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Comparison of Homogenizing, Shaking, and Blending on the Recovery of Microorganisms and Endotoxins from Fresh and Frozen Ground Beef as Assessed by Plate Counts and the *Limulus* Amoebocyte Lysate Test†

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Of three methods studied, brisk shaking of samples in dilution blanks by hand and homogenization by a stomacher were compared relative to their capacity to recover both endotoxins and viable bacteria; blending with a Waring blender was compared with these two methods only on the recovery of viable cells. Aerobic plate counts were essentially the same by the three methods for fresh meats, with the stomacher producing slightly higher aerobic plate counts and significantly higher gram-negative counts determined by violet red bile agar. The stomacher produced significantly higher aerobic plate counts and violet red bile agar results on frozen meats than did shaking. Endotoxins were determined by the *Limulus* amoebocyte lysate test; results by shaking and stomacher on 15 single samples of frozen meat were identical. Of *Limulus* amoebocyte lysate-negative beef which was spiked with known endotoxin, a higher percentage of recovery was obtained with the stomacher. Although both aerobic plate counts and violet red bile agar counts were found by shaking and stomacher to decrease significantly in frozen meats, endotoxin content was not significantly affected. The stomacher was found to be the better method overall, especially when meats are to be examined for their content of viable gram-negative bacteria, endotoxins, or both.

It is well established that gram-negative bacteria are the most important microorganisms in refrigerator-stored fresh meats from the standpoint of keeping quality. This is true perhaps also from the standpoint of food-borne pathogens, since the gram-positive food poisoning types do not fare well in the presence of the normal gram-negative meat flora.

In a previous report from this laboratory (4), the *Limulus* amoebocyte lysate (LAL) test was shown to be an excellent 1-h test of fresh ground beef quality. This test is the most sensitive method known for detecting endotoxins of gram-negative bacteria. Since fresh refrigerated meats are spoiled by gram-negative bacteria, use of the LAL was prompted as a rapid test of meat quality. Preparatory to the analysis of fresh meats for their endotoxin content as a means of assessing the microbiological quality of such meats and to the development of a 1-h test for estimating all microorganisms in meats, it is necessary to determine the efficacy of several methods for the recovery of endotoxins from meats.

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The most widely used method for the recovery of microorganisms from food samples such as ground beef is homogenization of samples with suitable diluent in a high-speed Waring blender. This method, however, presents two problems for the recovery of endotoxins from meats to be assessed by the LAL test. First, all glassware, utensils, and diluents must be pyrogen free. This state is achieved by heating in a dry-air oven at 185°C for 3 h, a process which is deleterious to the blade assembly gaskets of the Waring blender container. The second drawback to the use of blending for LAL is the problem of obtaining a particle-free sample for LAL as previously noted (4). Both of these drawbacks were overcome previously by employing the brisk shaking by hand of samples in dilution blanks. Previous results indicated that this procedure was preferable to blending, especially when spoiled beef samples were tested.

More recently, the stomacher has found widespread use and acceptance as a means of homogenizing various foods for microbiological analyses. This technique, first described by Sharpe and Jackson (7), has been reviewed relative to food use by Andrews et al. (1), Emswiler et al.

(2), and Sharpe and Dudas (6). Most investigators have found it to produce counts comparable to those obtained by blending and to provide advantages over blending in other ways. This technique seems ideal for extracting endotoxins from meats, since the sample is homogenized in a sterile and nonpyrogenic polyethylene bag. In the absence of reports on its efficacy in this regard, this study was undertaken. The study also compares the stomacher to shaking of sample by hand for the recovery of endotoxins, and to homogenization by Waring blender for viable plate counts.

MATERIALS AND METHODS

Samples. The fresh ground beef was obtained over the counter at large independent and chain supermarkets in the Detroit metropolitan area. Within 1 h of purchase, the meat was removed from its store packages, placed in pans lined with new aluminum foil, and mixed with flamed and cooled spatulas. Aliquots for testing were weighed on squares of aluminum foil and placed directly into pyrogen-free dilution blanks. The unused samples were placed in sterile and pyrogen-free Nasco Whirl-Pak bags and stored in a freezer at −20°C until used later. These frozen aliquots were used only after freezer storage for 30 to 45 days. Aliquots of frozen samples for testing were removed as described above after thawing by immersion in cold water.

