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Incidence of *Escherichia coli* in Black Walnut Meats

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Examination of commercially shelled black walnut meats showed inconsistent numbers of total aerobic bacteria, coliforms, and *Escherichia coli*; variation occurred among different meat sizes and within each meat size. The incidence of *E. coli* on meats of commercially hulled black walnuts depended on the physical condition of the nuts. Apparently tightly sealed ones contained only a few or none, whereas those with visibly separated sutures and spoiled meats yielded the most. This contamination was in part correlated to a hulling operation. Large numbers of *E. coli* on the husk of the walnuts contaminated the hulling water, subsequently also contaminating the meats by way of separated sutures. Chlorination of the hulling wash water was ineffective. Attempts were made to decontaminate the walnut meats without subsequent deleterious changes in flavor or texture. A treatment in coconut oil at 100 C followed by removal of excess surface oil by centrifugation was best.

The black walnut industry in California is dependent on walnuts derived from an agricultural crop under no organized farming operation. Many trees are situated in undesirable environments. From a sanitary point of view, there has been a perennial concern with the problem of *Escherichia coli* contamination in shelled nut meats (7, 10, 16, 17, 19, 25, 26). The presence of this organism in shelled nuts has long been held to be due to improper sanitary control during processing (7, 11-14), because for many years it has been the concensus that the unbroken nut is free from the coliform bacteria (13, 14). Only recently has this concept been questioned (21). Decontamination without altering appearance, flavor, or texture of the meats also has been a problem (3, 4, 18, 19). For some time, the epoxides, propylene and ethylene oxide, have been used for this purpose (4, 6, 15). Recently, it has been reported that these sterilants produce objectionable chlorohydrin reaction products in some foods (27).

These problems prompted a thorough study of the incidence of *E. coli* in in-shell black walnuts, the incidence of this organism in commercial black walnut meats, and methods for decontamination of meats bearing this bacterium.

MATERIALS AND METHODS

The unhulled, unshelled, black walnuts used in this investigation were collected from under trees in several localities of the Sacramento Valley of California during the 1967-1968 crop year by the senior author. Two different lots of the same were acquired from a commercial processor. Hulled, unshelled black walnuts and black walnut meats were also obtained from two California processors during the 1966-1967 and 1967-1968 seasons. In addition, waste water samples containing husk removed in the commercial hulling operation were taken during the 1967-1968 season.

All samples of meats and waste water were aseptically transferred at the time of collection to sterile glass jars. Whole walnuts were placed in new polyethylene bags. All samples were stored at 2.8 C.

Bacteriological analysis of in-shell meats. The meats of unshelled walnuts were aseptically removed from the shell. When the walnut samples (here the term "samples" refers to individual walnuts of the various lots) possessed a husk, they were hand-hulled immediately prior to use. The hulled samples were segregated into two groups according to whether they were tight ones or had hairline cracks (visible by the unaided eye) along the shell sutures. Those with significant suture cracks were discarded as being too difficult to sanitize without destroying the interior meat. To ensure that the microorganisms recovered were derived from the interior of the walnut, each nut was sanitized by rapid immersion in 3% hypochlorite and withdrawal, which was repeated 5 min later. A special aseptic cracking apparatus (Fig. 1) consisting of a solid brass cylinder fitting snugly into a matching cup provided a covered clamp in which the isolated walnut could be broken when the two metal parts were forced together with a blow from a lead hammer. The interior of the apparatus was washed with ethyl-alcohol and flamed; the nut was placed with the suture on the horizontal in the cracker and broken. Except for the very fine particles, the meats were extracted by small ethyl alcohol-flamed tweezers and transferred directly into a large tube containing 20 ml of the selected medium.

