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Determining Endotoxin Content of Ground Beef by the *Limulus* Amoebocyte Lysate Test as a Rapid Indicator of Microbial Quality

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Eighty-four samples of ground beef were placed into five half-log cycle groups based upon aerobic plate count (APC) results. Endotoxins were determined by the *Limulus* amoebocyte lysate test (LAL), and gram-negative viable counts were determined by a violet red bile agar overlay method. Ten samples with a log of APC of <5.50 had an APC mean of <5.24 and mean endotoxin content by the LAL of 51 ng/g. The 15 samples with APCs between a log of 5.50 and 5.99 had an APC mean of 5.79/g and an endotoxin mean of 103.8 ng/g. Twenty-eight samples had APCs between a log of 6.00 and 6.49 with a mean of 5.28/g and an endotoxin mean of 1106.4 ng/g. The 20 samples with APCs between a log of 6.50 and 7.00 had a mean of 6.77/g and an endotoxin mean of 5067.6 ng/g, while 11 samples had a log of APCs of >7.00 with a mean of 7.53 and an endotoxin mean of 7,472 ng/g. Correlation of half-log cycle mean APC and violet red bile agar counts with mean endotoxin content were both highly significant, indicating that LAL-determined endotoxin content can be used to make a rapid approximation of viable plate counts. Because results can be obtained by LAL in 1 h, the finding of low levels of endotoxins can be taken to indicate low-count meat. The use of additional tests of microbial quality may be necessary when high endotoxin levels are found because the LAL detects both viable and nonviable cells.

A large number of objective tests have been developed and proposed for assessing the microbial quality of fresh meats, especially ground beef. Among the simpler and more rapid techniques are several that were described in this laboratory such as the extract-release volume (ERV) method (2), water-holding capacity by filter paper press method (3), amino sugar determination (6), and others. More recently, the *Limulus* amoebocyte lysate test (LAL) for endotoxins of gram-negative bacteria was suggested as another simple and rapid objective test (4). In the latter report, LAL was shown to respond to increases in the numbers of gram-negative psychrotrophic bacteria as ground beef was held at 5°C until spoilage had occurred and to correlate highly with plate counts and ERV. Employing LAL reagents with a mean endotoxin sensitivity of around 1.0 ng, it was found that low-count beef produced a negative LAL at a 10^3 dilution (<1,000 ng of endotoxin per g). With the recent licensing by the U.S. Food and Drug Administration of several companies that make LAL reagents and endotoxin standards, it is now possible to obtain reagents that not only give more consistent results but which can be used to better quantitate endotoxin levels in various products.

LAL as a test of fresh meat microbial quality offers several advantages over other quality tests. It is simple to perform, requires only 1 h, and responds to the potentially harmful endotoxins. Some of the potential medical implications of the latter have been reviewed and discussed by Sullivan et al. (7). These substances are abundant in the gastrointestinal tract, and under normal conditions are prevented from entering the blood-stream of healthy individuals by permeability barriers. Under conditions of shock, however, permeability barriers may be decreased, making it possible for endotoxins to enter the bloodstream. In the rare event that the latter occurs, pyrogenic response and death may result.

In this study, LAL has been employed to measure the quantity of endotoxin in 84 samples of fresh ground beef. The LAL-determined values have been correlated with half-log cycle viable plate counts and with ERV test results.

MATERIALS AND METHODS

All samples of fresh ground beef employed were obtained from retail stores, and when in the laboratory they were treated as previously described (5). The 84 samples consisted of ground beef or hamburger meat (ca. 30% fat) and ground round and chuck (ca. 20%

fat). They were purchased from 31 large independent and chain supermarkets with no more than one purchase within a 30-day period from the same store.

The ERV test was employed as previously described (2). By this test, fresh beef of good microbial quality produces a value of 25 ml or more. The plating procedures, acquisition of LAL-negative beef, and the recovery and quantitation of endotoxins were all carried out as previously described (5). To recover cells and endotoxins, brisk shaking of samples by hand was employed for samples 001 to 073 and blending by stomacher for samples 074 to 085. Statistical analysis revealed that APC results and LAL titers on fresh meats by these two methods did not differ significantly (5).