Extraction. Three extraction or recovery methods were employed: shaking by hand, homogenizing by stomacher, and homogenizing by Waring blender. When shaking by hand was emplcyed, an 11-g sample of meat was placed in 99 ml of pyrogen-free water in a dairy dilution bottle and shaken briskly at three intervals (25 times within 10 s) over a period of about 5 min. When samples were extracted by stomacher, the above procedure was followed except that the sample was placed in a Stomacher 400, employing stomacher plastic bags, for 2 min. For LAL analyses, 5 ml of the particle-free homogenates was frozen at −20°C until used. Blending was carried out on samples prepared as above except that the 11 g of meat and 99 ml of water were placed in sterile Waring blender cups and blended for 2 min at high speed.

Recovery of LPS from spiked beef. To obtain pyrogen-free ground beef, fresh retail cuts of standing rib roast and sirloin tip were obtained. The outer cut surface was seared with a red-hot spatula essentially as previously described (5). After the careful removal of the seared layers by flamed surgical blades, small chunks of beef were removed and placed in a depyrogenized container. The meat was ground in a meat grinder whose mechanical parts were rendered pyrogen free in an oven as noted above. Before use, the pyrogen-free status of the meat was determined on 1:10 extracts as indicated below. Eleven-gram samples of the ground beef were placed in 50-ml beakers, followed by injection (spiking) of reconstituted *Salmonella typhimurium* lipopolysaccharide (LPS) (Difco) to give a concentration of 1,000 ng/g. After about 1 h, the spiked samples were added to a 99-ml dilution blank containing pyrogen-free water and extracted as above. LAL titers were determined by doubling dilutions.

Plate counts. Aerobic plate counts (APCs) were determined by plating in duplicate with Difco plate count agar with incubation at 30°C for 48 h. Viable gram-negative bacteria were determined by use of the agar overlay method of Hartman et al. (3). Briefly, petri dishes were poured first with about 15 ml of plate count agar and allowed to harden. Sample aliquots were planted onto the plate count agar surface, and then freshly boiled violet red bile agar was poured. Incubation was at 30°C for 48 h.

LAL reagents and titers. Most of the LAL reagent employed in this study was obtained from Associates of Cape Cod (Woods Hole, Mass.) in 5-ml multi-test vials (50 tests per vial); the rest was obtained from Difco Laboratories (Detroit, Mich.). Upon rehydration of the freeze-dried reagent, its endotoxin sensitivity was determined by doubling dilutions of Food and Drug Administration certified reference *Escherichia coli* endotoxin standard (Associates of Cape Cod) using pyrogen-free distilled water as diluent. The sensitivity of the LAL preparation was defined as the highest dilution of endotoxin standard that caused formation of a firm gel that did not break when the vial was inverted 180°. To determine endotoxin content of meat samples, aliquots of the particle-free area of homogenates were serially diluted with water. To tubes containing 0.1 ml of LAL reagent were added an equal quantity of the diluted sample (usually from 10^{-2} to 10^{-6}). After mixing of tubes, they were placed in a water bath at 37°C for 1 h, after which they were examined for gelling. The highest dilution of sample that caused firm gel formation was recorded as the titer. Quantity of endotoxin (nanograms) was determined by multiplying the titer by the previously determined sensitivity of the LAL preparation used.

RESULTS

It is essential that all glassware, utensils, and diluents that are used to assess endotoxins and gram-negative bacteria by the LAL method be free of pyrogens. Preliminary testing of Whirl-Pak and Stomacher 400 bags showed that they are pyrogen free (negative to 0.08 ng-sensitive LAL). All pipettes employed either were depyrogenized in an oven or were sterile disposables that were pyrogen free. Similarly, unused aluminum foil was found to be free of pyrogens. The pyrogen-free meats used were all negative to 0.32 ng-sensitive LAL reagent. The pyrogen-free water employed was that which is sold commercially by a number of companies as "water for irrigation."

