Journal of Food Protection, Vol. 58, No. 7, Pages 764-769

Microbial ATP Bioluminescence as a Means to Detect Contamination on Artificially Contaminated Beef Carcass Tissue†

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(MS# 94-257: Received 27 October 1994/Accepted 6 March 1995)

ABSTRACT

The use of microbial ATP bioluminescence was evaluated as a means to rapidly detect gross microbial contamination from feces on bovine-carcass surface tissue (BCT). Microbial ATP was selectively distinguished from nonmicrobial ATP by the assay procedure used. Regression analyses of microbial ATP and viable count scatterplots showed lean and adipose BCT artificially contaminated with bovine feces had the same regression line parameters ($P < 0.05$), and therefore, the microbial ATP responses were similar for both tissue types. Correlation coefficients (r) of these regression lines were >0.90 for both tissue types. Results indicated that swab samples can be held at 5°C for up to 6 h without compromising microbial ATP bioluminescence assay results. The microbial ATP bioluminescence assay shows potential for use as a means to rapidly detect fecal contamination on red meat carcasses and to gauge decontamination effectiveness and hence could monitor critical control points in a processing-plant HACCP plan.

Key words: ATP bioluminescence, rapid microbial assay

There exists a need for rapid microbiological assays to determine whether or not an animal carcass has been grossly contaminated with bacteria via fecal matter and if reconditioning procedures are effective for removing this contamination. The need for a science based carcass monitoring system was widely publicized following the outbreak of gastroenteritis and associated hemolytic uremic syndrome caused by *Escherichia coli* O157:H7 from consumption of undercooked ground beef burgers in January of 1993 in the state of Washington, USA *(1)*. The major reservoir of *Escherichia coli* O157:H7, and hence a source of infection and contamination, is the feces of warm-blooded animals, including the bovine *(10)*. In the process of converting animals to food, microbial contamination of the carcass is unavoidable. The major sources of contamination are the hide, hair, and hooves (all of which are likely to be encrusted with fecal matter), and intestinal tract contents (feces and ingesta). In the United States, inspection to detect these contaminant sources is based solely on sight. Reconditioning procedures are also validated by visual inspection. Although all carcasses with low generic microbial counts may not be pathogen free, reducing fecal contamination on carcasses should improve their microbiological safety. Therefore, an objective, rapid, sensitive, and accurate means to assay for the presence of microbial contaminants is needed by inspectors as well as processors. The emphasis on hazard analysis of critical control points (HACCP) for food processing has preceded establishment of the methodologies necessary to implement such programs in a timely manner. This study examined the use of microbial ATP bioluminescence as a means to fill this methodology void.

Because of the high numbers of bacteria (10^{11} to 10^{14} CFU/g) in feces, contamination of the carcass results in high microbial loads both in the specific areas of contamination and throughout the product as a consequence of processing *(7)*. Therefore, detecting areas of dense microbial numbers can be used as an index of fecal contamination. The use of microbial ATP to generate bioluminescence is an assay that has been used for many years in a variety of applications to estimate microbial load and/or actual bacterial populations *(24)*. Other workers have applied this technique to problems of estimating the microbial-population of ground beef and meats *(4, 15–17, 20, 25, 26)*. The research presented in this paper examines the microbial ATP bioluminescence assay as a method to detect fecal contamination and hence, gross microbial contamination, on the surface of bovine-carcass tissue.

