Internet Journal of Food Safety, Vol.5, 2005, p. 35-40

Longevity Studies of *Escherichia coli* on Apples from Tree

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The consumption of fresh apple juice and apple cider has been linked to increasing numbers of outbreaks associated with *Escherichia coli* O157:H7. Apples can become contaminated with *E. coli* O157:H7 not only during processing but also while still on the tree. This study was undertaken to ascertain if *E. coli* can survive and grow on apples in an orchard environment during the course of a growing season using three different *E. coli* strains. Four apple cultivars, Red Delicious, Golden Delicious, Gala, and Fuji, were sprayed with aqueous bacterial suspensions (ca. 10^5 CFU/ml) to compare their ability to promote or inhibit the survival of *E. coli* due to differences or similarities in structure or genetics. Spraying was performed at different fruit developmental stages from July through October 2001. Three apples of each cultivar were collected at 0, 1, and 3 days after spraying and shaken and rubbed for 1 min in 50 ml 0.2% peptone, then enumerated using Petrifilm (3M Corp) and Purple Base Broth containing 0.5% bile salts and 4-methylumbelliferyl-beta-D-glucuronide (MUG). When *E. coli* was enumerated immediately after spraying, the population on apple skin was around 10^{4-5} CFU/apple. However, these populations were reduced to undetectable levels (0.3 $\log_{10}$ CFU/apple) in apples collected 1 day after spraying. The same results occurred in both immature and mature apples sprayed at different times throughout the growing season. There were no significant differences relative to cultivar. Also, when *E. coli* was stored for a week at 4°C to produce more environmentally-resistant cells, they followed the same pattern of actively growing cells. These results show it is difficult for *E. coli* to survive and grow on apple fruit in an orchard environment, regardless of the fruit developmental stage.

Escherichia coli O157:H7 continues to be recognized as a foodborne pathogen of primary concern. The organism is responsible for hemorrhagic colitis, a condition manifested by severe abdominal pain and bloody diarrhea. The disease can be followed by life-threatening complications, the most common being hemolytic uremic syndrome (Tarr 1994). Apple juice and apple cider was not previously considered to present high risk of contamination due to the high level of acidity; however, *E. coli* O157:H7, unlike other *E. coli* serotypes, is able to withstand such levels due to the presence of lactic and malic acids. Due to high acid tolerance, not only can this pathogen survive in apples, it has been found that *E. coli* O157:H7 can also survive in apple juice and apple cider. Numerous researchers have described the exceptional survival of this bacterium in acidic apple beverages (Miiler et al 1994, Zaho 1993, Riordan 2000).

Recently, the consumption of fresh apple juice and apple cider has been linked to increasing numbers of outbreaks associated with *E. coli* O157:H7 (Douglas et al. 2000). The first reported outbreak of disease presumably caused by this pathogen and associated with the consumption of apple cider occurred in 1980 in Canada (Steele et al. 1982). In the United States, three reported outbreaks of disease associated with the consumption of apple cider have occurred in Massachusetts in 1991 and resulted in 23 reported cases of *E. coli* O157:H7 infection. The next two outbreaks (Connecticut and Washington State) occurred in the fall of 1996. These two outbreaks resulted in a total of 78 reported cases and the first reported death associated with consumption of this product and *E. coli* O157:H7 infection (Center for Disease Control and Prevention, 1996 and 1997). Consequently, the Food and Drug Administration (FDA) proposed rules control point principles to the production of hazard analysis critical control point principles to the production of fresh juices. As an interim measure, regulations have been promulgated by the FDA that require warning labels on fresh juice products that have not been treated in some manner to achieve a 5-log reduction in the target pathogen.

After outbreak of *E. coli* O157:H7 associated with unpasteurized apple cider, the source of the pathogen was tried to investigate by several researchers, but was not determined. The potential sources of *E. coli* O157:H7 for fruit contamination are numerous (Beuchat 1997). One possible source may be bird droppings, which are plentiful on apples during harvest, as many birds feed on the maturing fruit (Wallace 1997). Dropped apples may also be exposed to feces of domestic or feral animals (Rice 1995, Tauxe 1997, Zhao 1995). However, although manure has been suspected to be the primary source of some outbreaks, the definite route of contamination is unknown and no direct

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evidence linking the use of dropped apples to fecal contamination of cider has been presented (Douglas 2000). Cider manufactured using only tree-picked (i.e., obtained directly from the tree) fruit has been found to contain *E. coli* (Digman 1999). Other suspected environmental contamination sources are infestation by insects, run-off from nearby pastures, manure and contaminated irrigation systems (Janisiewicz 1999). If infiltration of internal structures and tissues of fruits and vegetables by pathogenic bacteria were happened, it is thought to occur when produce surfaces come in contact with cells suspended in water (Scott et al. 2000). In the field, this may occur when rain, dew, or irrigation water collects in the surface of produce or, in the event that fruit falls from trees, as a result of contact with ground water (Scott et al. 2000).