The relative efficacy of three procedures to recover endotoxins from spiked ground beef is presented in Table 1. Although the shaking of triplicate samples containing 0.391 ng each of added LPS gave uniform results, the percentage recovery was only about 82%. The shaking + stomacher samples were treated in the following way. The 11-g sample was placed first into 99 ml

of water and shaken. After removal of the 5-ml LAL sample, an equal volume of water was added to the blank, and the sample was then homogenized by stomacher. The percentage of recovery of LPS by this procedure was about 109%, indicating that shaking alone was insufficient to recover all of the added LPS. Samples that were added directly to the stomacher produced about a 136% recovery. Just why the stomacher samples yielded higher recoveries than the shaking + stomacher samples is not clear. Mean recovery by the three methods was around 109%. Overall, these results indicate that added LPS is not bound by fresh beef in such a way as to render it nonreactive or to reduce its extractability. It may be assumed that essentially all of the added toxin was recovered at least by two of the extraction procedures and that the 100%+ recovery figures are reflections of the doubling dilution procedure used to quantitate recovery. Also, the added LPS value of 0.391 ng was based on weight, whereas the recovered amounts are based on LAL determinations. Blending was not used to recover LPS because of the difficulty of rendering the containers pyrogen free without damaging the blade assembly gaskets.

TABLE 1. *Recovery of LPS from triplicate samples of spiked LAL-negative ground beef by shaking and by stomacher*

Method	Endotoxin (ng)		Avg	% Recovered
	Added	Recovered		
Shaking	0.391	0.32		
	0.391	0.32		
	0.391	0.32	0.32	82.0
Shaking + stomacher	0.391	0.32		
	0.391	0.64		
	0.391	0.32	0.427	109.0
Stomacher only	0.391	0.64		
	0.391	0.64		
	0.391	0.32	0.533	136.0
Average		0.427		109.0

The APCs and gram-negative counts obtained from 12 samples of fresh ground beef by shaking and by homogenization by Waring blender and stomacher are presented in Table 2. The LAL titers of endotoxins by shaking and by stomacher are also presented. The log APC mean of samples by shaking, stomacher, and blending was 6.38, 6.39, and 6.35, respectively, and the differences are not significant. The mean log plate counts of gram-negative bacteria were 5.79, 5.97, and 5.67/g, respectively, for shaking, stomacher, and blending. The violet red bile agar count differences are significant notably by shaking and stomacher ($P < 0.05$) and by stomacher and blending ($P < 0.02$). Since APC results by stomacher and blending did not differ significantly, this finding may indicate that gram-negative bacteria are more adversely affected by blending than gram-positives. The LAL titers by shaking and by stomacher were identical except for four samples. In three of these the titer was one decimal lower by stomacher, and the other was higher by one decimal. These differences, how-

TABLE 2. *APCs and gram-negative plate counts on fresh ground beef samples determined by shaking, blending, and homogenizing along with endotoxin titers by shaking and homogenizing*

Sample no.	Sample type[a]	APC			VRBA[b]			LAL titer[c]	
		Shake	Stomacher	Blender	Shake	Stomacher	Blender	Shake	Stomacher
074	HB	7.44	6.45	6.40	6.24	6.11	6.00	10^3	10^3
075	GR	5.72	5.68	5.76	5.07	5.13	5.15	10^2	10^2
076	HB	6.11	6.32	6.30	5.46	5.52	5.30	10^3	10^3
077	HB	6.95	6.98	6.92	6.15	6.49	6.59	10^4	10^4
078	GC	6.36	6.40	6.40	5.91	6.30	5.90	10^4	10^3
079	HB	6.45	6.60	6.52	6.61	6.95	6.28	10^3	10^4
080	GC	6.20	6.23	6.18	5.77	6.04	5.48	10^3	10^3
081	HB	6.26	6.40	6.34	5.42	6.00	4.85	10^4	10^3
082	HB	6.20	6.46	6.18	5.32	5.30	5.04	10^3	10^3
083	GR	5.45	5.28	5.20	4.08	3.90	4.08	10^2	10^2
084	HB	6.84	7.15	7.20	7.08	7.15	6.95	10^5	10^4
085	GR	6.58	6.73	6.81	6.40	6.77	6.46	10^4	10^4
Mean		6.38	6.39	6.35	5.79	5.97	5.67		

