edible	0.98	1.79	0.15	6.52	0.02	6.51
		Gloved hands	0.41	0.72	0.12	3.45	0.01	3.43
	Equator	Peel	35.99	47.32	6.64	207.30	0.03	207.27
		Edible	1.72	2.79	0.35	9.50	0.04	9.46
		Gloved hands	1.35	1.99	0.40	9.02	0.04	8.98
	Styler	Peel	23.43	30.76	3.47	119.49	0.09	119.39
		Edible	1.50	2.21	0.30	8.80	0.02	8.78
		Gloved hands	0.81	1.68	0.24	9.02	0.03	8.99

* Due to the variable nature of microbial counts calculated transfer exceeded 100% in some cases.

Figure legends

Figure 1. *Salmonella* log percent transfer frequency from Valencia fruit inoculated at the stem (A), equator (B), or styler (C) during hand peeling. Transfer was to the peel portion (●), or edible portion (○) of the fruit, or to the gloved hands (▼) of the individual doing the peeling. Frequency is the number of times a particular log percent transfer occurred within a target data set (n=36, except for edible portion where n=72).

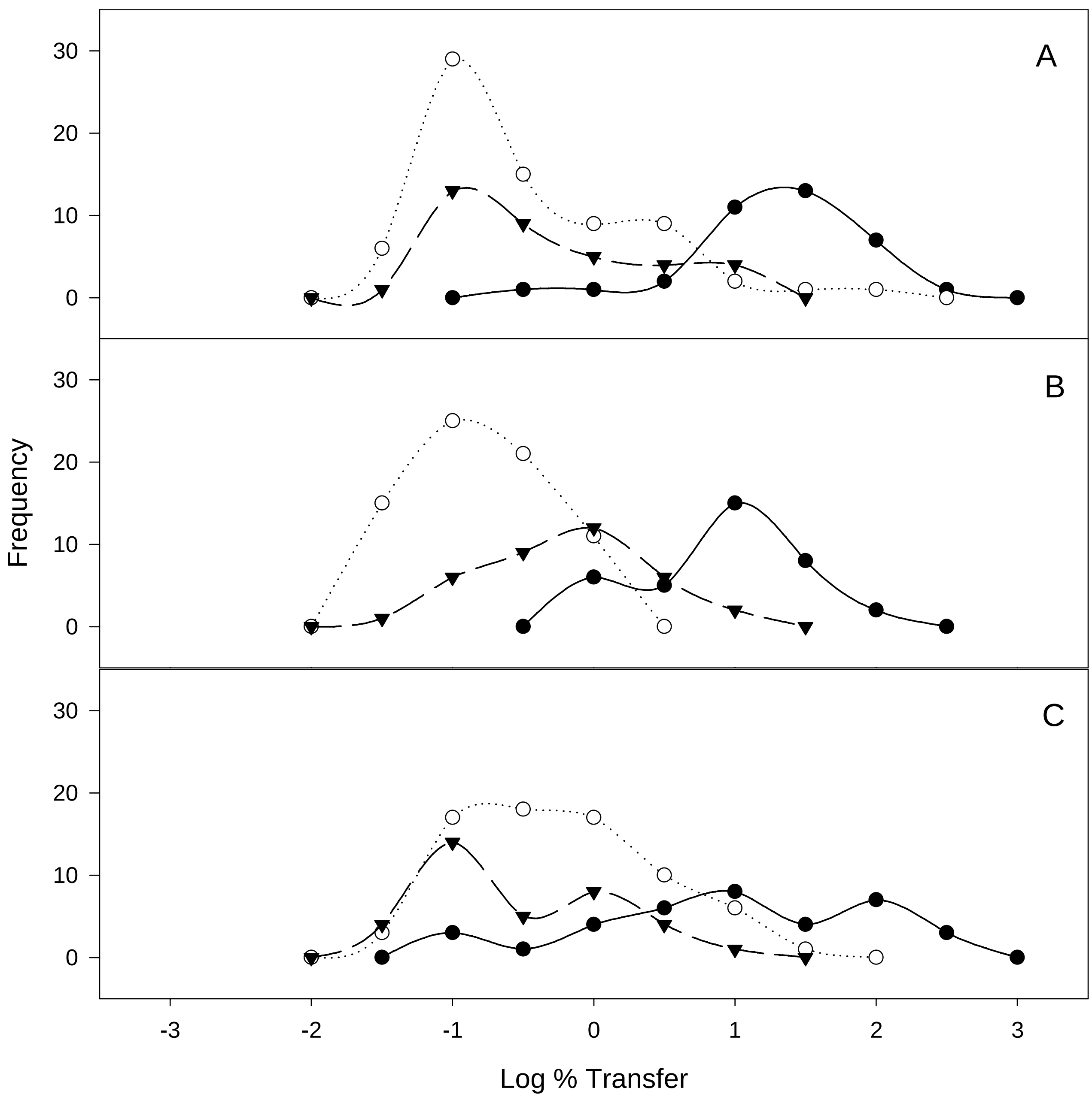
Figure 2. *Salmonella* log percent transfer frequency from Navel fruit inoculated at the stem (A), equator (B), or styler (C) during hand peeling. Transfer was to the peel portion (●), or edible portion (○) of the fruit, or the navel portion (△) or to the gloved hands (▼) of the individual doing the peeling. Frequency is the number of times a particular log percent transfer occurred within a target data set (n=36).