MATERIALS AND METHODS

Tissue and fecal inoculation

Beef carcass surface tissue (BCT) was obtained from the abattoir of the U. S. Meat Animal Research Center (Clay Center, NE) as described previously *(6)*. Briefly, surface tissue sections were excised from the cutaneous trunci (lean BCT) and the exterior rib area (adipose BCT) of the carcass. BCT was cut into 7.5 by 7.5 cm sections and stored frozen at -20°C until use. Prior to use, tissue was thawed to approximately 25°C. The exterior side of

†Mention of a trade name, proprietary product or specific equipment is necessary to report factually on available data; however, the USDA neither guarentees nor warrents the standard of the product, and the use of the name by USDA implies no appoval of the product to the exclusion of others that may also be suiable.

the BCT (with intact fascia representing the carcass surface) was inoculated as follows: samples of fresh bovine feces were obtained from cattle at the U. S. Meat Animal Research Center as they were defecated by the animal. All animals were fed corn and corn-silage rations and held in outdoor feedlot pens. Fecal samples were placed in individual Whirl Pak bags, transported within 5 min to the laboratory and diluted with sterile physiological saline. Diluted suspensions (1:2, 1:10, 1:100, and 1:1,000) were used to inoculate tissue sections. BCT pieces were individually placed, exterior side down, in a plastic weigh boat containing 10 ml of fecal suspension and held for 15 min at room temperature. The BCT sections were then removed using forceps and excess liquid was allowed to drain from the tissue. The BCT sections were then placed on aluminum foil (inoculated side up) until sampling.

Sampling

A 25-cm^2 section of the BCT piece was marked off using a flame-sterilized stainless steel 5 by 5 cm template. An ATP-free swab (Lumac, B.V., The Netherlands; purchased through Integrated BioSolutions, Monmouth Junction, NJ, USA) was moistened in a tube containing 1 ml of swab solution (physiological saline [pH 7.2] plus 0.05% [vol/vol] Tween 20) and swabbed over the 25-cm^2 area 10 times in both the vertical and horizontal directions with firm pressure. The swab was placed back into the swab buffer tube and the swab contents expressed into the solution by forcing the swab against the side of the tube before discarding the swab.

ATP bioluminescence assay

Determination of microbial ATP (mATP) was performed using the Lumac Industrial Microbial Control Kit™ (IMC kit) with a Lumac Model M-2500 Biocounter™ luminometer (Lumac, B.V.; through Integrated BioSolutions). All ATP-free reagents and plastic disposable cuvettes and swabs were obtained from the same manufacturer and source as the assay kit. Swab samples were vortexed and a 500-µl aliquot added to an ATP-free cuvette along with 500 µl of NRS (a nonionic quaternary ammonium surfactant, to release non microbial ATP) and 20 µl of SOMASE (a calcium-activated ATPase, to hydrolyze nonmicrobial ATP released by the NRS reagent). This mixture was vortexed for 2 s and allowed to incubate at 25°C for 45 min. A 100-µl aliquot of the mixture was then placed in a fresh ATP-free cuvette and assayed for microbial ATP content in the M-2500 Biocounter™ luminometer. NRB (100-µl; an ionic surfactant that releases microbial ATP) was injected, followed after 2 s with 100 µl of LUMIT-PM (luciferase, D-luciferin, bovine serum albumin, and dithiothreitol prepared in 0.025 M HEPES buffer containing $MgSO_4$). The resulting emission of light from the bioluminescence reaction was integrated over a 10-s period and read as relative light units (RLU). Internal standards for each sample were prepared by adding a known amount of ATP prepared in Lumit Buffer (pH 7.75, 0.025M HEPES, $MgSO_4$, EDTA, and sodium azide) and further diluted in bidistilled ATP-free water. These data were converted into picograms of microbial ATP in 1 ml of sample. ATP standard curves were constructed daily.

Viable microbial counts

The same swab samples obtained from fecally contaminated tissue used for mATP analysis were diluted in buffered peptone water (Difco Laboratories, Detroit, MI, USA) and spiral plated using a Model D spiral plater (Spiral Systems Instruments, Bethesda, MD, USA). For anaerobic and aerobic counts, anaerobic agar with GasPak II anaerobic system incubation (BBL, Becton Dickinson Microbiology, Cockeysville, MD) and tryptic soy agar (BBL) were used, respectively. Plates were incubated at 37°C for 36 h. The number of colony forming units (CFU) per cm^2 was calculated and converted to $\log_{10}$. The total microbial count (TOT) was calculated as the log of the sum of the aerobic (AER) and anaerobic (ANAER) counts.

