RAPID BIOLUMINOMETRIC ENUMERATION OF MICROORGANISMS IN GROUND BEEF

by

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(ABSTRACT)

Use of the bioluminometric ATP assay was evaluated for estimating total bacterial counts in ground beef. Minimum sensitivity was found to be 10^6 cfu/g using a double filtration procedure for sample preparation. Although ATP content per cfu decreased approximately 10 fold during storage, correlation of total aerobic plate count (APC) with microbial ATP content was 0.96.

Selective non-microbial ATP extraction with ATPase treatment was evaluated for use in conjunction with the double filtration procedure to increase assay sensitivity. The new method was effective for removing additional non-microbial ATP without reducing ATP in bacteria. Estimated APC values were generally accurate to within $\pm$0.50 log for ground beef samples above the detection limit of 5×10^4 cfu/g. ATPase treatment increased sensitivity of the ATP assay and APC estimation by about 1 log while increasing assay time by 40 minutes, for a total of 60 minutes for 4 samples assayed in triplicate.

The ATP assay was evaluated for use with ground beef patties inoculated with mixed ground beef spoilage flora,

Pseudomonas, or Lactobacillus and stored at $2^{o}C$ or $10^{o}C$ using oxygen permeable or impermeable (vacuum) packaging. Excellent correlation (r^2=0.95) was obtained for each inoculum and storage condition over the range of 5×10^4 to 1×10^9 cfu/g, when estimated APC values were compared with experimentally observed APC values.

Usefulness of the ATP assay for estimating APC values of frozen ground beef was evaluated. Retail ground beef and Lactobacillus- and Pseudomonas-inoculated beef were frozen and thawed at different rates and examined for APC and microbial ATP content. Results indicated that, although freezing and thawing lowered numbers of Pseudomonas, APC values and microbial ATP content closely correlated. APC estimates were generally accurate to within 1/2 log.

The importance of using an ATP assay standard to correct for variable enzyme activity and presence of quenching factors was demonstrated, and improved formulae were developed for optimum assay standard use. Alternate regression methods were evaluated for estimation of APC values but did not yield enhanced accuracy.

Only one regression equation was needed for estimating APC values of ground beef containing different types of bacteria stored in various ways. Therefore, little knowledge of ground beef history is needed in order to rapidly and accurately estimate microbial numbers in ground beef using the bioluminometric ATP assay.

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Introduction

The food industry needs a rapid method for assessing microbial quality. Microbial concentration in food is often used as an index of quality and current plating methods used for determining microbial numbers usually take 48 hours or longer to complete. Waiting days for plate count results may cause delays with using ingredients or increased holding time for products needing clearance prior to shipment. In addition, the long time required for plate count results makes it impossible to judge incoming ingredients for microbial quality prior to acceptance at the loading dock, or assess microbiological situations during ongoing production.

In addition to rapidity, other attributes must be considered when choosing an analytical method. The method must have adequate sensitivity and accuracy, as well as applicability to the food being analyzed. Also, cost of the test and technical skill required to perform the test may be important. The use and development of rapid methods for microbiological analysis have increased greatly in recent years. Methods may be grouped according to their purposes: 1) Detection of specific organisms 2) Identification of isolates 3) Enumeration of microorganisms.

Detection methods include enzyme-linked immunosorbent assay ELISA and DNA-probe techniques. These methods do not require pure microbial culture but need time-consuming enrichment steps in order to increase numbers of specific organisms to detectable levels. Another detection method is the One-Two test for Salmonella (BioControl Systems, Inc., Bothell, WA) for which a pure culture is not needed, but which requires at least 32 hours for cell multiplication and diffusion through a gel before cells can be detected by antibodies.

Identification method development has been aimed at miniaturization and automation. Isolation of pure cultures is required, which requires time for colonial growth on agar. Diagnostic kits are numerous, and include API (Analtab Products, Inc., Plainview, NY), Minitek (BBL Microbiology Systems, Cockeysville, MD), Spectrum 10 (Austin Biological Laboratories, Austin, TX) and MicroID (Organon Teknika, Morris Plains, NJ). Automated systems such as the Auto Microbic System (Vitek Systems, Inc., Hazelwood, MO) rapidly identify isolates by monitoring differential biochemical reactions.

