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Use of a Rapid Microbial ATP Bioluminescence Assay to Detect Contamination on Beef and Pork Carcasses†

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ABSTRACT

A new microbial ATP bioluminescence assay was shown to be an accurate and rapid method to determine the levels of generic bacterial contamination on beef (n = 400) and pork (n = 320) carcasses sampled in commercial processing plants. Based on in vitro fecal dilution studies, the rapid microbial ATP (R-mATP) assay is as accurate as the standard plate count method for estimating bacteria in bovine or porcine fecal samples. The correlations *(r)* between the R-mATP assay and the standard aerobic plate count for beef and pork carcasses sampled in commercial processing were 0.91 and 0.93, respectively. A segmented-model statistical approach to determine the lower limits of assay sensitivity was developed. By using this model to analyze the in-plant data, the R-mATP test responded in a linear fashion to levels of microbial contamination of $> \log_{10}$ 2.0 aerobic CFU/cm^2 on beef carcasses and of $> \log_{10}$ 3.2 aerobic CFU/cm^2 for pork carcasses. The R-mATP assay requires approximately 5 min to complete, including sampling. Given the rapidity and accuracy of the assay, processors interested in monitoring critical control points in the slaughter process could potentially use the R-mATP assay to monitor microbiological prevention and intervention procedures for minimizing carcass contamination.

Key words: ATP bioluminescence, rapid microbial testing, HACCP monitoring

The microbial profile of an animal carcass is dictated by events that occur during the process of converting the whole animal into carcass halves for further processing. Meat animal processors are concerned with the microbial safety of their products and often use traditional culture methods to monitor levels of generic aerobic mesophilic bacteria as well as specific pathogens. Such monitoring is useful; however, it usually requires at least 36 h to obtain results. By then, the affected carcasses have been processed and shipped, rendering plate count data useful only from a retrospective viewpoint.

A major source of microbial contamination on the carcasses of red meat animals is feces. Feces are also the major reservoir for human enteric pathogens such as *E. coli* O157:H7 *(6)*. It has been proposed that all meat packers implement an HACCP approach *(1)* to control pathogen levels and incidence on raw animal carcasses. Such an approach requires real-time monitoring to verify proactive control measures and identify possible sites of microbial contamination.

The microbial ATP (mATP) bioluminescence assay is a technique for measuring levels of total generic bacteria. This technique has a history of use in a variety of industrial applications, including foods and sanitation or hygiene monitoring *(7, 12, 16, 17)*. Assaying mATP has been shown to be efficacious for detecting generic microbial loads directly on beef carcass tissue inoculated with feces *(15)*. An advantage in using mATP as a microbial monitor of carcass processing is rapidity. To be an effective (HACCP) monitoring tool in a processing plant, a carcass assay should require substantially less than 1 h to complete. Previous work *(15)* validating an mATP test for carcass tissue required approximately 55 min to complete the assay.

The objective of the current research was to develop a rapid mATP assay for measuring levels of generic bacteria and to correlate results with standard viable plate count methods. The sponge method of sampling was chosen for its rapidity since it contributes very little carcass tissue components that might interfere with the assay. This rapid mATP assay was evaluated both in the laboratory and in beef- and pork-processing facilities.

MATERIALS AND METHODS

In-plant carcass samples

Beef carcass samples were obtained from two different commercial processing plants. One plant produced graded beef (from heifers and steers) and the other plant, ground beef (from cows and bulls). Samples were taken both immediately before and after the final spray wash, but before chilling. Randomly chosen carcass sites were sampled mainly from the brisket area. Sampling was done by one of two means. First, a 500-cm^2

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

sampling area was delineated on the brisket area with a rectangular stainless steel template. Otherwise, the second means to specify the sampling area was to use edible ink to draw an outline of the area to sample and to measure the dimensions with a ruler. Samples from the marked areas were taken using an ATP-free, sterile, microbiological sampling sponge (NASCO, Fort Atkinson, WI, USA) packaged in a Whirlpak™ bag. The sponge was moistened with 25 ml of a sterile sponge solution composed of 0.085% (wt/vol) NaCl + 0.05% (vol/vol) Tween 20 adjusted to pH 7.8. The solution was expressed from the sponge as it was removed from the Whirlpak™ bag using a sterile glove. The sponge was wiped firmly over the sample area 10 times in both the vertical and horizontal directions. The sponge was then placed into the bag containing the residual sponge solution and held in an ice chest at approximately 8 to 10°C. Analyses were performed within about 2 to 3 h of taking the sample.