Beef-carcass surface tissue decontamination

Inoculated tissues were decontaminated using a pilot scale model carcass washer as described previously *(6)*.

Data analysis

Microbial ATP data, direct RLU readings, and viable cell count data were analyzed using the General Linear Models procedure and ANOVA of SAS (SAS Institute, Cary, North Carolina, USA) and the InStat2 version 2.0 statistical software package (GraphPad Software, San Diego, CA, USA). Other data analysis methods are described in the results section.

TABLE 1. *Means of $\log_{10}$ transformed parameters from adipose and lean BCT inoculated with bovine feces.*

Parameter[a,b]	Adipose	Lean	(P)[c]
ATP	2.47	2.95	0.26
RLU	2.89	3.54	0.12
AER	3.46	3.54	0.86
ANAER	3.58	4.29	0.18
TOT	3.85	4.39	0.30

[a] ATP, log pg of microbial ATP/cm^2; RLU, log relative light units/cm^2; AER, ANAER, TOT, log aerobic, log anaerobic, log total plate count (log CFU/cm^2).

[b] $n = 108$.

[c] Probability that the means in a row are not significantly different ($P > 0.05$).

RESULTS

Microbial ATP bioluminescence response from lean and adipose BCT

A range of inoculum levels from approximately 10^2 to 10^6 CFU/cm^2 was obtained by the fecal dilutions used. This was an appropriately large range for evaluation of the microbial ATP (mATP) *(8)*. The mean RLU values of the mATP responses from both lean and adipose inoculated BCT, as well as the plate counts calculated across the range of inoculum dilutions, are shown in Table 1. The arithmetic means of the measured parameters were lower in the case of the adipose BCT; however, these values were not statistically different ($P > 0.05$).

Regression lines were determined from the scatterplots of the log mATP (or RLU)/cm^2 versus the log plate count CFU/cm^2 for each tissue type (Table 2). Comparisons of the slopes and the intercepts of the regression lines by analysis of covariance (SAS, Cary, NC, USA), with tissue type as the classification variable, indicated no significant differences in either the slope or intercept for lean versus adipose tissue response ($P > 0.05$). Therefore, the response of the mATP test for fecal inocula on the different tissue types was the same. On the basis of these analyses, the data obtained from lean BCT could be combined with that from adipose BCT (Figures 1 and 2). Regression parameters and correlations could be determined with either the $\log_{10}$ RLU/cm^2 or the log picograms of mATP per cm^2 as given in Table 2.

Statistical estimation of mATP threshold of response

The sensitivity or threshold of quantitative linear response between mATP and bacterial numbers is a direct reflection of the

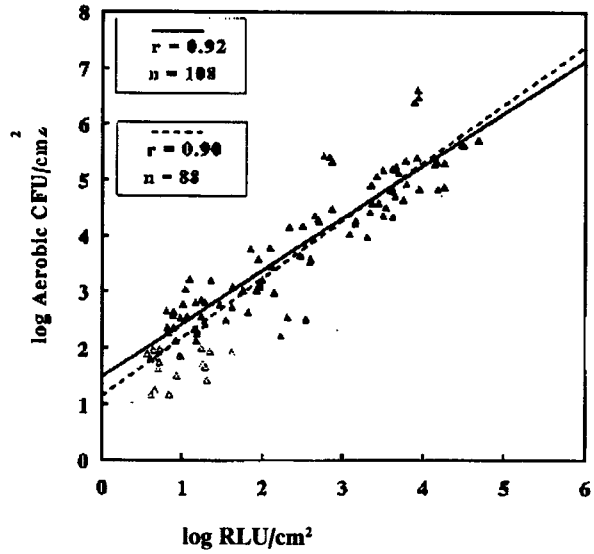

Figure 1. *Scatterplot of mATP (RLU) and aerobic plate counts (AER) from inoculated beef-carass tissue sections; (——); Regression through all datapoints; (——); regression line through datapoints above the sensitivity cutoff plate count of the assay; points below the sensitivity cutoff,▲.*