Enumeration methods can be subdivided into 3 categories. The first category includes methods which make enumeration more convenient but still require incubation time for microbial colony development. Examples are the

Spiral Plater with laser colony counter (Spiral Systems, Inc., Bethesda, MD), the Isogrid hydrophobic grid membrane system (QA Laboratories Ltd., Toronto, Canada), Petrifilm (3-M company, St. Paul, MN) and Redigel (Conviron, Inc., Goshen, IN).

Faster enumeration methods (category two) which require microbial growth but not colony development, can take 6 to 72 hrs to complete. These measure cell growth or metabolites produced during incubation. Electrical impedance has been developed for use with foods (Firstenberg-Eden, 1983; Phillips and Griffiths, 1985; Hardy et al., 1977; Sorrells, 1981; Bishop et al., 1984; Martins et al., 1982; Gnam and Luedecke, 1982; Bossuyt and Waes, 1983). This method is based on electrical conductance properties of liquid media in which microorganisms grow. Numbers of organisms are estimated from the time required to cause inflection in an impedance curve during microbial growth. Results are obtained in 6 to 72 hours depending on the initial microbial concentration present in the food. Other methods used to measure microbial metabolic activity are radiometry (Stewart et al., 1980; Rowley et al., 1979; Lampi et al., 1974; Caslow et al., 1974) in which radiolabeled CO_2 is measured following production by bacteria in food samples metabolizing radioactive nutrients, and microcalorimetry (Gram and Sogaard, 1985; Lampi et al.,

1974) which is used to detect small temperature changes due to microbial growth. These methods have been reported to give results in 10-48 hours, depending on microbial numbers present.

The third category of enumeration methods includes the very rapid (less than 2 hrs) techniques of microbial ATP quantitation (luminometry), catalase activity measurement or catalimetry (Bio Engineering Group, New Haven, CT), the Limulus Amoebocyte Lysate assay (LAL) and the Direct Epifluorescent Filtration Technique (DEFT). These methods, which measure numbers of cells or amount of specific cell component, require no incubation for cell growth. Catalimetry is a means to measure catalase-positive organisms in foods using a disk flotation principle in which gas produced by catalase reaction with H_2O_2 is trapped in an absorbent disk. Time required for flotation through an aqueous column is dependent on the amount of catalase present and can be used to estimate microbial numbers (Wang and Fung, 1986; Charbonneau et al., 1975; Dodds et al., 1983; Gagnon et al., 1959). The LAL assay can be used to estimate microbial quality of foods by detecting endotoxin of Gram negative bacteria (Jay and Margitic, 1979; Hansen et al., 1982; Jay, 1977; Jay et al., 1979). The LAL does not distinguish between live and dead cells. DEFT is a microscopic method in which microorganisms in food are

filtered, stained and counted using a fluorescent microscope (Pettipher et al., 1983; Pettipher and Rodrigues, 1981; Rodrigues and Pettipher, 1984). The method is extremely rapid, but subject to difficulty in distinguishing live cells from dead cells.

Another very rapid method, luminometry, can be used to quantitate numbers of microorganisms in foods by measurement of microbial ATP. The subjects of luminometry and bioluminesence have been reviewed thoroughly (McElroy and Deluca, 1983; Strehler, 1978; Shimura, 1982; Seitz and Neary, 1976; McElroy and Seliger, 1966; Herring, 1978; DeLuca and McElroy, 1981). An excellent review has been recently written dealing specifically with food microbiology applications (LaRocco et al., 1986). The assay is based on use of enzyme and substrate extracted from firefly (Photinus pyralis) tails. Light is produced quantitatively in direct proportion to the amount of ATP present in test samples using these reagents . Numbers of microorganisms can be estimated based on the amount of microbial ATP present.

Original work with the bioluminescent ATP assay was reported by McElroy (1947). Much work has been done since to further characterize the reaction mechanism and properties of luciferase and luciferin, enzyme and substrate for the reaction (DeLuca, M., 1976, 1978; McElroy, W. D. and DeLuca, M. A., 1983). Applications have been mainly

concentrated in the clinical field, such as analysis of bacteremia, bacteriuria and dental plaque (Alexander et al., 1976; Beckers and Lang, 1983; Conn et al, 1975; Curtis et al, 1981; Gutekunst et al, 1977; Kemp, 1979; Mackett et al, 1982; Schrock et al, 1976; Thore et al, 1983). Environmental studies have also been numerous. Microbial analysis using ATP quantitation include water, soil and activated sludge applications (Afghan et al, 1977; Azam and Hodson, 1977; Bulleid, 1978; Christian et al, 1975; Doxtader, 1969; Guinn and Eidenbock, 1972; Holm-Hanson, 1970; Karl and Bossard, 1985; Lee et al, 1977; Picciolo et al, 1976; Paul et al, 1977).