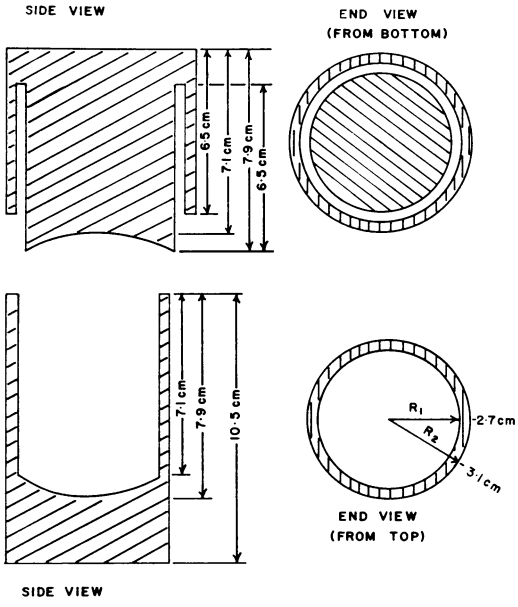

FIG. 1. *Cross-section drawings of the two parts of the nut cracker.*

The medium used for selective enrichment of *E. coli* was a buffered boric acid-lactose broth (BALB; 20, 22, 23). Each inoculated tube, containing a Durham tube, was incubated in a water bath at 43 ± 0.5 C for 4 days. Every 24 hr, the tubes were examined for gas formation.

In some instances, Difco lactose broth (LB) was the primary enrichment medium. The tubes, incubated at 37 C, were checked for gas formation at 24 and 48 hr. For detection of *E. coli*, gas-positive tubes were inoculated (1 ml) into the boric acid medium and incubated as described. The method of using lactose broth as a primary enrichment followed by the secondary borate broth for *E. coli* detection was favored by Levine et al. (8).

Confirmed and completed tests for coliform bacteria were made by the procedure given in *Standard Methods for the Examination of Water and Wastewater* (1). All positive tubes of BALB and LB were streaked, and, if necessary, restreaked on Levine E M B Agar (Difco), incubated at 37 C for 24 hr, and examined for typical coliform colonies. Pure cultures of possible *E. coli* were selected and placed on nutrient agar (Difco) slants and inoculated into LB fermentation tubes. The Gram stain and the differential tests (indole, methyl red, Voges-Proskauer, citrate) were made from 24-hr nutrient agar slant cultures.

Analysis of waste water. Samples of effluent waste water with the suspended husk fragments from one commercial black walnut hulling operation were examined with 2 to 4 hr after collection, to minimize any adverse effects of tannin (7, 9). Tubes of BALB were inoculated with 10-, 1-, and 0.1-ml portions of the effluent samples in replicates of five. The most probable number (MPN) of *E. coli* was estimated from *E. coli*-positive tubes by reference to a table (1). Incubation and identification of *E. coli* was as before. Appropriate dilutions (with 0.9% sodium chloride blanks) of the waste liquid were also prepared, from which triplicate spread plates of E M B Agar and tryptone-glucose-yeast extract (TGY)-agar (1) were inoculated to give the average numbers of coliform and viable bacteria per milliliter of waste water. The influent freshwater and the discharge were tested for residual chlorine content by the *o*-tolidine method (1).

Induced contamination of in-shell meats. Attempts were made to contaminate in-shell meats with *E. coli*. The particular culture used was isolated from a walnut meat sample (sample 33b) and was typical for *E. coli* in all tests applied. Enough organisms, grown on nutrient agar, were aseptically transferred to 1 liter of sterile phosphate-buffered (0.1 M) water and distributed by use of a sterile blendor to make an even suspension. In a few cases, the inoculum was a 24-hr *E. coli* population grown in 1 to 1.5 liters of nutrient broth. The whole, unbroken walnuts were submerged in the bacterial suspension and held at 2.8 C. Because walnut husk contains tannin, the viable count of *E. coli* was determined periodically to ensure that viable organisms were always present. At intervals, samples were removed and examined by the BALB enrichment method.

Visual determination of shell separation in whole walnuts. Methylene blue (0.4%, aqueous) was used as a stain to determine the degree to which unshelled "unbroken" walnuts were sealed. Hulled walnuts were selected which, to the naked eye, appeared to have tight sutures. They were immersed in the dye for various periods of time and then withdrawn; excess surface moisture was removed. Visual observation was made as to dye penetration, particularly along the shell suture, after each nut was cracked.

Bacteriological examination of commercial black walnut meats. Walnut meats (20 g) were aseptically weighed and added to 500-ml Erlenmeyer flasks containing 180 ml of sterile physiological saline solution; the flasks were then vigorously handshaken several times during 10 min. Decimal dilutions were prepared to 0.00001 ml by transferring 10 ml of inoculum to 90-ml saline blanks. Each dilution was again handshaken for 1 min prior to the next transfer. Triplicate pour plates of E M B Agar and TGY agar were prepared from dilutions of 0.01 though 0.00001 ml. BALB was inoculated in replicates of five from the 1-, 0.1-, and 0.01-ml dilutions; LB, also in replicates of five, was inoculated from the same dilutions. Presumptive tests subsequently confirmed for *E. coli* and for coloforms were used to estimate the MPN of these organisms.