Pork carcass samples were obtained from three large swine processors. The method of sampling for pork carcasses was the same as for beef, except that a 50-cm^2 template was used. All pork carcass samples were from skinned rather than scalded carcasses.

To determine the levels of microbial contamination on beef carcasses immediately after hide removal, a set of in-plant samples were taken from the two different plants within 45 s after the hide was removed and assayed for aerobic plate counts. Microbial contamination levels were determined from samples from two different plants taken randomly from carcasses with (n = 237) and without (n = 163) signs of visible fecal contamination, both before and after the final carcass wash. Upon returning to the laboratory (usually within 3 h), the sponge samples and their contents were aseptically transferred to a filtered Stomacher bag (International BioProducts, Redmond, WA, USA) and stomached for 2 min in a Stomacher Model 2000 (Tekmar, Inc., Cincinnati, OH, USA). The filtered stomachate was transferred to a sterile tube and held at room temperature until analyzed. Repeatability of the in-plant sample tests was determined as the correlation coefficient of the regression line between the duplicate determinations.

Rapid microbial ATP (R-mATP) assay

The major challenge in using mATP as a means of determining total microbial populations in food samples is the separation of nonmicrobial ATP from microbial ATP. The basis of the described R-mATP assay is the use of a filtration device in which somatic ATP is extracted; then within the same device, extraction of bacterial ATP is followed by its quantification.

The steps of the R-mATP assay were as follows. The carcass sponge sample (50 µl) was added to a Filtravette™ (New Horizons Diagnostics, Columbia, MD) followed by 100 µl of a somatic ATP extraction reagent, NRS™ (nucleotide-releasing agent for somatic cells; Lumac B.V., Netherlands; purchased through Integrated BioSolutions, Monmouth, NJ). The fluid was then suctioned through the Filtravette™ using a vacuum manifold (New Horizons Diagnostics, Columbia, MD) and the filtrate captured in a vacuum flask. Another 150 µl of NRS™ were added and pulled through the device. At this stage, the Filtravette™ retained bacteria and other cellular debris on the filter' s surface. Somatic cell ATP and free ATP were removed by the action of the somatic cell extractor, NRS. The Filtravette™ was placed in the holding drawer of the Model 3550 Microluminometer (New Horizons Diagnostics, Columbia, MD and ILC Dover, Inc., Frederica, DE). Thirty microliters of the microbial ATP extractor, NRB™ (nucleotide-releasing agent for bacteria; Lumac B.V., Netherlands; purchased through Integrated BioSolutions, Monmouth, NJ) were added, followed immediately by 30 µl of luciferin/luciferase reagent (New Horizons Diagnostics, Columbia, MD). The fluid was mixed by rapidly aspirating three times with the micropipettor, the drawer to the luminometer was closed and light emission integrated over 10 s. The mATP level was recorded as relative light units (RLU) taken directly from the luminometer's digital readout. All reagents were used at room temperature and were checked for contaminating ATP before use. The microluminometer internal calibration was checked before each experiment by performing a blank count with no filtravette. The time required to perform these steps of the R-mATP assay is approximately 90 s.

Viable bacterial plate counts

Samples to be cultured were serially diluted in buffered peptone water (BBL, Cockeysville, MD) and plated using a Model D Spiral Plater (Spiral Systems Instruments, Bethesda, MD, USA). For aerobic plate counts, samples were plated on tryptic soy agar (BBL, Becton and Dickinson, Cockeysville, MD, USA) and incubated 48 h at 35°C. Anaerobic counts were made by plating samples on anaerobic agar (BBL) and incubating in a GasPak jar with a GasPakII anaerobic atmosphere generator (BBL) for 48 h at 35°C. Bacterial counts were converted from colony-forming units (CFU) per ml to $\log_{10}$ CFU/cm^2, based on the area sampled. The total bacterial count (TOT) was defined as the $\log_{10}$ sum of the aerobic and anaerobic counts.