To date, an outbreak of *E. coli* O157:H7 infection associated with consumption of raw apples has not been reported (Stephen et al. 2001). However, because of the increasing frequency of outbreaks associated with cider, there has been growing concern about the potential presence of *E. coli* O157:H7 on raw apple. And, the potential for infection caused by the consumption of apples harboring *E. coli* O157:H7 exists. Because the number of cells needed to cause illness is low, a single contaminated apple may result in infection, making identification of the source of *E. coli* O157:H7 difficult. The objective of this study was to ascertain if *E. coli* can survive and grow on apple surfaces in an orchard environment during the course of a growing season using three different *E. coli* strains. Four apple cultivars, Red Delicious, Golden Delicious, Gala, and Fuji, were sprayed with aqueous bacterial suspensions (ca. 10^5 CFU/ml) to compare their ability to promote or inhibit the survival of *E. coli* due to differences or similarities in structure or genetics.

MERTERIALS AND METHODS

Cultures and cell suspension. The surrogate species, *E. coli* ATCC 25922 was purchased from Microbiologics, Inc. (MN, USA). *E. coli* WADDL # 2701, isolated from ground beef and *E.*coli K-2 2B were obtained from the Food Science and Human Nutrition bacteria collection at Washington State University (Pullman, WA). These three strains of *E. coli* were used spraying in apple trees instead of *E. coli* O157:H7 in order to protect the natural environment from the pathogenic organism. Each strain of *E. coli* was cultured in Tryptic Soy Broth (TSB: Difco laboratory. Detroit, MI, USA) at 37°C for 24 h and harvested by centrifugation at 4.000×g for 20 min at 4°C and washed three times. The final pellet was suspended in buffered peptone water (Difico), corresponding to approximately 10^{8-9} CFU/ml, then, three suspended cells were mixed to construct culture cocktail. The mixed culture was diluted with distilled water for spraying.

Preparation of resistance cells. In order to make resistance *E. coli* cells, the cultured cells in TSB for 24 h were stored in 4ºC for 7 days. After storage, the cultures were serially 10-fold diluted with 9 ml buffered peptone water and survived *E. coli* were enumerated using Petri Film(3M Co). As described above, the mixed culture cocktail, corresponding to approximately 10^{8-9} CFU/ml of *E. coli* was made for spraying cell suspension.

Spray and Sampling. Two of each type of apple tree, Red Delicious, Golden Delicious, Gala, and Fuji, were sprayed with contaminated spray wash onto each individual apple as a known concentration of bacteria using sprayer. One apple from unsprayed trees and three individual apples from sprayed tree of each variety were picked up and were separately putted into stomaching bag. These sampling was performed immediately, 1 day, and 3 days after spray.

Bacterial enumeration. Each stomacher bag containing individual apple was added 50ml buffered peptone water and was intensely shook with buffered peptone water in order to isolate *E. coli* from apple skin. After shacking, the liquid sample was serially 10-fold diluted with 9 ml buffered peptone water. And then, sprayed *E. coli* were enumerated using Petri Film (3M co.) and ? (PBB) containing 0.5% bile salt and (MUG) and incubated at 37°C for 24 h.

RESULTS

The spray treatment of apple trees with a 3 strain mixture of *E. coli* on was performed in two separate seasons, 2-3 months before harvest and about the time of harvest and each spraying in one season was repeated three times. This permitted us to examine the effects of environment and the apple's growth upon the survival of *E. coli* on apple skins. The first *E. coli* spraying was performed three times during July. Immediately after each spraying, apples weighing between 30g and 50g, representing Day 0, were sampled. These data are shown in tables 1, 2, and 3. Immediately after spraying, the *E. coli* population on each apple skin was about 10^{3-6} CFU/apple (Table 1, 2, and 3). After only 24 hours, *E. coli* could be detected on the skins of only three of the 36 apples tested (Golden Delicious I (table 1), Gala I (table 2), Gala III (table 3)). After 3 days, no *E. coli* was found on any of the apple skins sampled (Table 1, 2, and, 3). The second spraying was performed three times near time of harvest, in September and October. These results are shown on table 4, 5, and, 6. Immediately after spraying, the *E. coli* population on each apple skin was about 10^{3-6} CFU/apple (Table 4, 5, and 6). After 1 day, *E. coli* was detectable on just 4 of the 36 apples tested. *E. coli* was found on 5 apples 3 days after spraying.(Of the positive samples, 8 were ≤ 1.00 log CFU/apple, 1 sample was 2.73 log CFU/apple).