Recently the ATP assay has been applied to foods for determining microbial quality. Although early food analysis attempts failed because of inferference by ATP present in the food (Sharpe et al, 1970; Williams, 1971), others have used the technique with varying degrees of success. Analysis of beverages for yeast content has been particularly successful, largely due to filterability of the products and the large quantities of ATP relative to bacteria present in yeast cells (Galligan et al, 1984; Graumlich, 1985; LaRocco et al, 1985; Littel and LaRocco, 1985; Patel and Williams, 1983; Stannard and Wood, 1983a). Use of luminometry for determining microbial quality of milk (Britz et al, 1980; Bossuyt, 1978, 1981, 1982a, 1982b; Issen

and Tsai, 1985; Kaneko et al, 1984; Theron et al, 1986a, 1986b; Waes and Bossuyt, 1981; Waes et al, 1984) and meats (Baumgart et al, 1980b; Kennedy and Oblinger, 1985; Stannard and Smith, 1982; Stannard and Wood, 1982, 1983b) have been investigated with varying degrees of success.

In general, the use of luminometry is viewed by members of the food industry as being limited by interference by ATP present in foods and by the amount of skilled manipulation required to perform the numerous steps in the assay. The objective of the present research was to evaluate methods for determining levels of microorganisms in ground beef using luminometry. The problem of interference by ATP present in food was specifically addressed, and individual steps were studied to determine how much they affected sensitivity and/or accuracy of the assay. For this work various ground beef storage and packaging conditions were used in order to evaluate the scope of usefulness. Although the agar plate count has accuracy limitations, it is the currently accepted method for enumerating bacteria, and was therefore used for comparison with the ATP assay throughout this study.

Chapter 1

Rapid enumeration of bacteria in ground beef using luminometry

ABSTRACT

Microbial ATP content was determined using a rapid bioluminescent method in order to estimate numbers of microorganisms in ground beef. Microbial ATP content as well as mesophilic ($35^{o}C$) and psychrotrophic ($20^{o}C$) plate counts were determined periodically during $7^{o}C$ storage of retail ground beef and laboratory-prepared ground beef. Microbial ATP content was found to correlate well with psychrotrophic counts in the range of about 5×10^{5} to 1×10^{9} cfu per gram. Although ATP content per cfu decreased approximately 10 fold during sample storage, results indicate that the ATP assay can be used to quickly and accurately predict microbial numbers in ground beef over a wide range of contamination levels.

INTRODUCTION

Recent interest in rapid methods for determining the microbial quality of foods has stimulated research efforts to evaluate the usefulness of the firefly luciferin/luciferase ATP assay. The assay has potential as a rapid method for monitoring the microbial quality of ground beef since microbial numbers may be estimated in less than 30 min, as compared with traditional plate counts which take 24 to 72 hr to complete.

The bioluminescent ATP assay is well established and has been successfully used to estimate ATP concentrations in water (Baumgart et al., 1980a), urine (Johnston et al., 1976a;b), blood cultures (Molin et al., 1983), sewage sludge (Lehtokari et al., 1983) and laboratory broth media (Sharpe et al., 1970; Karl, 1980). The basic steps in the assay are: 1. Separation of microorganisms from the sample (not necessary in some cases), 2. Extraction of microbial ATP, 3. Quantitative determination of microbial ATP, and 4. Estimation of microbial numbers, given the concentration of microbial ATP. The bioluminescent reaction involves:

$$1.\ \text{ATP} + \text{luciferin} \xrightarrow[\text{luciferase}]{Mg^{+2}} \text{luciferin-AMP} + PP_i$$

$$2.\ \text{luciferin-AMP} + O_2 \longrightarrow \text{AMP} + CO_2 + \text{oxyluciferin} + \text{light}$$

The amount of light emitted per sec is proportional to the quantity of ATP present. This reaction is dependent on pH, temperature and ionic strength; therefore assay conditions must be carefully controlled. ATP solutions of known concentration are used to standardize the assay and serve as internal standards (Strehler, 1968; Wood and Gibbs, 1982; DeLuca and McElroy, 1981).