Experimental reduction of E. coli on black walnut meats. Commerically shelled large-size black walnut meats were inoculated with the typical *E. coli* culture recovered from walnut meat sample 33b. The suspension of the organism prepared as described above (400 ml) was mixed with 400 g of meats and soaked, with intermittant stirring, for 20 min. The liquid was drained and the meats were spread and dried on sterile cheesecloth placed in a dehydrator at 28 C with

an airflow of 650 ft/min for 1 hr. The semi-dry meats were then left at room temperature for 2 days to return them to the moisture content approximately equivalent to that found in the initial samples.

Immersion of inoculated meats in boiling water was carried out by exposure of 20-g portions for various times. The treated meats were drained and aseptically transferred to a saline dilution blank for detection of *E. coli* survivors. To check the effect of necessary drying of the wet meats, other meat samples were identically treated but dried at 88 C. After 5, 10, and 15 min of drying, the meats were checked for appearance, moisture, taste, and texture.

A second treatment involved immersion of the inoculated meats (20 g) in coconut oil at temperatures of 100, 125, and 150 C for different times. Subsequently, the excess surface oil was removed from the warm meats by a moderate centrifugation for 30 sec; asepsis was used to detect *E. coli* survivors.

Enumeration of *E. coli* in all cases was determined by using triplicate pour plates of E M B Agar and MPN in BALB medium.

RESULTS

Populations on in-shell meats. A total of 1,234 individual walnuts were examined for *E. coli* contamination of meats. Of these, 408 were collected from the field and still possessed a husk. In all instances, there appeared to be no visible cracks in the sutures when the husks were removed. The remaining 826 walnuts were commercially machine-hulled, with 220 being collected before being dried. The wet samples appeared to have tight sutures, whereas those dried possessed a high percentage of cracked shells. Consequently, this latter group was segregated as to: no visible suture separation; visible suture separation; and probable spoilage as evidenced by oil which had soaked through the shell or the presence of mold on the shell surface. All samples were further differentiated as to the meat condition once the walnuts were shelled.

E. coli was not recovered from any of the 408 field samples with husks (Table 1). When LB was used as the primary enrichment medium rather than BALB, it was possible to recover coliform organisms. (Approximately one-half of such samples had coliform organisms on the meats even though the sutures appeared intact.) Although *Aerobacter* types predominated, several were confirmed as *E. intermedia*. These samples, examined with LB, were collected from a pasture in current use; the pasture had been subjected to recent wet weather.

The 302 walnuts with no visible suture separations (Table 2) yielded 284 edible meats, of which only one sample resulted in recovery of *E. coli*; 18 spoiled meats yielded one recovery for a somewhat higher rate of incidence. In the second group of 208 nuts possessing separated sutures, the edible total of 192 yielded three with *E. coli* and the 16 spoiled gave two such recoveries. The third group of obviously spoiled walnuts yielded six recoveries from 96 samples.

TABLE 1. *Examination for E. coli on meats of field-run whole black walnuts with husk*

Enrichment medium	Lot no.	Edible meats		Spoiled meats		Recovery	
		Gas-positive	Gas-negative	Gas-positive	Gas-negative	Coliform	*E. coli*
BALB	1(50)[a]	0	50	0	0		0
	2(20)	0	20	0	5		0
	3(16)	0	16	0	10		0
	4(26)	0	26	0	14		0
	5(54)	0	59	0	0		0
	6(30)	0	60	0	8		0
	7(30)	4	26	0	0		0
	8(42)	0	42	0	3		0
	9(21)	0	21	0	9		0
LB	10(15)	8	7	0	0	7	0
	11(20)	15	5	0	0	12	0

[a] Number of individual nuts is given in parentheses.

Further analysis of the commercially hulled and dried samples disclosed that, when segregated as to their commercial source, the samples from one processor had a higher overall incidence of *E. coli* (2.86%) than did those of the other processor (0.33% of 300 samples). This led to an examination of commercially hulled walnuts collected immediately after being hulled as well as of the waste water being produced at the hulling plant; the waste water had previously indicated a greater incidence of *E. coli* contamination. A total of 220 wet samples (Table 3*a*) were checked, from which two recoveries of *E. coli* were made, both from the same lot (21b). Additional analysis of this contaminated lot by using LB for the primary enrichment indicated that 8 of 20 samples of meats contained coliform organisms.