As the results above, to estimate the survival efficacy of *E. coli* in mature apples, the spray of apple trees with *E. coli* was performed in two separate season related with the mature levels of apples in apple trees. However, there is no significant difference of survival of *E. coli* cell on apple skins between immature and mature apples in apple trees.

To investigate the survival efficacy of resistant *E. coli* on the apple skins in apple trees, the final spraying was performed in October using environmentally-resistant *E.coli.*

TABLE 1. Survival of *Escherichia coli* on the surface of apple fruits after spraying in apple trees on the (A) July 05, (B) July 12, (C) July 18, (D) September 6, (E) September 11, and (F) October 02, 2001

A.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C[a]	I[b]	II[b]	III[b]	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm[d]	0	ND[c]	5.94	5.20	4.40	ND	4.46	4.81	2.30	ND	3.28	3.23	5.11	ND	5.28	4.64	4.56
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PBB-MUG[e]	0	ND	<6	<6	<5	ND	<5	<6	<4	ND	<2	<4	<6	ND	<6	<6	<5
	1	ND	ND	ND	ND	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

B.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm	0	ND[c]	5.94	5.20	4.40	ND	4.46	4.81	2.30	ND	3.28	3.23	5.11	ND	5.28	4.64	4.56
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PBB-MUG	0	ND	<6	<6	<5	ND	<5	<6	<4	ND	<2	<4	<6	ND	<6	<6	<5
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	<1	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

C.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm	0	ND[c]	5.11	5.26	4.70	ND	5.18	5.28	5.46	ND	5.30	4.90	5.76	ND	4.48	4.48	4.60
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.63	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PBB-MUG	0	ND	5	6	4	ND	4	5	5	ND	5	4	5	ND	4	4	6
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

D.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm	0	ND[c]	5.20	5.60	5.30	ND	5.60	4.48	5.60	ND	4.48	5.60	5.60	ND	5.34	5.61	5.26
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	1.00	ND	ND	ND	ND	0.30	ND	ND	ND	ND	ND	ND	1.00	ND	ND
PBB-MUG	0	ND	<5	<6	<4	ND	<4	<5	<5	ND	<5	<4	<5	ND	<4	<4	<6
	1	ND	ND	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<1	ND	ND

E.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm	0	ND[c]	5.07	4.70	5.43	ND	5.00	5.08	4.85	ND	5.00	5.48	5.08	ND	4.87	5.11	5.48
	1	ND	ND	2.73	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.00	ND	ND
PBB-MUG	0	ND	<5	<4	<5	ND	<5	<5	<5	ND	<5	<6	<5	ND	<5	<5	<5
	1	ND	ND	<2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

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F.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm	0	ND[c]	5.70	5.62	5.76	ND	5.61	5.31	5.34	ND	5.02	5.73	5.63	ND	5.53	5.52	5.52
	1	ND	ND	ND	1	ND	2.34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.00	ND	ND
PBB-MUG	0	ND	<5	<4	<5	ND	<5	<5	<5	ND	<5	<6	<5	ND	<5	<5	<5
	1	ND	<1	<1	<1	ND	<2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Control apple picked from unsprayed apple tree.
[b] Each three apples (I, II, and III) for four various apple trees were sampled for enumeration of *E. coli*.
[c] Not detected; detection limits when *E. coli* were enumerated with Petrifilm was 1.28 log CFU/ apple, and PBB-MUG was 2.28 log CFU/ apple.
[d] Petrifilm (3M co. USA)
[e] Purple Base Broth containing 0.5% bile salt and 25mg/L 4-methylumbelliferyl-beta-D-glucuronide (MUG).

These cells were produced through storage for a week at 4°C. After spraying, sprayed *E. coli* on apple skin of 0 and 1 day were investigated on Petri Film. They exhibited the same pattern observed in actively growing cells (Table 7). In all spray experiments, there were no significant differences relative to cultivar. And the results show that it is difficult for *E. coli* to survive and grow on apple fruit in an orchard environment, regardless of the fruit developmental stage.