Laboratory accuracy and repeatability studies with diluted fecal samples

The relative accuracy of both the R-mATP assay and the plate count methods was determined by performing the respective tests on a set of diluted fecal samples. Fresh bovine feces was obtained from 31 feedlot cattle all fed a standard corn, corn-silage ration. Each sample was diluted 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in sponge solution (see in-plant carcass samples section). The R-mATP test and aerobic and anaerobic plate counts were performed in duplicate as described above. An unbiased test would be expected to have a slope of -1.0. Therefore any deviation (positive or negative) from -1.0 would indicate a bias. Accuracy was defined as the slope of the regression line between dilution as an independent variable and either plate count (aerobic or total) or mATP values as the dependent variable relative to the expected (unbiased) slope of -1.0. Estimates of accuracy were indicated by the deviation of the slope of the regression line of the reciprocal dilution plot from -1.0.

Repeatability was calculated by statistical analysis of the data obtained from the same reciprocal dilution plots. Data were analyzed by analysis of variance (SAS) techniques fitting the dilution, the animal, and the animal-by-dilution interaction. Estimates of variance components for the animal and the animal by dilution interaction were obtained and combined with the residual variance to calculate the repeatability of fecal dilution assays according to the following formulae:

Repeatability = $(\sigma^2_e + r\sigma^2_{A\times D}) / (\sigma^2_A + \sigma^2_{A\times D} + \sigma^2_e)$;
Error mean square (EMS) for animal = $\sigma^2_e + dr\sigma^2_A = r\sigma^2_A + 8\sigma^2_A$;
EMS for animal by dilution interaction = $\sigma^2_e + r\sigma^2_{A\times D} = r\sigma^2_e + 2\,r\sigma^2_{A\times D}$;
where (A) = no. of animals; (D,d) = no. of dilutions; (r) = no. of replicates; and σ^2_e = residual error variance.

The same analyses described above were used to estimate the accuracy and repeatability of the R-mATP assay with porcine fecal samples.

Statistical analysis of in-plant carcass data

Linear regression analysis was performed on scatterplots of the mATP values (RLU) versus the viable microbial counts using either the SAS (Cary, NC, USA) or InStat2 version 2.0 statistical analysis package (GraphPad Software, San Diego, CA, USA). The segmented-model method to determine the lower

limit of sensitivity or linear response of the R-mATP assay was used and described previously *(15)*. Briefly, the segmented model, using the nonlinear procedure of SAS, fits and optimizes two regression lines simultaneously to the data. The point ($\log_{10}$ CFU/cm^2) at which the slope of the R-mATP assay response (RLU) line from the lower end of the scatterplot ceased to equal zero was taken as the lower limit of linear response. Data falling below that specific value ($\log_{10}$ CFU/cm^2) are indicated on the scatterplot figures as open symbols. Linear regression was performed only on the data falling within the linear range of the R-mATP assay (i.e., using samples with plate counts greater than that value).

RESULTS

Initial assay development

The R-mATP assay is the result of a progression of research already shown to be effective in detecting high levels of bacterial contamination on beef carcasses *(15)*. Initially, a 90-min mATP assay was developed and tested on beef carcasses from a commercial plant (n = 278). The correlation *(r)* between mATP ($\log_{10}$ RLU/cm^2) and plate count ($\log_{10}$ total CFU/cm^2) in the 90-min assay was 0.88. This assay was based on a method previously described *(15)* and included a 30-min centrifugation (10,000 × g, 4°C) and decantation step to eliminate interfering substances. The accuracy estimate from fecal dilution experiments of the 90-min mATP assay protocol was -1.23, compared to -1.40 for the total bacterial plate count method (n = 20 animals). Repeatability determined from fecal dilution experiments for the 90-min mATP assay was 0.99.