The LAL reagent employed throughout most of the study was obtained from Associates of Cape Cod (Woods Hole, Mass.) and had a sensitivity of 0.32 ng when tested with Food and Drug Administration-certified reference *Escherichia coli* endotoxin. Some LAL reagent was obtained from Difco Laboratories (Detroit, Mich.), and this had a sensitivity of 0.08 ng. In each case, the reagents were used per manufacturer's instructions.

RESULTS

The relationship between the growth of a fluorescent pseudomonad in LAL-negative beef at 5°C over a 13-day period along with LAL titers is presented in Fig. 1 from which it can be seen that LAL titers paralleled the growth curve during the logarithmic phase. LAL titers remained high on days 10 and 11 when the viable count began to decrease because LAL measures endotoxins from both viable and nonviable cells. The beef employed was obtained from a standing rib roast, and by the special handling procedures employed the ground beef that was obtained had an APC of <10/g and was negative to LAL with an endotoxin sensitivity of 0.08 ng. The LAL preparations employed in these titrations for the initial and day-2 samples had a sensitivity to *E. coli* endotoxin of 0.08 ng, while that which was employed from days 4 to 13 was 0.32. The quantity of endotoxin present can be determined by multiplying these values by the respective titers. For example, 80 ng of endotoxin was present at day 2 ($0.08 \text{ ng} \times 10^3$), 3,200 ng was present at day 4 (0.32×10^4), etc. The correspondence of endotoxin titers with growth rate indicates the feasibility of employing endotoxin detection as a rapid estimate of numbers of gram-negative bacteria, assuming that the bacterial cells are in the logarithmic phase of growth. The correlation coefficient (r) between viable cells and endotoxin over the 13-day period was highly significant ($r = 0.907$, $P < 0.01$).

The 84 retail store ground beef samples have been placed into five groups based upon APC. The 10 that had a log of APCs of <5.50/g are presented in Table 1. The mean ERV of these samples was 32.2 with a range of 26 to 40. The log of APC range was <4.30 to 5.48 with a mean of <5.24, while the VRBA range was <3.00 to 5.04 with a mean of <4.41/g. In regards to endotoxin, the range was 3.13 to 313 with a mean of 51 ng/g. Only 2 of these 10 samples were hamburger meat.

The 15 samples that had APCs of a log of 5.50 to 5.99 are presented in Table 2. ERV values ranged from 27 to 54 with a mean of 32.9. The APCs ranged from a log of 5.53 to 5.94 with a mean of 5.79, while VRBA counts ranged from 4.20 to 5.35 with a mean of 4.97. Endotoxin content of the 15 samples ranged from 3.13 to 313 with a mean of 103.8 ng/g. Although this range is the same as that for the 10 samples whose log APCs were 5.50, only 1 sample in this group had 3.13 ng/g. As is the case with the 10 lowest count samples, the majority of these 15 consisted of ground round and chuck.

The largest number of the 84 samples had APCs that fell within the range of 6.00 to 6.49, and these 28 samples are presented in Table 3. The ERV ranged from 20 to 42 with a mean of 32.1. While an ERV of 20 is below the value that edible quality ground beef normally produces, other parameters of this sample suggested that it was of edible quality, and, consequently, it was not rejected. APCs ranged from a log of 6.05 to 6.58 with a mean of 6.28, while VRBA counts ranged from a low of 3.54 to 6.61 with a mean of 5.47. Endotoxin content of these samples cov-

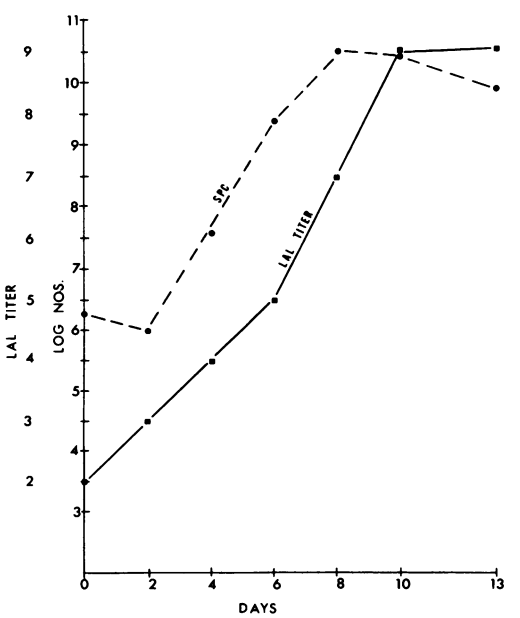

FIG. 1. *Growth and endotoxin production of a fluorescent pseudomonad in LAL-negative ground beef at 5°C for 13 days.*