DISSCUSSION

Intact tree fruit had significantly lower counts than the other fruit samples collected. The hypothesis that the orientation of the fruit on the tree might be associated with the potential for internalization of microflora was not borne out. However, tree fruit was associated with *E. coli* contamination on two occasions (one intact and one damaged sample). Previously, it had been thought that contamination of fruit with *E. coli* was a consequence of contact with the ground or humans, as this organism is rarely found on plant in nature (Sumner, et al. 1997). It is possible that *E. coli* contamination of tree fruit may come from insects and birds; Wallace et al. (1997) reported that birds, mainly gulls, can harbor *E. coli* O157:H7. Further studies are ongoing at this laboratory to characterize situations in which *E. coli* can contaminate tree fruit. Janisiewicz et al. (1999) showed that flies inoculated with the pathogen resulted in a high incidence of contamination of surface wounds in the apple. Once the bacteria are inoculated onto the surface of the apple, it is possible that they can then spread further into the apple's flesh. In this case, topical sanitizers will not be affective.

The other possibility which apples in the tree were contaminated with *E. coli* was thought from water contamination. Riordan et al (2001) has reported about the study of potential sources of *E. coli* O157:H7 in U.S. orchards. Their results showed that the irrigation water in three orchards of the high-risk orchards was contaminated with *E. coli* and is an obvious route of contamination for all fruit. Other researcher reported about attachment and infiltration of *E. coli* cell into apple surfaces and texture. Their studies showed that infiltration of internal structures and tissues of fruits and vegetables by pathogenic bacteria is thought to occur when produce surfaces come in contact with cells suspended in water. In the field, this may occur when rain, dew, or irrigation water collects in the surface of produce or, in the event that fruit falls from trees, as a result of contact with ground water. After harvest, wash and flume waters used to clean fruits and vegetables may provide a vehicle to facilitate the infiltration of microbial cells (Bartz 1982, Bartz 1981, Zhuang 1995). The potential for infiltration of viable cells is highest if the water is contaminated and antimicrobial agents such as chlorine ate ineffective due to low concentration or pH (FDA 1998).

However, in our study, even though cell suspension with high concentration of three mixture of *E. coli* was sprayed on the apple tree, the cells were not able to survive long time and were significantly reduced after 1 day. However, we sampled only unwounded and intact apple from apple tree. If the wounded or dropped apples were used in production of apple juices or apple cider, they might have dangerous factor with surviving and growing in the apple texture. Beside, several researchers have reported about entrance and growth of bacterial cells in apple fruits though injuries in the skin. However, to date, there is no report whether internalization of *E. coli* can be happen into apple of apple tree in the orchard or not. In our preliminary experiment, we investigated survived *E. coli* not only apple surface but also flesh, outer core, and inner core of randomly apple samples. We could not find any *E. coli* cells in whole apples after directly plating on Petri film and enrichment (data now shown). From this result, it shows not easy and normal internalization of *E. coli* into the normal apples of apple

TABLE 2. Survival of *Escherichia coli* having resistance through storage for 6 days at 4°C on the surface of the mature apples after spraying in apple trees.

		Apple cultivars															
		Red Delicious				Golden Delicious				Gala				Fuji			
	Days	C[a]	I[b]	II[b]	III[b]	C	I	II	III	C	I	II	III	C	I	II	III
Petrifilm[d]	0	ND[c]	5.04	4.60	4.72	ND	4.81	4.56	4.35	ND	4.36	4.36	3.20	ND	4.54	4.23	4.20
	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.00	1.00	ND	ND	ND	ND

[a] Control apple picked from unsprayed apple tree.
[b] Each three apples (I, II, and III) for four various apple trees were sampled for enumeration of *E. coli*.
[c] Not detected; detection limit was 1.28 log CFU/ apple.
[d] Petrifilm (3M co. USA)

trees in the orchard. And, even though internalization of *E. coli* may occur, it is thought that very few numbers of cells with limit to hardly detect is involved in internalization.

Whether *E. coli* cells can enter and grow in apple texture in apple tree will be investigated as a future work. Also, factors which able to facilitate the attachment and infiltration of *E. coli* in apple skin and texture in apple tree should be estimated.

And, in our results, the sprayed *E. coli* cells rarely survived within short time in apple skins of apple tree in the orchard regardless of the degree of growing and difference of cultivar. The reason thought to be possible is numerous. UV light is thought the first thing that can kill significantly *E. coli* cell. Extremely dry condition after spraying and drying also can affect. The dramatically temperature change between day and night can also inhibit to survive and grow bacterial cells on the apple skins. Several reasons as mentioned above or not might be affect multiply to inactive and kill the *E. coli* on the apple skin. These potential reasons which can be explained the failure of surviving and growing of *E. coli* on the apple skins in the orchards also should be investigated as a future work.

Base on our results, the contamination and growth of *E. coli* in damaged apples during harvesting, storage, and processing of apple poses a greater risk for contamination of apple juice or cider than surface contamination of only apple in the orchards during culture.

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