[a] HB, Hamburger meat; GC, ground chuck; GR, ground round.
[b] VRBA, Violet red bile agar.
[c] LAL sensitivity was 0.32 ng.

ever, are not significant. LAL reagent from the same lot was used for titration; each paired sample tested was incubated in the water bath at the same time. LAL titers were not done from blended samples for the reason stated above. Although shaking was less effective than the stomacher in recovering LPS from spiked beef, the two procedures did not differ greatly in the recovery of gram-negative bacteria. This suggests that endotoxins in meats occur as gram-negative cells more than as LPS fractions.

Table 3 presents data on the recovery of bacteria and endotoxins from 15 samples of frozen ground beef. Mean APCs by stomacher were slightly higher than shaking (log 5.97/g versus 5.70/g), and the difference is significant ($P < 0.01$). In a similar manner, gram-negative numbers by stomacher were slightly higher than by shaking (log 5.11/g versus 4.86/g), and the differences are significant ($P < 0.01$). In the case of LAL titers, shaking and homogenization by stomacher gave identical titers on paired samples in spite of the higher stomacher counts. With these 15 samples, the same aliquots were subjected to shaking and to stomacher (shaking + stomacher). The identical LAL titers here suggest that the different titers noted in Table 2 are the result of sampling more than of differences between the shaking and stomacher methods.

Since the LAL results of frozen samples were all identical by shaking and by stomacher, 21 paired fresh and frozen samples of ground meat were tested by shaking alone in an effort to determine both the effect of aliquot variance and that of freezing on counts and titers. It can be seen from Table 4 that the APCs of fresh and frozen samples differed significantly (mean of log 6.32 for fresh and 5.86 for frozen, $P < 0.01$). Gram-negative counts differed also but to a lesser extent (log 5.59 for fresh versus 5.15 for frozen). With respect to LAL titers, four samples showed a lower titer after freezing, whereas two increased in titer, all by one dilution. Overall, these differences in titer are not significant. Since freezing is not known to affect endotoxins and since all of these were done by the same procedure, the six samples that produced different titers between fresh and frozen may be presumed to be the result of aliquot differences, since separate aliquots of fresh and frozen meats were tested. Although freezing resulted in significant decreases in viable counts, the endotoxin content remained essentially the same.

The reproducibility of the LAL test in determining endotoxin titers of ground meats was excellent. Generally a frozen homogenate of previously determined titer was run each time a new series of meat homogenates was titrated, and in each instance the titers were identical as long as LAL reagent of the same lot number was used. In a similar manner, frozen homogenates

TABLE 3. *Relative efficacy of shaking by hand and using a stomacher on recovery of bacteria and endotoxins from frozen ground beef*

Sample no.	Sample type[a]	PCA[b]		VRBA[c]		LAL titer[d]	
		Shaking	Stomacher	Shaking	Stomacher	Shaking	Stomacher
057	HB	6.32	6.70	6.11	6.26	10^4	10^4
058	GR	6.49	7.00	6.30	6.51	10^4	10^4
059	HB	5.28	5.36	3.71	3.73	10^2	10^2
060	GR	6.62	6.68	5.83	6.11	10^4	10^4
061	HB	6.51	6.78	6.08	6.42	10^4	10^4
062	GR	5.78	6.20	4.28	5.08	10^2	10^2
063	GC	5.81	6.11	4.51	4.99	10^3	10^3
064	HB	3.90	4.11	3.43	3.34	10	10
065	GR	4.91	5.54	4.58	4.76	10	10
066	GC	4.60	5.08	4.04	4.28	10	10
067	HB	6.18	6.28	4.51	4.70	10^3	10^3
068	GR	5.72	5.77	4.84	5.04	10^2	10^2
069	GC	6.15	6.40	4.64	4.99	10^3	10^3
072	HB	5.08	5.20	4.60	4.90	10^2	10^2
073	GR	6.08	6.34	5.43	5.51	10^3	10^3
Mean		5.70	5.97	4.86	5.11		

[a] See Table 2, footnote *a*.
[b] PCA, Plate count agar.
[c] VRBA, Violet red bile agar.
[d] LAL sensitivity was 0.32 ng.