TABLE 1. *ERV, microbial numbers, LAL titers, and endotoxin content of 10 ground beef samples with APCs of less than a log of 5.50*

Sample no.	Type[a]	ERV	APC	VRBA	LAL titer	Endotoxin (ng/g)
018	GC	33	5.08	4.75	10^2	31.3
031	HB	40	5.48	4.86	10^2	31.3
032	GC	37	<5.30	4.63	10^2	31.3
035	GR	30	<5.48	<4.48	10^2	31.3
054	GR	33	<5.30	<3.85	10^3	313
064	HB	26	<4.30	<3.00	10	3.13
065	GR	28	<5.32	4.64	10	3.13
066	GC	28	<5.23	4.81	10	3.13
073	GR	35	5.42	5.04	10^2	31.3
083	GR	—[b]	5.45	4.08	10^2	31.3
Mean	—	32.2	<5.24	<4.41	—	51.0
Standard deviation	—	4.6	0.35	0.62	—	—

[a] GC, ground beef; HB, hamburger meat; GR, ground round.
[b] —, No data.

TABLE 2. *ERV, microbial numbers, LAL titers, and endotoxin content of 15 ground beef samples with APCs of a log of 5.50 to 5.99/g.*

Sample no.	Type[a]	ERV	APC	VRBA	LAL titer	Endotoxin (ng/g)
012	GC	27	5.81	5.04	10^3	313
015	HB	37	5.61	5.02	10^2	31.3
019	GR	30	5.85	4.66	10^2	31.3
024	GC	31	5.87	5.35	10^3	313
028	GR	29	5.79	5.32	10^3	313
036	HB	54	5.85	4.87	10^2	31.3
039	GR	30	5.91	4.93	10^2	31.3
040	GC	33	5.94	4.79	10^2	31.3
042	GR	35	5.79	5.32	10^2	31.3
049	HB	31	5.53	5.15	10^3	313
052	GC	28	5.90	4.72	10^2	20
053	HB	36	5.78	4.94	10^2	31.3
059	HB	30	5.56	4.20	10	3.13
068	GR	29	5.94	5.11	10^2	31.3
075	GR	—[b]	5.72	5.07	10^2	31.3
Mean	—	32.9	5.79	4.97	—	103.8
Standard deviation	—	6.8	0.13	0.29	—	—

[a] See footnote *a* of Table 1.
[b] —, No data.

TABLE 3. *ERV, microbial numbers, LAL titers, and endotoxin content of 28 ground beef samples with APCs of a log of 6.00 to 6.49/g*

Sample no.	Type[a]	ERV	APC	VRBA	LAL titer	Endotoxin (ng/g)
004	GR	35	6.32	(4.81)	10^4	3,125
006	HB	36	6.48	(5.46)	10^3	313
007	HB	38	6.11	(4.56)	10^3	313
008	GR	36	6.40	(5.65)	10^3	313
011	HB	39	6.42	5.85	10^4	3,125
016	GC	30	6.39	5.57	10^4	3,125
034	GC	29	6.08	5.81	10^4	3,125
037	GR	29	6.32	5.23	10^3	313
041	HB	31	6.38	5.93	10^3	313
043	HB	30	6.25	5.69	10^3	313
044	GC	36	6.05	5.17	10^3	313
045	GR	39	6.06	5.54	10^3	313
047	GC	29	6.08	5.23	10^3	313
048	GR	20	6.28	5.32	10^3	313
056	GC	38	6.15	5.24	10^3	313
062	GR	32	6.30	5.30	10^3	313
063	GC	27	6.42	5.32	10^4	3,125
067	HB	27	6.36	5.15	10^3	313
069	GC	29	6.36	5.18	10^3	313
070	GR	28	6.42	3.54	10^2	31.3
071	GC	26	6.38	5.86	10^4	3,125
072	HB	42	6.20	5.85	10^3	313
076	HB	—[b]	6.11	5.46	10^3	313
078	GC	—	6.36	5.91	10^4	3,125
079	HB	—	6.45	6.61	10^3	313
080	GC	—	6.20	5.77	10^3	313
081	HB	—	6.26	5.42	10^4	3,125
082	HB	—	6.20	5.32	10^3	313
Mean	—	32.1	6.28	5.47	—	1,106.4
Standard deviation	—	5.4	0.13	0.54	—	—