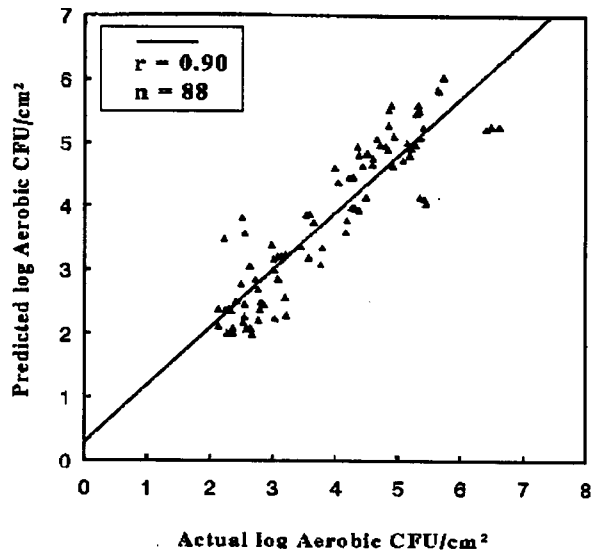

Figure 2. *Plot of predicted aerobic plate counts (AER) calculated from the regression line of mATP (RLU) vs. AER using only data above the sensitivity cutoff plate count of the assay (AER >log_{10} 2.0 CFU/cm²).*

TABLE 2. *Regression parameters and cutoff points for minimum detection levels of bacterial populations derived from mATP and culture method microbial count scatterplots of bovine-carcass surface tissue inoculated with bovine feces*

Parameters[a]	Data[b]	(*n*)	Regression Equation	(*r*)	(Sy.x)[c]
AER vs. ATP	All	108	AER = 1.02 (ATP) + 0.73	0.93	0.53
AER vs. ATP	AER ≥ 2.1	88	AER = 1.10 (ATP) + 0.92	0.91	0.51
AER vs. RLU	All	108	AER = 1.04 (RLU) + 1.16	0.92	0.54
AER vs. RLU	AER ≥ 2.0	88	AER = 1.47 (RLU) + 0.95	0.90	0.51
TOT vs. ATP	All	108	TOT = 1.11 (ATP) + 0.97	0.93	0.55
TOT vs. ATP	TOT ≥ 2.7	88	TOT = 1.65 (ATP) + 0.97	0.92	0.49
TOT vs. RLU	All	108	TOT = 1.14 (RLU) + 1.59	0.93	0.55
TOT vs. RLU	TOT ≥ 2.7	88	TOT = 2.06 (RLU) + 1.00	0.92	0.52

[a] AER, log_{10} AER CFU/cm²; TOT, log_{10} TOT CFU/cm²; RLU, log_{10} RLU/cm²; ATP, log_{10} pg of mATP/cm².

[b] Parameters were calculated from all points in the dataset as well as from the truncated dataset determined as described in Materials and Methods.

[c] Sy.x, standard deviation of residuals from regression line.

combined effects of the luminometer's sensitivity, the efficacy of the reagents for mATP extraction, and the bacterial cell energy status. The sensitivity of the current assay for measuring mATP (i.e., RLU) and relating it to viable plate count was analyzed using two methods. The limit of detection determination method described by Nieuwenhof and Hoolwerf *(21)* has been applied to similar data obtained from mATP measurements of raw milk samples *(2, 28)* and was one method used for the present study. This estimate of the detection limit is predicated on the chosen interval size and, therefore, is subjective. If the same interval sizes are used, however, it is a reproducible threshold determination procedure. The estimated cutoff by the aforementioned method was log_{10} 2.8 CFU/cm² for the estimation of AER by RLU when interval sizes of 0.25 log_{10} units were chosen (data not shown).