Figure 3. *Salmonella* log percent transfer frequency from Satsuma mandarin fruit inoculated at the stem (A), equator (B), or styler (C) during hand peeling. Transfer was to the peel portion (●), or edible portion (○) of the fruit, or to the gloved hands (▼) of the individual doing the peeling. Frequency is the number of times a particular log percent transfer occurred within a target data set (n=36).

Figure 4. *Salmonella* log percent transfer frequency from Minneola fruit inoculated at the stem (A), equator (B), or styler (C) during hand peeling. Transfer was to the peel portion (●), or edible portion (○) of the fruit, or to the gloved hands (▼) of the individual doing the peeling.

Frequency is the number of times a particular log percent transfer occurred within a target data set (n=36).

Figure 5. *Salmonella* log percent transfer frequency from Marsh grapefruit inoculated at the stem (A), equator (B), or styler (C) during hand peeling. Transfer was to the peel portion (●), or edible portion (○) of the fruit, or to the gloved hands (▼) of the individual doing the peeling. Frequency is the number of times a particular log percent transfer occurred within a target data set (n=36).



A
B
C
30
20
10
0
Frequency
-3
-2
-1
0
1
2
3
Log % Transfer

Figure 2.

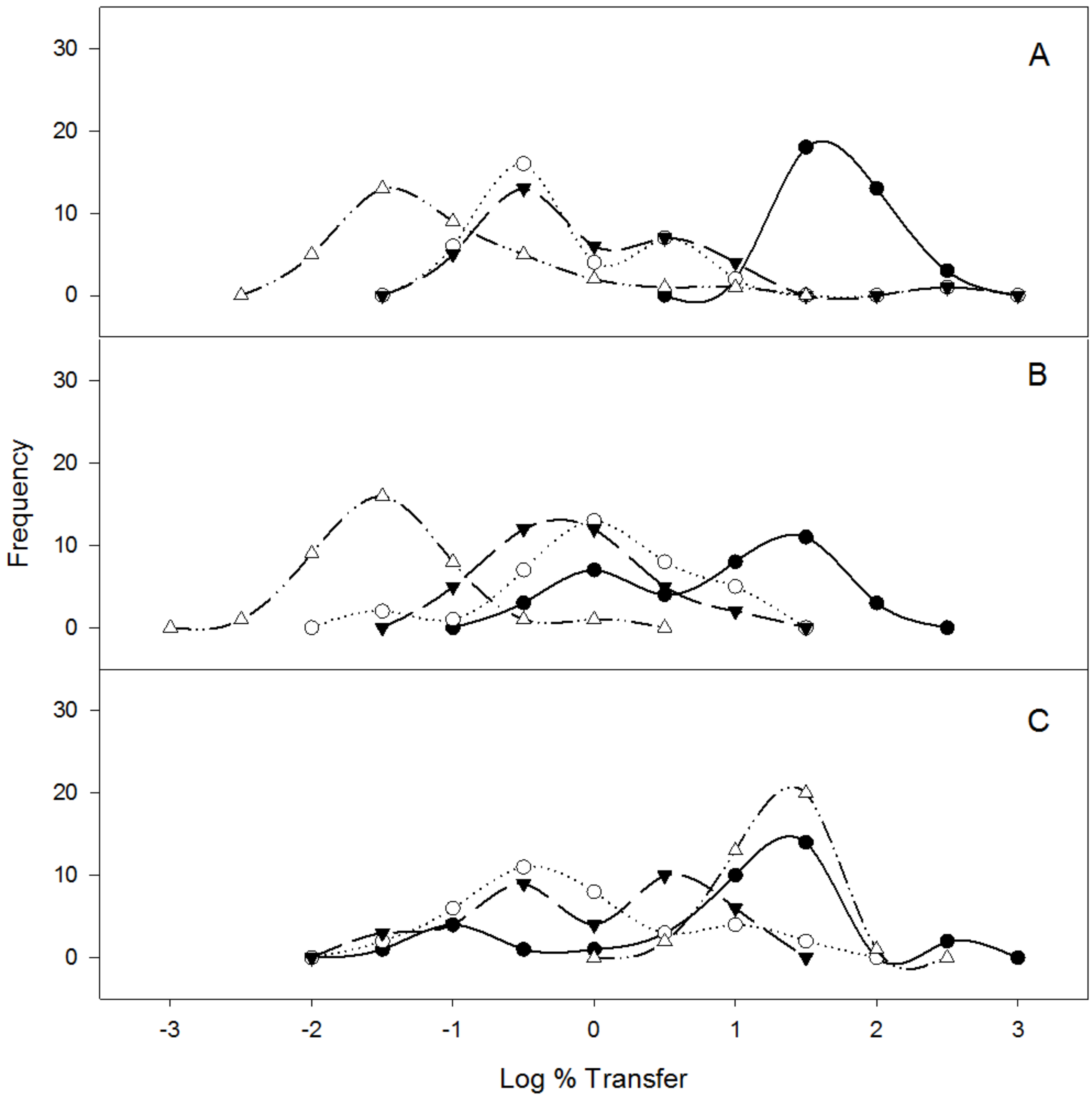


Figure 3.

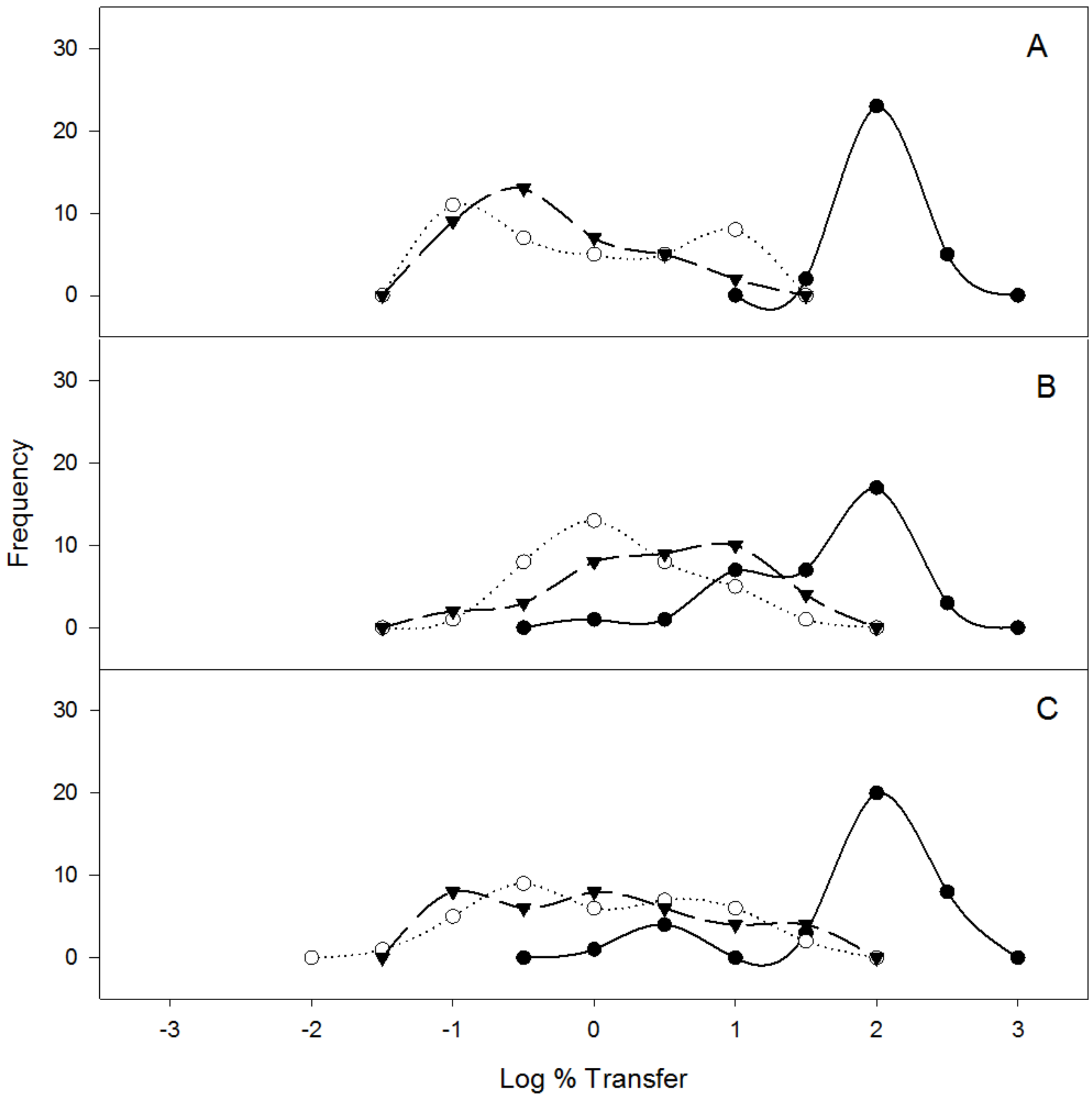


Figure 4.