Analysis of waste water samples. Waste water samples (Table 3*b*), correlated to several of the wet walnut samples, were examined for standard plate count (TGY), E M B plate count, and MPN of *E. coli* (BALB medium). The sample correlated to walnut lot 20b indicated approximately 360,000 bacteria per ml (standard count), 50,000 bacteria per ml on E M B Agar, and no *E. coli* recovered in the BALB. The second sample, collected 60 min after sample 20b and correlated to walnut lot 21b, from which *E. coli* was recovered in the two instances cited previously, contained so many *E. coli* that the MPN was indeterminate. However, typical *E. coli* colo-

TABLE 2. *Examination for E. coli on meats of whole dried black walnuts, husk commercially removed, samples enriched in boric acid-lactose broth*

Lot no.	No. of "sealed" sutures				No. of separated sutures				Whole walnuts, visibly spoiled		*E. coli* recovery (in order across)				
	Edible meats		Spoiled meats		Edible meats		Spoiled meats								
	Gas +	Gas −	Gas +	Gas −	Gas +	Gas −	Gas +	Gas −	Gas +	Gas −					
12a[a]	0	43	0	0	1	48	2	6	5	5	0	0	0	0	5
13a	1	68	0	0	2	29	0	0	0	19	0	0	0	0	0
14b	2	46	1	3	1	42	1	4	0	0	1	1	1	1	0
15b	2	38	0	5	2	40	1	2	0	0	0	0	0	1	0
16a	0	0	0	0	0	0	0	0	5	62	0	0	0	0	1
17a	1	70	0	9	1	19	0	0	0	0	0	0	1	0	0
18b	0	13	0	0	2	5	0	0	0	0	0	0	1	0	0
Total	6	278	1	17	9	183	4	12	10	86	1	1	3	2	6

[a] Designations "a" and "b" represent different commercial sources.

TABLE 3*a*. *Examination for E. coli on meats of hulled wet whole black walnuts*

Enrichment medium	Lot no.	No. of "sealed" sutures				No. of cracked sutures				Coliform recovery	*E. coli* recovery
		Edible meats		Spoiled meats		Edible meats		Spoiled meats			
		Gas +	Gas −	Gas +	Gas −	Gas +	Gas −	Gas +	Gas −		
BALB	19b	2	77	0	0	0	1	0	0		0
	20b	0	30	0	0	0	0	0	0		0
	21b	2	23	0	5	0	0	0	0		2
	22b	0	30	0	0	0	0	0	0		0
	23b	0	29	0	0	0	0	0	1		0
LB	21b	10	10							8	

TABLE 3*b*. *Examination for E. coli in waste water*

Sample no.	Time	Standard plate count	E M B plate count	MPN of *E. coli*/ml
	min			
20b	0	3.6×10^5	5.1×10^4	None
21b	55	3.5×10^6	1.3×10^5	All[a]
22b	80	1.8×10^6	5.0×10^5	3.5
23b	105	3.0×10^6	1.6×10^6	2.78

[a] All tubes positive (2×10^4 on EMB).

nies on the E M B Agar, with representative colonies yielding positive tests for *E. coli*, indicated approximately 20,000 *E. coli* per ml. Standard counts increased to 3,500,000; the E M B count increased to 130,000 bacteria per ml. Water samples collected at 80 and 105 min, correlating to walnut lots 22b and 23b, both yielded MPN of *E. coli* of 3.5 and 2.8 per ml, respectively. Plate counts remained high with the last sample giving counts of 3,000,000 (Standard) and 1,600,000 (E M B) bacteria per ml.

The residual chlorine concentration of the clear incoming water supply was approximately 30 μg/ml; in a matter of seconds, the chlorine was dissipated in the effluent waste water owing to the high organic content.

Induced contamination of in-shell walnuts. The possibility that in-shell walnut meats were becoming contaminated with coliform bacteria under certain environmental conditions suggested that the whole nuts were not as impervious as they would appear to be. Experiments were then performed to determine whether coliforms could penetrate to the meats of walnuts that by all outward appearances were sealed. Care was taken to select walnuts from lots that previously were free from *E. coli*.