A different procedure developed by our lab and used to estimate the cutoff was not based on chosen interval sizes, and hence, was objective. This cutoff determination procedure was performed using the non-linear procedure of SAS (SAS Institute; Cary, NC, USA). Briefly, data from scatterplots of mATP versus plate counts are fitted to a segmented non-linear model. The segmented nonlinear model consisted of two components: a line with a slope of zero at the lower end of the scatterplot data, and another line with a measurable slope. The non-linear procedure optimized the fit by minimizing the sums of squares for the segmented non-linear model. Data below the threshold of sensitivity of the assay (i.e., below the numbers of bacteria needed to obtain a linear response in the mATP assay) describe a line with a slope of

zero. Data on the scatterplots which are derived from samples with adequate bacterial counts describe a second line with a slope greater than zero. From the model, the x value of the point at which these two lines intersect is the minimum number of bacteria which must be present in a sample to be measured by the mATP assay. The cutoff points for RLU data using the second method were $\log_{10}$ 2.0 to 2.1 CFU for estimation of AER and $\log_{10}$ 2.7 CFU for estimating TOT (Table 2).

Effect of holding swab samples at 5°C on mATP measurements

To determine the effect of short term refrigerated storage of samples already taken, swabs were made from the inoculated BCT as described earlier, held at 5°C, and sampled for mATP and aerobic plate counts at 0, 2, 4, and 6 h. Results are shown in Table 3. In the case of $\log_{10}$ mATP per CFU, no measurable differences were observed from 0 to 6 h of refrigerated storage. Even though the viable plate counts did decrease by 0.37 $\log_{10}$, it was not a significant reduction ($P > 0.05$). The case was the same if the data were expressed as $\log_{10}$ RLU/CFU (data not shown). Thus, samples could be refrigerated without a significant drop in mATP for between 0 and 6 h.

TABLE 3. *Effect of holding swab samples at 5°C prior to measuring mATP*

Hours at 5°C	RLU[a,b]	ATP[b,c]
0	3.34	3.04
2	3.25	3.59
4	3.25	3.04
6	3.20	3.15

[a] $\log_{10}$ RLU per ml.
[b] Means in columns are not statistically different ($P < 0.05$).
[c] $\log_{10}$ ATP per ml.

TABLE 4. *Effects of holding inoculated BCT at 5°C prior to sampling*

Hours at 5°C	RLU[a,b]	ATP[b,c]
0	4.20	3.64
2	3.87	3.39
4	3.80	3.26
6	3.80	3.21

[a] log RLU per cm^2.
[b] Means in columns are not statistically different ($P < 0.05$).
[c] log pg of microbial ATP per cm^2.

Effect of holding inoculated BCT samples at 5°C on mATP measurements

To determine the effect on the mATP bioluminescence assay after holding inoculated tissues at refrigerated temperatures before assaying, swab samples were taken from inoculated tissue held for 0 to 6 h at 5°C sampled over a 25-cm^2 area, and assayed for mATP as described. Results are presented in Table 4. Over the 6-h period, no significant decrease in $\log_{10}$ RLU values were observed ($P >$ 0.05). However, while holding samples under refrigerated conditions for this short period of time should not greatly affect the results, it is still advisable that the mATP determinations be conducted as soon as possible after obtaining the samples.