In an effort to shorten the assay, a 35-min procedure (which included a centrifugation step prior to the standard R-mATP assay described in the rapid microbial ATP assay section) was developed and tested on beef carcasses from a commercial plant (n = 158). The correlation *(r)* between mATP ($\log_{10}$ RLU/cm^2) and plate count ($\log_{10}$ total CFU/cm^2) in the 35-min mATP assay was 0.88. The accuracy estimate from fecal dilution experiments of the 35-min protocol was -1.18 compared with the accuracy estimate of the total bacterial plate count method, which was -1.03 for that particular set of beef carcass samples. Repeatability determined from fecal dilution experiments for the 35-min assay was 0.97. Despite the effectiveness of these two initial assays, their time and equipment (centrifuge) requirements precluded them from further evaluation as viable process microbiological monitors that approach real time.

Laboratory accuracy and repeatability studies

The accuracy and repeatability estimates for the R-mATP assay for bovine and porcine diluted feces are given in Table 1. Although neither the viable plate count (Figure 1) nor the R-mATP (Figure 2) assay gave perfect accuracy values, the R-mATP result was slightly less accurate than the viable plate count methods whether calculated from the aerobic or the total plate count (Table 1). The R-mATP assay and the aerobic plate and total plate count methods were, for practical purposes, equally repeatable (Table 1).

TABLE 1. *Accuracy and repeatability estimates from* in-vitro *fecal dilution studies.*

Method	Species	Accuracy	Repeatability
R-mATP[a]	Bovine	-0.88	0.99
Aerobic plate count[b]	Bovine	-1.01	0.96
Total plate count[b]	Bovine	-1.17	0.96
R-mATP[a]	Porcine	-0.84	0.93
Aerobic plate count[b]	Porcine	-1.22	0.96
Total plate count[b]	Porcine	-1.10	0.98

[a] $\log_{10}$ RLU/ml.
[b] $\log_{10}$ CFU/ml.

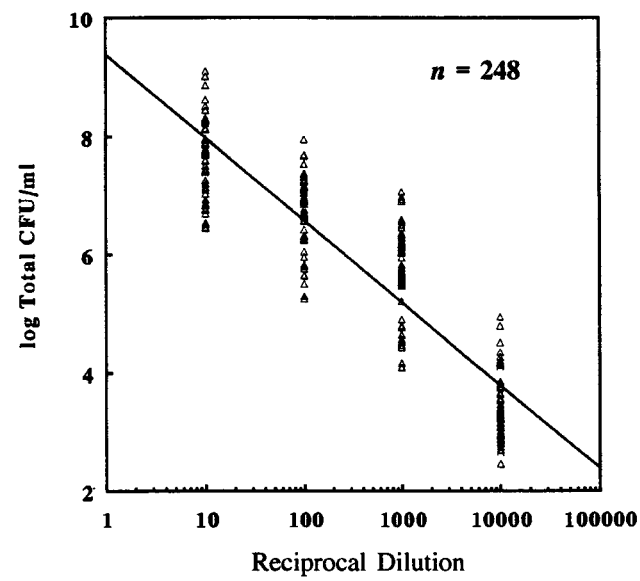

Figure 1. *Reciprocal dilution plot of total plate count assay of diluted bovine fecal specimens from 31 different feedlot cattle.*

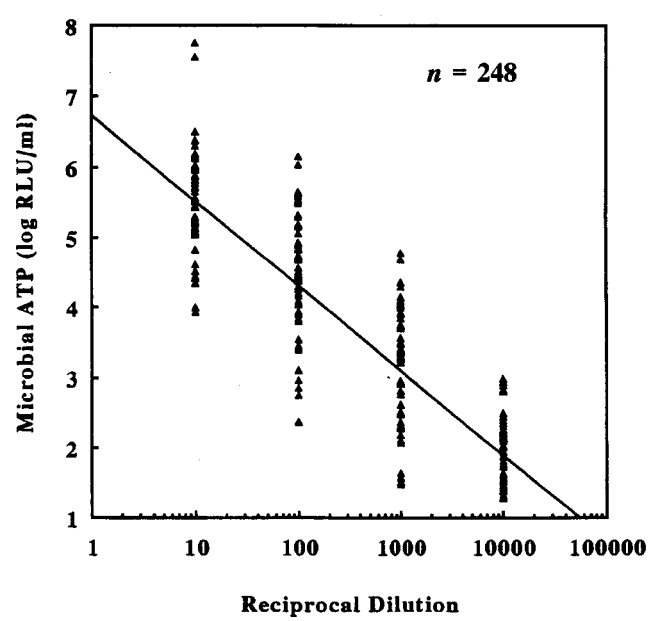

Figure 2. *Reciprocal dilution plot of R-mATP assay of diluted bovine fecal specimens from 31 different feedlot cattle.*

In-plant carcass samples

The amount of time required to both collect the sample and perform the assay as described in the materials and methods section was approximately 5 min per sample.