The initial lot of 20 hand-hulled walnuts (1 year old) were exposed to *E. coli* for several days at 2.8 C by soaking in a turbid nutrient broth suspension. All meats were positive for *E. coli*. A second lot of 24 hand-hulled wet walnuts (from the current crop) were thus soaked, and four walnuts were examined every 4 hr; one was

positive after 4 hr, four at 8 hr, three at 12 hr, and all after 16 hr.

The next check was made by using walnuts with husks in various stages of deterioration. First, nine walnuts with wet, soft, deteriorating black husks were soaked in the suspension. Three samples removed after 48 hr. were all contaminated; six removed after 72 hr yielded five positive results. Three walnuts with black but intact firm husks were also soaked but were negative for *E. coli* when checked 48 hr later. The intact husk appeared to act as a barrier.

To simulate field conditions, walnuts were divided into two groups: (a) there was soft black husk due to deterioration; (b) the husk was firm and very green with no breaks in the skin. Each group was soaked in separate *E. coli* (buffered) suspensions containing approximately 10,000,000 organisms per ml. Samples were checked after 6, 12, 24, 32, 59, and 264 hr of soaking. With the deteriorated group, all examinations (four nuts at a time) indicated that the meats were contaminated. Tests of two walnuts at a time from the green husk group yielded only two that were positive for *E. coli* (one at 24 hr and one at 59 hr). All others, including those at 264 hr, were not contaminated. Ten green walnuts, with the husk removed, were also soaked. These were checked at 24 hr and two were positive for *E. coli*. These results suggest that both intact hull and low incidence of shell separation contribute to the prevention of meat contamination of green mature nuts.

Visual determination of shell separation in whole nuts. The presence of coliform bacteria on the meats in whole walnuts indicated that the assumption of sealed walnuts was not always valid, and consequently the most likely route of penetration would be the separated suture. In the first substantiating check, 10 hand-hulled and dried walnuts and 5 semi-dry nuts with deteriorated husks were soaked for 10 min in a methylene blue solution. All 10 of the well-dehydrated and 4 of the semi-dry walnuts showed dye penetration to the meats at some point along the suture. A second hulled and dried lot was soaked. Ten of 12 nuts removed after 30 sec, and all 8 removed after 1 min, had been penetrated. A final check involved hulled walnuts which had not been dried. Four removed after 1 min had two penetrated and spoiled, eight removed after 3 min had two penetrated and spoiled, and eight removed after 5 min had no penetration or spoilage.

The dye penetration was greatest at the apex, extending along the suture in both directions towards the stem end. In the larger eastern variety walnuts, the dye also penetrated frequently at the stem end. The final check also demonstrated that, when a separated suture existed, spoilage organisms had penetrated the walnuts prior to this examination, causing the observed spoilage. It is certain that the suture, even in an apparently sealed walnut, is a primary route by which microorganisms enter and contaminate the walnut meats.

Bacteriological examination of commercial nut meats. Fourteen black walnut meat samples, consisting of commercial grade sizes Midget, Small, Medium, and Large, were tested (Table 4).

When all grades are considered, the standard plate count ranged from 31,000 to 2,000,000 bacteria per g, with both extremes found in the Medium grade. The high count of Midget was 180,000, a count comparable to 175,000 in Large. The usual expectation that the smaller sizes tend to have the greater number of bacteria was not evident from these results. However, the number of samples examined may not have been sufficient.

The number of bacteria obtained on E M B Agar was generally one-half, with a range of one-third to two-thirds, the standard count. The number on E M B Agar was usually 10 times the MPN obtained in LB, with exceptions of over 200 times the MPN in LB.

The boric acid medium results were perhaps more significant. The MPN of *E. coli* per gram (average of positive samples for each grade) of nuts from one processor was 7.9 in Midget, 2.8 in Medium, and 2.3 in Large. The results with nuts from another processor were 3.3 in Midget, 25 in Small, 2.4 in Medium, and 0.7 in Large. (This processor also had the lowest incidence of *E. coli* in the whole black walnuts.) Here it is evident that

TABLE 4. *Bacteriological examination of black walnut meats*[a]