TABLE 4. *Effect of freezing of fresh ground beef on aerobic and gram-negative plate counts and on endotoxin titers as determined by shaking*

Sample no.	Sample type[a]	PCA[b]		VRBA[c]		LAL titer[d]	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
027	HB	8.28	6.79	6.92	6.61	10^3	10^4
029	HB	6.90	6.72	6.83	6.75	10^4	10^4
030	GC	7.40	6.89	7.34	6.82	10^4	10^4
032	GC	<5.30	4.61	4.63	4.15	10^2	10^2
033	LP[e]	<5.43	4.80	<4.30	3.79	10^2	10^2
034	GC	6.08	5.68	5.81	4.99	10^4	10^3
035	GR	<5.48	3.94	<4.48	2.98	10^2	10^2
036	HB	5.85	5.76	4.87	4.65	10^2	10^3
037	GR	6.32	5.82	5.23	5.11	10^3	10^3
038	HB	7.11	7.11	6.92	6.95	10^5	10^5
039	GR	5.91	5.76	4.93	4.52	10^2	10^2
040	GC	5.94	5.62	4.79	4.53	10^2	10^2
041	HB	6.38	5.94	5.93	5.61	10^3	10^3
042	GR	5.79	5.36	5.32	4.60	10^2	10^2
043	HB	6.25	5.42	5.69	4.78	10^3	10^3
044	GC	6.05	5.68	5.17	5.04	10^3	10^3
045	GR	6.06	5.84	5.54	5.26	10^3	10^3
046	HB	6.88	6.71	6.00	5.85	10^4	10^4
048	GR	6.28	6.08	5.32	4.02	10^3	10^2
055	HB	6.90	6.70	6.20	5.87	10^4	10^3
056	GC	6.15	5.77	5.24	5.20	10^3	10^2
Mean		<6.32	5.86	<5.59	5.15		

[a] See Table 2, footnote *a*.
[b] PCA, Plate count agar.
[c] VRBA, Violet red bile agar.
[d] LAL sensitivity was 0.32 ng.
[e] LP, Lamb patties.

which were thawed and refrozen at least three times over a period of several weeks all produced identical titers.

DISCUSSION

The data generated by this study indicate that the stomacher produces higher endotoxin recovery and slightly higher viable plate counts on fresh and frozen ground meats than do blending with a Waring blender and brisk shaking of dilution bottle by hand. The latter, however, produced microbial counts very similar to those of the stomacher. In regard to LAL titers on meats, shaking and stomacher results appear to be similar, although the latter method was more efficient in recovering spiked endotoxins. The 4 of 12 fresh samples that produced titers which differed by one tube by the two methods were most likely the result of aliquot variance, since a similar degree of variance was found between 21 paired fresh and frozen samples all examined by shaking (Table 3). When aliquot variance was eliminated, all 15 samples produced identical LAL titers by shaking and by stomacher (Table 2). Although freezing resulted in viable plate count decreases, endotoxin titers remained essentially unchanged. This suggests that the LAL method can be used to assess the degree of freezer death of gram-negative bacteria in frozen meats.

Overall, the stomacher is an excellent device for extracting both microorganisms and endotoxins from fresh and frozen ground meats. It offers the advantage of more consistency of treatment of samples than shaking by hand. Also, the 2-min treatment is shorter than the overall time used for shaking, and the disposable pyrogen-free bags lend themselves to freezer storage of samples for repeat testing better than do dairy dilution bottles. On the other hand, homogenates from the stomacher are difficult to pipette, as are those after blending. However, the use of large-bore pipettes minimizes this problem.

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