The bioluminescent ATP assay has been shown to be accurate and rapid when used for a number of applications. In order for the method to become accepted as routine for estimating numbers of microorganisms in foods, procedures must be developed which are less labor intensive, more sensitive and higher in throughput.

The objectives of this research were to: 1) Investigate the efficacy of a double filtration procedure for separating bacteria from retail and laboratory-prepared ground beef stored for various periods, 2) Estimate the number of bacteria in samples by using the firefly bioluminescence ATP assay, and 3) By these procedures

determine the minimum number of bacteria detectable, the correlation of counts estimated using luminometry with actualmicrobial plate counts, and the influence of ATP content per cfu on this correlation.

MATERIALS AND METHODS

Ground Beef

For this study 3 samples of fresh ground beef with pull dates differing by a least one week were purchased from each of 3 local retail grocery stores. In addition, 3 samples of beef bottom round were purchased and ground in the laboratory to prepare ground beef with lower initial counts. Preparation of ground beef in the laboratory was done using a sterile grinder by passing the meat twice through a 3/16 inch plate. Meat ground in this manner was first surface-flamed until charred with a bunsen burner. After flaming, the meat surfaces were trimmed off using a sterile knife and the raw interior was used for ground beef preparation. Portions (25g) of each retail or laboratory-prepared ground beef sample were placed into sterile petri dishes and stored at $7^{o}C$. Ground beef was sampled periodically for microbial ATP assay and plate count determination until plate counts reached 10^{9} cfu/g.

Sample Preparation for Microbial ATP Assay

The general procedure for sample preparation and ATP determination is shown in Figure 1.1. Ground beef samples were diluted 1:10 (w/v) using Butterfield's 0.0003 M potassium phosphate buffer and homogenized using a Stomacher