[a] See footnote *a* of Table 1.
[b] —, No data.

ered a 2-log cycle range of 31.3 to 3,125 with a mean of 1,106.4. Only 1 sample contained 31.3 ng, while 19 samples contained 313 ng and 8 samples contained 3,125 ng/g. Eleven of the samples were hamburger meat, while the other 17 were ground round and chuck.

The second largest number of the 84 samples had APCs between a log of 6.50 and 7.00/g (Table 4). The ERV of the 20 samples had a range of 23 to 42 with a mean of 33.4, a value that is slightly higher than for the lower count samples. The one sample whose ERV was below 25 did not show any signs of organoleptic offness. In regards to APCs, the range was a log of 6.51 to 7.00 with a mean of 6.77, while VRBA counts ranged from 5.20 to 7.08 with a mean of 6.23. Although endotoxin values for these 20 samples had the widest range (20 to 31,250), only two

TABLE 4. *ERV, microbial numbers, LAL titers, and endotoxin content of 20 ground beef samples with APCs of a log of 6.50 to 7.00/g*

Sample no.	Type[a]	ERV	APC	VRBA	LAL titer	Endotoxin (ng/g)
001	HB	30	6.62	(5.48)	10^3	313
009	HB	31	6.51	(5.25)	10^3	313
013	GR	31	6.81	6.26	10^4	3,125
014	GC	37	7.00	6.24	10^4	3,125
017	HB	33	6.69	5.66	10^3	313
020	HB	39	6.88	6.45	10^4	3,125
021	GC	38	6.67	5.99	10^4	3,125
022	HB	38	6.57	5.48	10^4	3,125
023	HB	38	6.86	5.94	10^4	3,125
025	HB	34	6.81	6.20	10^4	3,125
029	HB	42	6.90	6.83	10^4	3,125
046	HB	29	6.88	6.00	10^4	3,125
051	GR	23	6.64	5.76	10^2	20
055	HB	32	6.90	6.20	10^4	3,125
057	HB	27	6.72	6.40	10^3	80
060	GR	30	6.85	6.54	10^3	313
061	HB	36	6.76	6.52	10^5	31,250
077	HB	—[b]	6.95	6.15	10^4	3,125
084	HB	—	6.84	7.08	10^5	31,250
085	GR	—	6.58	6.40	10^4	3,125
Mean	—	33.4	6.77	6.23	—	5,067.6
Standard deviation	—	4.9	0.14	0.38	—	—

[a] See footnote *a* of Table 1.
[b] —, No data.

were below 313 ng/g, while 12 or 60% had 3,125 with a mean of 5,067.6 ng/g. Repeat testing of frozen aliquots of the extremely low and high samples in this group, as well as of other groups, confirmed the values indicated. Unlike the lower count samples, 14 or 70% of the 20 in this APC range consisted of hamburger meat.

The 11 highest counts of the 84 samples are presented in Table 5. The APC range of these samples was from a log of 7.08 to 8.28/g with a mean of 7.53. ERV ranged from 24 to 36 with a mean of 31.4, only slightly lower than that for the lower-count meats. The VRBA counts on seven of these averaged a log of 6.89/g. VRBA counts on the first four samples in the table were done by direct pour plating with sterilized VRBA, a procedure later shown to give lower counts of gram-negative bacteria than the overlay procedure employed for all others. Endotoxin titers for these high count meats were higher than for the other groups with a range of 313 to 31,250 and a mean of 7,472 ng/g.