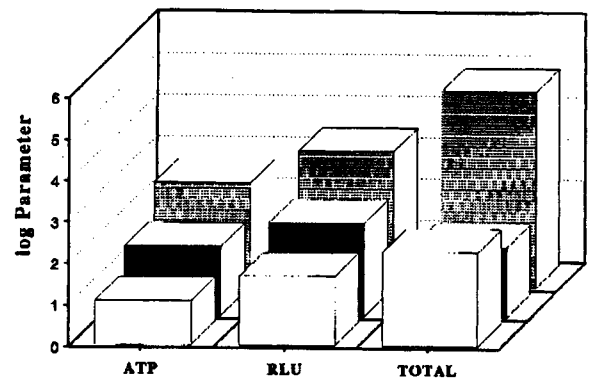

Figure 3. *Effects of spray washing inoculated BCT with water (▭) or 5% (vol/vol) acetic acid (■) or no wash (▩) on the total microbial plate count (AER+ANAER) and the mATP levels (RLU and ATP). Parameter units are as follows: ATP, $\log_{10}$pg of mATP/cm^2; RLU, $\log_{10}$RLU/cm^2; TOT, total $\log_{10}$ CFU/cm^2.*

Use of mATP test to gauge decontamination of inoculated BCT

Inoculated tissue was spray washed with water or 5% vol/vol acetic acid using a model carcass washer *(6)*. Bacterial loads of tissues so treated were assayed for their viable plate counts and mATP values. Decreases in the viable microbial total count were detectable concomitantly by both the viable plate count method or the mATP test (Figure 3).

DISCUSSION

To our knowledge, no other published reports are available on the use of this assay to detect generic microbial levels on bovine carcasses resulting from fecal contamination or on the use of this assay to determine the efficacy of carcass decontamination procedures.

Similar mATP assay methods have been used by other workers *(9, 11–14, 18, 19, 22, 23, 25, 29)* for a variety of purposes. The current research demonstrates that the mATP assay can be used to determine the relative levels of generic bacterial contamination on bovine carcass surface tissue in about 1 h, as compared to the viable plate count, which can take 36 to 48 h to complete.

The level of ATP per bacterial cell is not constant and is affected by several factors *(5, 14, 27)*. When measuring the ATP content of mixed populations of bacteria, it must be considered that the different species are possibly in different growth phases and different nutritional states, and may have different concentrations of intracellular ATP pools. The numbers of different bacterial species estimated to occur in cattle feces is at least 400 *(7)*. These organisms can be present in clumps or may not even be culturable using current protocols. These factors must be considered when attempting to correlate the mATP assay results and microbial numbers from carcasses with microbial loads derived mainly from feces.

Bovine fecal matter was selected as the inoculum in this experiment for two reasons. First, fecal matter is the major source of microbial and pathogen contamination on meat-animal carcasses. Second, bovine feces contain 10^{11} to 10^{14} viable bacteria per g *(7)*. In this study, aerobic and anaerobic viable counts were performed in an attempt to more fully account for bacterial numbers in feces. Since many of the organisms detected by the anaerobic plate count method used are probably facultative, it is possible that they were counted twice. However, the mATP assay correlated well with the aerobic mesophilic viable counts as well as with the total estimate of aerobic plus anaerobic bacteria (Tables 1 and 2). Fecal bacteria are anaerobic species, not all of which can be cultured even under the strictest conditions of anaerobiosis. It is therefore likely that some portion of the microbial population of the swab samples from this study were not cultured. This hypothesis is supported by the observation that direct microscopic counts of bacteria in feces are greater than the populations counted by culturing *(7)*. Therefore, this study correlated the measurable bacterial ATP content of samples to only a subpopulation of the total microflora.

Removal of nonmicrobial (somatic) ATP is a major challenge to using this technique as a bacterial enumeration method for samples with high intrinsic or nonmicrobial ATP contents. Sharpe et al. *(23)* reported that in the case of beef and beef product samples, the ratio of intrinsic ATP to bacterial ATP ranges from 1,500 to infinity.