Scatterplots of the beef and pork R-mATP data sets are shown in Figures 3 and 4, respectively. The lower limit of assay sensitivity for beef was determined by the segmented-model method to be $\log_{10}$ 2.0 aerobic CFU/cm^2. Correlation *(r)* between the R-mATP values and the aerobic plate count for the beef study overall was 0.92 (n = 400), without taking into consideration the data below the assay sensitivity. Taking into consideration the lower limit of assay sensitivity of $\log_{10}$ 2.0 aerobic CFU/cm^2, the correlation *(r)* was 0.91 (n = 365). Repeatability between the duplicate determinations on field samples was 0.96 for $\log_{10}$ RLU/cm^2 and 0.99 for $\log_{10}$ aerobic CFU/cm^2. In the case of pork in-plant samples, the lower limit of sensitivity was determined by the segmented-model method to be $\log_{10}$ 3.2 aerobic CFU/cm^2. Correlation *(r)* between the R-mATP values and the aerobic plate count for the pork study overall was 0.93 (n = 320), without taking into consideration the data below the assay sensitivity. Taking into consideration the lower limit of assay sensitivity of $\log_{10}$ 3.2 aerobic CFU/cm^2, the correlation *(r)* was 0. 89 (n = 242). Regression parameters for the beef and pork data sets are given in Table 2.

Aerobic plate counts of in-plant carcass samples taken from beef carcasses immediately after hide removal (< 45 sec) ranged from < 0.1 to 0.6 $\log_{10}$ CFU/cm^2 (n = 22). In-plant beef samples taken from carcasses with visible fecal contamination had average aerobic plate counts of 4.5 $\log_{10}$ CFU/cm^2 (n = 237) versus 2.8 $\log_{10}$ CFU/cm^2 (n = 163) from samples with no signs of visible fecal contamination. Of those samples with visible fecal contamination, 71% had aerobic plate counts of ≥ 4 $\log_{10}$ CFU/cm^2 and 9% had aerobic plate counts of ≥ 6 $\log_{10}$ CFU/cm^2, while only 4% of the samples with no visible fecal contamination had aerobic plate counts of ≥ 4 $\log_{10}$ CFU/cm^2.

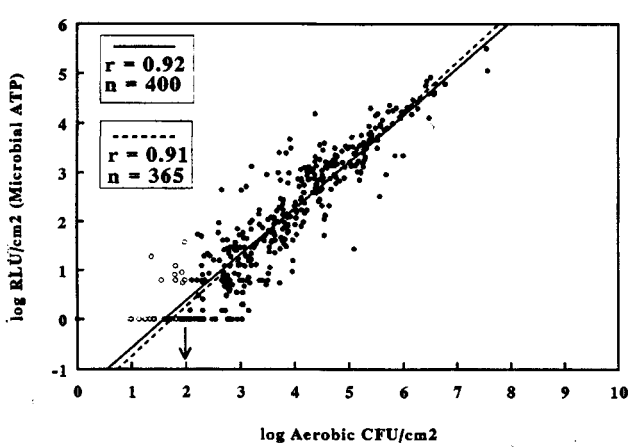

Figure 3. *Scatterplot of R-mATP assay values (microbial ATP) and aerobic plate counts from beef carcass in-plant samples. The solid line is the regression including all data points. The dashed regression line is calculated from data points above the lower limit of assay sensitivity ($\log_{10}$ 2.0 aerobic CFU/cm^2, indicated by the arrow). Data points below the threshold are open symbols and those above the threshold are solid symbols.*