A summary of the 84 samples by APC range relative to ERV, mean APC, mean VRBA, and mean endotoxin content is presented in Table 6. It can be seen that mean ERV values for the five APC ranges were quite similar and consequently of no value in predicting endotoxin content. On the other hand, the aerobic and VRBA plate count means both correlated significantly with mean endotoxin content (for mean APC and endotoxin, $r = 0.945$, $P < 0.02$; for mean VRBA and endotoxin, $r = 0.972$, $P < 0.01$). These data suggest that when endotoxin values are determined by LAL, the values can be used to estimate the approximate APC range for the product.

DISCUSSION

As a rapid test of fresh ground beef microbial quality, LAL shows excellent potential. In a previous report (4), ERV was shown to correlate highly with LAL titers of ground beef when it was held from freshness to spoilage at refrigerator temperatures. In the present study, ERV did not correlate with either LAL titers or viable plate count results. ERV is clearly more effective as an indicator of microbial quality of fresh meats in which psychrotrophic gram-negative bacteria have actually grown than for fresh meats where a sufficient amount of low-temperature growth has not occurred.

TABLE 5. *ERV, microbial numbers, LAL titers, and endotoxin content of 11 ground beef samples with a log of APCs of >7.00/g*

Sample no.	Type[a]	ERV	APC	VRBA	LAL titer	Endotoxin (ng/g)
002	HB	35	8.26	(7.75)	10^4	3,125
003	HB	34	7.94	(7.23)	10^4	3,125
005	HB	36	7.24	(6.16)	10^4	3,125
010	HB	30	7.18	(5.99)	10^3	313
026	GR	24	7.32	7.20	10^4	3,125
027	HB	29	8.28	6.92	10^3	313
030	GC	28	7.40	7.34	10^4	3,125
038	HB	34	7.11	6.92	10^5	31,250
050	GR	36	7.57	7.11	10^5	31,250
058	GR	28	7.08	6.52	10^4	3,125
074	HB	—[b]	7.44	6.24	10^3	313
Mean	—	31.4	7.53	6.89	—	7,472
Standard deviation	—	4.1	0.44	0.39	—	—

[a] See footnote *a* of Table 1.
[b] —, No data.

TABLE 6. *Summary of endotoxin content of 84 samples of ground beef by APC range relative to ERV and a log of APC and VRBA means*[a]

APC range	ERV	No. of samples	Mean APC	Mean VRBA	Mean endotoxin (ng/g)
<5.50	32.2	10	<5.24	<4.41	51.0
5.50–5.99	32.9	15	5.79	4.97	103.8
6.00–6.49	32.1	28	6.28	5.47	1,106.4
6.50–7.00	33.4	20	6.77	6.23	5,067.6
>7.00	31.4	11	7.53	6.89	7,472.0

[a] r between APC and endotoxin = 0.945 ($P < 0.02$); r between VRBA and endotoxin = 0.972 ($P < 0.01$).

To assess the relative amounts of endotoxin, APC values were employed to group the samples of beef rather than VRBA counts. Ideally the latter counts would be used because endotoxins are produced by gram-negative bacteria for which the VRBA was employed. Preliminary results showed that VRBA alone was inhibitory to at least part of the meat gram-negative flora and that much of this inhibition could be overcome by use of the VRBA overlay method of Hartman et al. (1) as previously described (5). Except for the highest count samples in Table 5, APC results produced consistently lower standard deviations than did VRBA. Further work relating viable gram-negative counts more accurately to endotoxin content must await the development of plating procedures that better permit the growth of all meat-borne gram-negative bacteria.

The amounts of endotoxin recorded for the 84 samples of beef are higher than can be accounted for by viable plate counts, and this is because the LAL reagent detects both viable and nonviable cells. It may be expected that some nonviable gram-negative cells exist in products of the type in question, but the relative incidence of nonviable to viable cells is not known at this time. From the growth curve in Fig. 1, it can be seen that an endotoxin content of 3,200 ng/g occurred with a viable plate count of a log of 7.56 after 4 days. After 6 days, 32,000 ng of endotoxin occurred with a viable plate count of a log of 9.34/g. Because these are logarithmic phase cells, it may be assumed that essentially no nonviable cells were present. These viable plate counts are higher than those from retail store ground beef for comparable endotoxin levels. The existence of nonviable cells in the store samples is one possible explanation for this. It is also possible that this reflects a difference in the LAL sensitivity of members of the more varied flora that normally exists in ground beef.