Sample	Grade	Standard plate count	E M B count	LB MPN	BALB MPN (*E. coli*)
24a	Midget	180,000	96,000	11,000	3.3
23b	Midget	68,000	48,000	17,000	7.9
26a	Small	1,090,000	510,000	5,420	25.0
27a	Small	95,000	27,000	17,000	0
28a	Medium	31,000	15,200	2,300	0
29a	Medium	68,000	46,000	54,200	0.4
30b	Medium	33,000	17,000	1,300	3.3
31b	Medium	2,040,000	1,680,000	7,900	0
32b	Medium	153,000	55,000	3,300	4.9
33b	Medium	410,000	220,000	34,800	2.3
34b	Medium	50,000	28,000	3,300	0.8
35a	Large	47,000	38,000	330	0
36a	Large	175,000	124,000	3,480	0.7
37b	Large	43,000	30,000	1,720	3.2

[a] Results expressed as number of organisms per gram.

TABLE 5. *Bacteriological examination of walnut meats after heating in water or coconut oil*

Time of exposure	Boiling water		Coconut oil (100 C)		Coconut oil (125 C)		Coconut oil (150 C)	
	MPN/g	EMB count/g	MPN/g	EMB count/g	MPN/g	EMB count/g	MPN/g	EMB count/g
min								
0	2.4×10^5	9.8×10^5	—[a]	3.6×10^6	2.4×10^5	9.8×10^5	—	1.0×10^6
0.25							—	1,230
0.50			—	3,400	22.1	900	0	0
0.75	0	0			3.3	230	0.8	0.33
1.00	0	0	—	1,200	0.2	4	0	0
1.25					0	0	0	0
2.00	0	0	34.8	490				
3.00	0	0	1.70	54.6				
3.50	0	0	0	0				
4.00	0	0	0	0				
4.50			0	0				
5.00	0	0						
6.00	0	0						

[a] All tubes *E. coli*-positive.

the smallest sizes contained the greatest numbers of *E. coli* per gram. High standard plate counts did not necessarily indicate that *E. coli* would also be present.

Methods for decontamination of meats. The results of the two methods of treatment attempted for the reduction of *E. coli* from walnut meats are summarized in Table 5.

Black walnut meats were immersed in boiling water for periods of 45 sec in two trials, 60 sec in two trials, and 2, 3, 4, 5, and 6 min in one trial each. All treatments caused destruction of *E. coli* To prevent the development of mealiness due to the soft wet meats, they were rapidly dried in circulating dry heat. Exposures of 5, 10, and 15 min all resulted in meats with identical physical appearances. The broken meat surfaces appeared translucent; the skin was partially bleached, particularly along the veins and skin edges. After a few days of storage, the white meat surface changed to a yellow-brown color, a probable result of water-soluble tannin being deposited on the broken meat surfaces during drying. The flavor of these heat-treated meats was also milder than that of the original untreated meats.

E. coli was recovered from black walnut meats heat-treated by immersion in coconut oil at 100 C in all cases except after heat exposure of 3.5 min or more. The broken meat surfaces were slightly translucent, and the entire meat had an oily appearance that disappeared later. There was no change in the flavor as far as could be ascertained. At 125 C, the 60-sec exposure was inadequate but 75 sec was lethal. After treatment at 150 C, viable *E. coli* remained after 30 and 45 sec. These two higher temperatures resulted in a distinct roasted odor and a noticable number of loose skin fragments in addition to the translucence noted after the 100 C treatment.

DISCUSSION

The results presented here suggest that a tight suture and firm husk provide the walnut with an effective barrier to meat contamination. After husk deterioration, the shell also begins to dehydrate; during this process the two halves tend to separate, a separation that is not always visible to the eye. Following deterioration of the protective husk, and development of hairline suture separations, the walnut meats can be exposed to a diversity of microbes.

The movement of contaminated liquid into the walnut could be by capillary action or by internal aspiration resulting from a lower ambient temperature in the environment, as when warm walnuts are exposed rapidly to cold water during the hulling operation. The waste water samples from commercial hulling operations clearly demonstrate that significant numbers of *E. coli* and other microorganisms do contaminate the walnut husk. With a high count of *E. coli* in the exterior liquid, only a comparatively short period of time is required for susceptible walnuts to become contaminated in-shell.

Since good manufacturing practice with strict in-plant sanitary measures, including chlorination of hulling and wash waters, did not prevent in-shell contamination of meats, another method of control is indicated. Two possibilities exist: use of hot (80 to 90 C) hulling water or gas fumigation of the unhulled nuts prior to the hulling operation. Neither has been applied under commercial conditions to our knowledge.

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