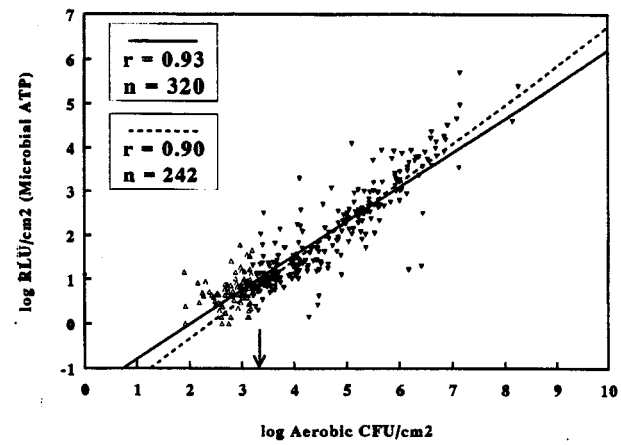

Figure 4. *Scatterplot of R-mATP assay values (microbial ATP) and aerobic plate counts from pork carcass in-plant samples. The solid line is the regression including all data points. The dashed regression line is calculated from data points above the lower limit of assay sensitivity ($\log_{10}$ 3.2 aerobic CFU/cm^2, indicated by the arrow). Data points below the threshold are open symbols and those above the threshold are solid symbols.*

DISCUSSION

The R-mATP assay provided an accurate measure of generic microbial levels on beef and pork carcasses. This assay provides a means to rapidly separate nonmicrobial ATP from microbial ATP in the same vessel. Additionally, the microbial ATP content of the sample can be quantified in this vessel. This unique feature allows the assay to be conducted in a minimal amount of time (5 min) as compared to the standard plate count (36 to 48 h).

To our knowledge, this is the first report of the use of this particular rapid mATP assay; however, other workers have used a variety of mATP tests to enumerate generic bacteria in meat samples. Stannard and Wood *(18)* reported correlations *(r)* as high as 0.94 for mATP content ($\log_{10}$ fg of mATP/g) versus 30°C plate count ($\log_{10}$ CFU/g) in beef homogenates. Kennedy and Oblinger *(9)* published correlations *(r)* of 0.95, 0.98, and 0.98 for mATP content ($\log_{10}$ fg of mATP/g) versus 35°C, 20°C, and 7°C plate counts ($\log_{10}$ CFU/g), respectively, in ground beef homogenates. Bulte and Reuter *(2)* obtained correlations *(r)* of 0.66 to 0.87 between mATP ($\log_{10}$ ATP {mol/l}) and 30°C plate count ($\log_{10}$ CFU/ml) obtained for beef homogenates. Labots and Stekelenburg *(10, 11)* reported correlations *(r)* of 0.91, 0.91, and 0.94 for raw meat, minced meat, and vacuum-packaged meat products, respectively, between plate counts ($\log_{10}$ CFU/g) and mATP ($\log_{10}$ RLU). Littel et al. *(13)* observed correlations *(r)* of 0.96 between 25°C plate count data ($\log_{10}$ CFU/g) versus mATP ($\log_{10}$ fg of mATP/g) from homogenized beef incubated to obtain differing degrees of microbial spoilage. These previous reports *(2, 9, 10, 11, 13)* described

TABLE 2. *Regression parameters of regression lines from R-mATP (RLU) assay data and aerobic plate count (AER) culture method scatterplots from beef and pork in-plant carcass samples. Data sets are composed only of data above the calculated assay sensitivity cutoff.*

Parameters[a]	Data[b]	(n)	Regression Equation	(r)	(Sy.x)[c]
AER vs. RLU	Beef (AER > 2.0)	365	AER = (0.836) RLU + 2.126	0.91	0.52
AER vs. RLU	Pork (AER > 3.2)	242	AER = (0.895) RLU + 2.839	0.89	0.50

[a] AER, $\log_{10}$ aerobic CFU/cm^2; RLU, $\log_{10}$ RLU/cm^2.
[b] Parameters were calculated from points in the data set above the lower limit of sensitivity.
[c] Sy.x, standard deviation of residuals from regression line.

experiments performed using different mATP assays and different sample preparation methods and reported the content of mATP in different units and on specific bases. In addition, many of the previously published reports were based on aerobic mesophilic bacterial counts ranging from 10^5 to 10^9 CFU/g. Therefore, it is not possible to directly compare previously reported results with the current data that is expressed as the RLU (from mATP) per area basis from surface sponge samples. However, Kennedy and Oblinger *(9)* did report that correlations between plate counts obtained from different incubation temperatures and either the mATP content ($\log_{10}$ fg of mATP/g) or the RLU value ($\log_{10}$ RLU) were equally as high.