With respect to the use of endotoxin content to make a half-log cycle estimate of APC, the results indicate that this is possible even though the degree of error is a bit high with this limited number of samples. Of the 25 samples that had APCs of <6.00 (Tables 1 and 2) where the mean endotoxin content was 83 ng/g, 5 had 313 ng of endotoxin per g. Most samples with 313 ng fell within the APC range of a log of 6.00 to 7.00. On the other hand, only 3 of the 48 samples that were within the range of a log of 6.00 to 7.00 contained 313 ng of endotoxin per g (mean content per gram of these 48 samples was 2,757 ng). The use of decimal dilutions for endotoxin titrations is one reason for some of these discrepancies. Standard deviations for endotoxin values would be larger than the mean values largely because of decimal differences. Doubling dilutions would undoubtedly lead to less variable results, but this procedure would be considerably more expensive. Decimal dilutions were chosen primarily because they are used in standard plate count procedures. The use of more precise gram-negative viable counts to relate endotoxin content rather than APC would perhaps make for less variability within half-log cycle ranges. Also, a much larger number of samples would provide a broader base and make the application of statistical treatments more valid.

Some of the problems that may be encountered in the use of LAL to quantitate endotoxins in specimen have been raised and discussed by Sullivan et al. (7). The variations that exist in the sensitivity of different endotoxins to the same LAL preparation is one of these problem areas. For example, the above authors found that the quantity of endotoxin from 21 different cultures required to produce a positive LAL ranged from 0.06 to 0.31 ng. For 10 strains of *E. coli* the range was 0.15 to 0.31 ng. This problem was overcome in the present study by employing only one reference endotoxin.

In regards to the relative costs of doing serial versus decimal dilution analyses of endotoxins by LAL, the latter is the least expensive because fewer tests are run. When 5-ml multitest vials of LAL reagent are employed, 50 test vials of 0.1 ml can be prepared at a cost of about $0.70/vial. (Some commercially available single-test vials currently cost about $3.00 each). When multitest vial preparations are used, five decimal dilutions can be run in duplicate by using 10 vials at a cost as low as $7.00. On the other hand, if serial doubling dilutions are used for duplicate determinations, from 24 to 30 vials are needed to reach a dilution of 10^6.

Overall, fresh ground beef that had low endotoxin levels had low aerobic and gram-negative plate counts. Because LAL produces results in only 1 h, the finding of low levels of endotoxin would suggest a product of excellent microbial quality. Although the finding of higher levels of endotoxin by LAL may not reflect poor quality meat, further tests of microbial quality would be indicated. The determination of more precise values for edible and inedible quality meats must await more data from a larger sample base where the same reference endotoxin is used. Further work along these lines is in progress.

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LITERATURE CITED

1. **Hartman, P. A., P. S. Hartman, and W. W. Lanz.** 1975. Violet red bile 2 agar for stressed coliforms. Appl. Microbiol. **29**:537–539.
2. **Jay, J. M.** 1964. Beef microbial quality determined by extract-release volume (ERV). Food Technol. **18**:1636–1641.
3. **Jay, J. M.** 1965. Relationship between water-holding capacity of meats and microbial quality. Appl. Microbiol. **13**:120–121.
4. **Jay, J. M.** 1977. The *Limulus* lysate endotoxin assay as a test of microbial quality of ground beef. J. Appl. Bacteriol. **43**:99–109.
5. **Jay, J. M., and S. Margitic.** 1979. 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. Appl. Environ. Microbiol. **38**: 879–884.
6. **Shelef, L. A., and J. M. Jay.** 1969. Relationship between amino sugars and meat microbial quality. Appl. Microbiol. **17**:931–932.
7. **Sullivan, J. D., F. W. Valois, and S. W. Watson.** 1976. Endotoxins: the *Limulus* amoebocyte lysate system, p. 218–236. *In* A. W. Bernheimer (ed.), Mechanisms in bacterial toxinology. John Wiley & Sons, New York.