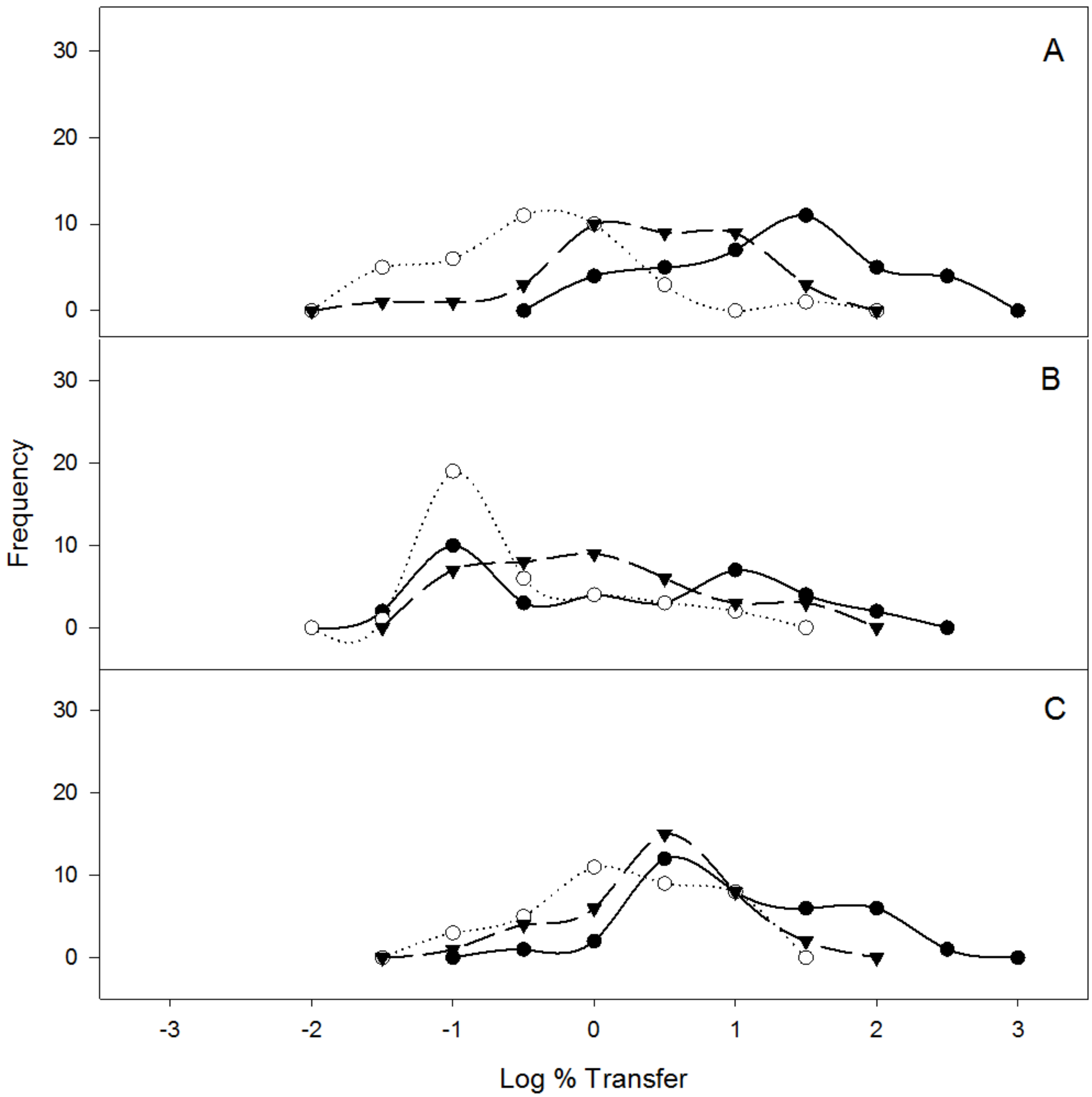


Figure 5.