Assessing the efficacy of the R-mATP test requires that samples with an adequate range of aerobic bacterial populations be tested. Eden and Eden *(5)* recommended that only ranges of at least 4 $\log_{10}$ cycles be used for evaluating the correlation between standard plate count methods and impedance methods of bacterial enumeration. That criterion was met in both the beef and pork data sets of the present study.

The segmented model is an objective means to estimate the lower limit of sensitivity (i.e., cutoff valve) of the mATP assay. Other means to accomplish this analysis have been devised and used *(14, 20)*. Previously, the method described by Van Crombrugge *(20)* was found to give a cutoff estimate very close to the value calculated using the segmented model *(15)*. Because the segmented model determines the lower end of assay sensitivity, it should provide researchers with a more reproducible means to evaluate mATP versus standard plate count scatterplots.

For monitoring the microbiological status of a commercial process, the R-mATP assay will provide an accurate and rapid means to determine relatively high levels of generic microbial contamination on beef or pork carcasses. Once R-mATP baseline data for a plant is established, this information will alert a processor to a point or points at which significant microbial contamination is entering the processing chain or whether a processing procedure is resulting in significant or unusual microbial contamination of the carcass. Additionally, if accidentally contaminated carcasses are segregated for reconditioning by trimming or spray washing, the R-mATP assay provides an objective means to judge if the carcass is sufficiently decontaminated. Ultimately, each plant must establish baseline plate count data with corresponding R-mATP assay values to use the R-mATP assay by itself. Such a database can be easily obtained and updated by most plants that already perform cultural microbiological analyses on carcasses.

Central to the efficacy of the R-mATP or any mATP assay in predicting bacterial numbers is the content of ATP per microbial cell. Although the average range of ATP content of bacterial cells is remarkably narrow between species *(2)*, the amount of ATP is a property of bacteria that is highly influenced by a number of factors *(2, 3, 8)*. These factors include bacterial type, nutrient status, growth phase, temperature, and the presence of antimicrobials. Media composition and temperature were demonstrated to greatly affect the per cell content of ATP in *Enterobacter aerogenes (19)*. Because of the current popularity of antimicrobial spray washes (e.g., organic acids, trisodium phosphate, and chlorine) the effect of such treatments on the R-mATP assay will require more research.

Carcass contamination, largely fecal in origin, is composed of a highly diverse population of different bacteria, not all of which are known to be culturable *(4)*. In using mATP as a measure of bacterial levels, mATP (RLU) values were correlated to the aerobic mesophilic plate count. The bacteria composing the microbial population of fecal contamination very likely contains a large portion of constituent bacteria that do not fall into this category and hence were not counted in the plate count method. Relating the mATP value back to the aerobic plate count does not consider the bacterial groups excluded by the aerobic plate count. Bulte and Reuter *(2)* stated it is problematic to use an average value of the ATP content per cell to predict the bacterial population from an ATP measurement. Also, the same workers noted that the predominant microflora may influence the measured mATP. In the current case, the dominating fecal bacterial flora on beef carcass samples are likely to be *E. coli*, fecal streptococci, lactobacilli, and clostridia *(4)*. In the case of pork carcasses, *Bacteroides* spp., *E. coli*, fecal streptococci, lactobacilli, and clostridia are likely to predominate *(4)*. Because of the short time frame between contamination and sampling, the ultimate predominating microflora of the carcass will not have had time to establish itself. Although the contaminating bacteria are on a fairly hospitable surface for growth and maintenance, they most likely are still a highly mixed population, with each group in different growth phases having different ATP contents.

An additional point to consider in the use of this assay

is the sampling method. As stated in the introduction, although sponging may not be the most efficient means to remove bacteria from carcasses, it is nondestructive, rapid, reproducible, and contributes little interference to the assay. Although mATP testing is subject to these drawbacks, it remains perhaps the most rapid means to estimate bacterial populations and shows great potential for the animal processor to monitor the microbiology of carcasses.

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