Two methods reported for removing somatic ATP are (i) differential ATP extraction and enzymatic hydrolysis and (ii) physical filtration of bacteria and differential ATP extraction followed by physical removal of somatic ATP. Other workers have combined the enzymatic removal of differentially detergent-extracted somatic ATP with multiple filtration to remove unbound somatic ATP *(2, 11, 19, 20, 26)*. Such methods appear to enhance the accuracy of the method when compared to plate counts, as evidenced by higher correlation coefficients, but require up to 30 min of additional incubation.

Another challenge in using the mATP bioluminescence assay is the current lack of sensitivity below about 10^3 to 10^4 CFU/ml. At this threshold, samples must be concentrated by centrifugation and/or filtration. Both manipulations take additional time and are known to be cell stressors that can reduce cellular mATP levels *(5)*. However, concentration by either of these methods does remove interfering substances from the sample. The threshold of detection is also affected by the sample menstruum. To compensate for this quenching effect, the internal standardization procedure, as used in this assay, was employed. This method accounts for individual sample response and allows a determination of the actual quantity of ATP per cell. One objective of this study was to determine the efficacy of this assay as a means to gauge high levels of generic fecal bacteria on carcasses. Since there were high correlations between both the amounts of ATP (picograms of microbial ATP per cm^2) or the direct readings from the luminometer (log_{10} RLU per cm^2), and the log viable CFU per cm^2 (Table 2), additional time to obtain actual ATP quantities is neither necessary nor advantageous in gauging high levels of bacterial contamination. Because the response correlations were similar for both parameter plots (Figures 1 and 2), log_{10} RLU per cm^2 were used to express the mATP values as reported previously *(2, 3, 15, 17, 30)*. In the current study, the correlation between the log picograms of microbial ATP per cm^2 and log RLU per cm^2 was 0.98.

Because of the rapidity of the assay, ATP bioluminescence has been recognized as a promising means to obtain results that are useful to the processor. Previous studies on the use of the ATP bioluminescence assay for meat testing have centered on comminuted products, but some data from surface-excision samples have been published *(4, 15, 17, 20, 26, 29)*. The previously published reports include a variety of mATP test methods and report a range of correlations between standard plate counts and mATP from 0.66 to 0.99 for ground beef, fish, and excised beef samples. The previously reported methods required from 45 min to 3.5 h to complete, excluding sampling times. Although the data presented in this study centers on carcass tissue that was fecally contaminated and sampled non-destructively, the correlations are in close agreement with data obtained by other methods used for comminuted products or excision sampling of beef products.

It is important to note that mATP assays measure an intrinsic yet variable metabolite of the bacterial cell, as opposed to a growth-based assay such as the viable plate count method. These are clearly two different bases for quantifying microbial numbers, so it is unreasonable to expect perfect correlations between a plate count method and the mATP test *(24)*. Karl *(14)* stated that although the accuracy of the mATP bioluminescence techniques has been questioned, and its difficulties cited, it is still by far the most convenient and reliable method for measuring total microbial biomass in most environmental samples. The current study provides evidence that the mATP bioluminescence assay will accurately detect high levels of microbial contamination on beef-carcass tissue and can gauge the efficacy of protocols to recondition accidentally contaminated carcasses. For monitoring the presence of high microbial loads and possible fecal contamination on carcasses, a 1-h assay is a considerable improvement over the standard plate count, but is still not rapid enough for the modern plant setting. More research is needed to develop quicker tests to gauge high levels of fecal or microbial contamination on meat animal carcasses. Such an assay could be used to segregate animal carcasses with gross microbial or fecal contamination for reconditioning and to test the microbial reductions on the reconditioned carcasses on a real time basis. The microbial ATP bioluminescence assay has the potential to offer an objective, rapid, and sensitive means for inspectors, as well as food microbiologists, to rapidly monitor carcass processing at critical control points of a HACCP plan.

ACKNOWLEDGMENTS

We wish to thank Mrs. Carole Smith and Mrs. Jane Long for their expert technical assistance. For statistical analyses and statistical methods development we thank Mr. James Wray, and for statistical methods development, Dr. Kreg Leymaster.

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