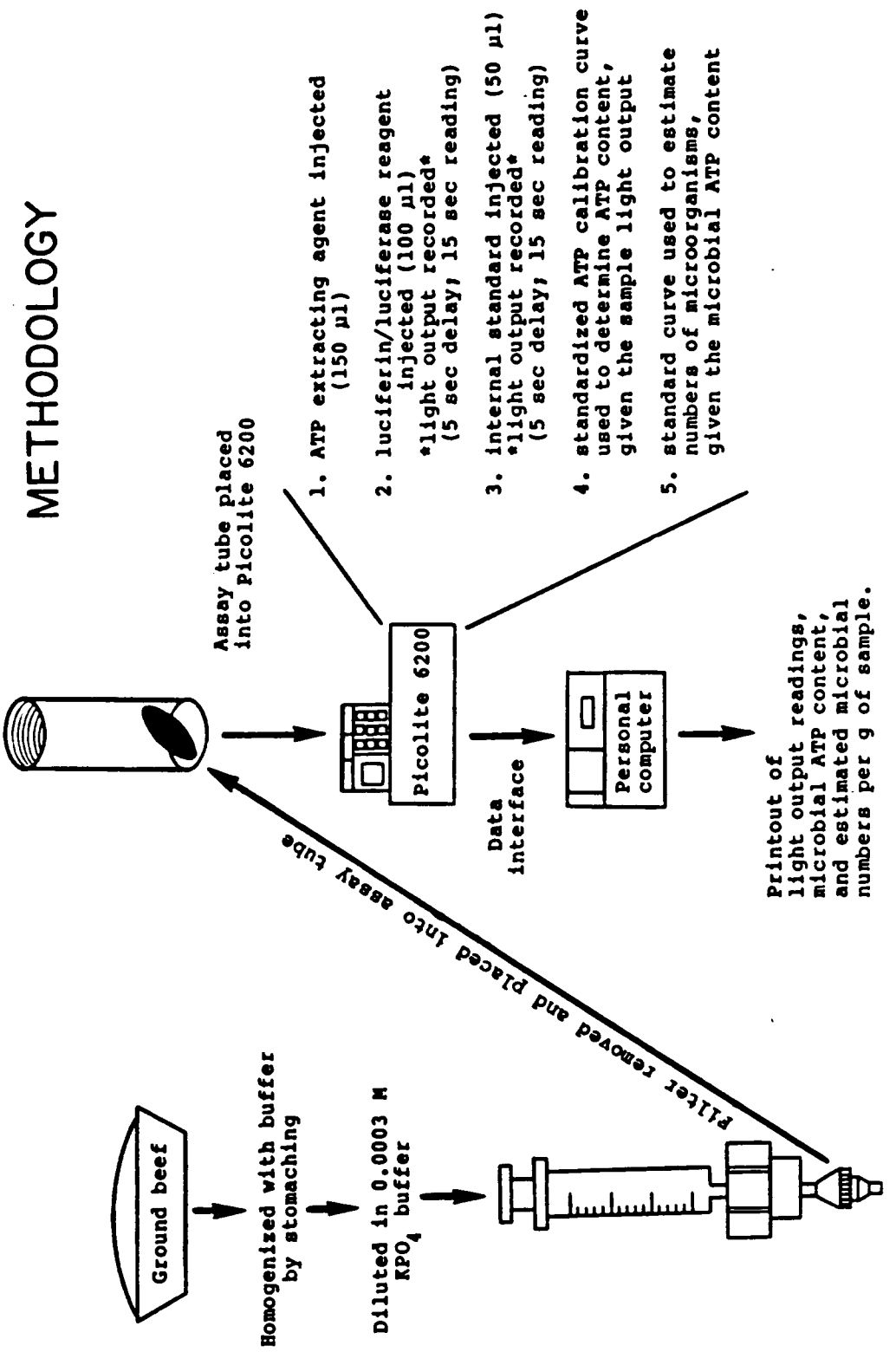

Figure 1.1. Procedure for sample preparation, ATP determination, and estimation of microbial numbers in ground beef.

400 (Tekmar Co., Cincinnati, OH). Homogenates were further diluted with the same buffer so that light readings were within the range of the ATP calibration curve. Ten ml of each diluted sample was filtered through a 3 μm polycarbonate filter (Nucleopore, Pleasanton, CA) held in a Swin-lok assembly (Nucleopore) to remove non-microbial particles, and then filtered through a 0.4 μm polycarbonate filter (Nucleopore) in a Swinney filter holder (Gelman, Ann Arbor, MI) to collect microbial cells. Homogenizing for at least 15 to 30 sec resulted in maximum plate count values for 6 ground beef samples but that homogenizing for longer than 90 sec caused filtration difficulty for 1:100 dilutions of ground beef with 30% fat. This may have been due to breakage of meat cells which were then capable of passing through the 3μm filter and clogging the 0.45 μm filter. Higher dilution or lower fat percent allowed easier filtration. Therefore, 90 sec was chosen as the stomaching time. Evaluation of this double filtration technique for collecting and concentrating microorganisms revealed high efficiency. Microbial numbers of 6 ground beef homogenates were the same before and after filtration through the 3 μm filter while no bacteria were recovered after filtration through the 0.4 μm filter. After filtration, filters retaining bacteria were placed into 17 x 60 mm glass tubes for ATP assay. Each ground beef sample was assayed in

quadruplicate and the results were averaged.

Reagents and Microbial ATP Measurement

All reagents and potassium phosphate diluents were prepared using distilled deionized water which was filter sterilized and autoclaved in order to reduce background ATP contamination and remove dissolved gasses. Removal of excess dissolved gasses was necessary to achieve consistent volumes of reagents when pumped by the luminometer. ATP content was determined using a model 6200 Luminometer (Packard Instrument Company, Downers Grove, IL). During the assay, 300 µl of extracting agent (PicoexB, Packard Instrument Company) was first used to disrupt cells and release ATP. An assay reagent containing luciferin, luciferase and Mg^{2+} (Picozyme F, Packard Instrument Company) was then used to measure ATP by causing quantitative light output detected by a photomultiplier tube. A standard ATP solution was used during ATP assay to help correct for differences in light output - altering properties among individual samples. These may be differences in opaqueness, presence of light output reaction inhibitors or other properties. Light response was expressed as a ratio of light output due to sample (C1-blank) divided by light output due to the standard ATP solution (C2).

Standard solutions of ATP between 10^{-10} M and 10^{-7} M were used to calibrate the assay so that results could be

expressed in terms of ATP concentration rather than arbitrary light output values. Standard curves of ATP concentration v.s. light response were made each day and used for calculation of ATP concentration in ground beef samples, given the light response during assay.

During preliminary experiments, brighter laboratory lights were found to cause increased background counts when glass tubes were used. The longer the tubes were exposed to light before being placed into the luminometer, up to 5 minutes, the higher the background counts became. This did not occur with polystyrene plastic tubes used for experiments reported in Chapters 2 through 5. All subsequent experiments using glass tubes were done in subdued light so that background counts remained low.

Plate Counts

Microbial plate counts were done using the pour plate method with Standard Methods Agar. Plates were incubated at 20°C for 4 days for psychrotrophic counts, and at 35°C for 2 days for mesophilic counts. Dilutions of the initial homogenates were made using 0.1% peptone. Preliminary work showed that maximum plate counts for ground beef stored at 7°C were obtained at 20-25°C and that plate count results were the same whether Trypticase Soy Agar or Standard Methods Agar were used.

Estimation of Microbial Numbers

Plate count results were compared with microbial ATP concentrations. Linear regression curves of log ATP concentration vs log plate count were made and the coefficients later used to estimate numbers of microorganisms per gram of meat, given the concentration of microbial ATP in each sample.

RESULTS AND DISCUSSION

Retail ground beef had initial psychrotrophic plate counts of approximately 10^6 cfu/g and mesophilic counts in the 10^5 cfu/g range. (Tables 1.1-1.3). Throughout storage at 7°C, psychrotrophic counts were higher than mesophilic counts, and therefore were the better estimates of total numbers of microorganisms. Within 5 days of storage, psychrotrophic counts reached 10^9 to 10^{10} cfu/g (Tables 1.1-1.3).

Microbial ATP levels in the meat increased from approximately 5×10^{-12} to 1×10^{-9} moles per gram during storage. In this period, the amount of ATP per cfu decreased approximately 10 fold, although the amount of decrease varied among meat samples. Counts in the 10^6, 10^7 and 10^8 ranges averaged 1.7, 1.1 and 0.43 fg (1 fg = 10^{-15} g) of ATP per cfu, respectively. Stannard and Wood (1983b) reported a similar decrease in ATP per cfu during storage of ground beef, although their values were somewhat lower than those reported here. The decrease in ATP level per cell may have been due to depletion of available nutrients for cell growth as cell density increased in the meat. Another explanation is that the number of bacteria per cfu may have decreased during storage of the beef. This could occur due to less chain formation or less clumping.

Table 1.1. Plate counts and microbial ATP contents for retail ground beef (Store A) stored at 7°C.

Sample	Storage Time (Days)	Plate Counts (CFU/g) 20°C[a]	Plate Counts (CFU/g) 35°C[b]	Microbial ATP (Moles/g meat)	Microbial ATP (fg per CFU)[c]
I	0	1.1×10^{6}	6.8×10^{5}	7.4×10^{-12}	4.0
	1	4.0×10^{6}	2.1×10^{6}	7.4×10^{-12}	1.0
	2	3.0×10^{7}	8.0×10^{6}	4.0×10^{-11}	0.74
	3	2.2×10^{8}	4.3×10^{7}	2.0×10^{-10}	0.49
	4	7.0×10^{8}	1.2×10^{8}	3.2×10^{-10}	0.25
II	0	6.5×10^{6}		1.1×10^{-11}	0.93
	1	6.0×10^{7}		6.0×10^{-11}	0.55
	2	2.5×10^{8}		3.8×10^{-10}	0.82
	3	5.5×10^{9}		2.8×10^{-9}	0.28
III	0	1.6×10^{6}		7.2×10^{-12}	2.5
	1	7.1×10^{6}		2.0×10^{-11}	1.5
	2	6.8×10^{7}		4.2×10^{-11}	3.4
	3	3.0×10^{9}		4.5×10^{-9}	0.82
	4	8.6×10^{9}		2.4×10^{-9}	0.15

[a]Plated on Standard Methods Agar and incubated 4 days at 20°C.

[b]Plated on Standard Methods Agar and incubated 2 days at 35°C.

[c]Calculated using the 20°C plate counts.

Table 1.2. Plate counts and microbial ATP contents for retail ground beef (Store B) stored at 7°C.

Sample	Storage Time (Days)	Plate Counts (CFU/g) 20°C[a]	Plate Counts (CFU/g) 35°C[b]	Microbial ATP (Moles/g meat)	Microbial ATP (fg per CFU)[c]
I	0	1.9×10^{6}	6.2×10^{5}	2.7×10^{-12}	0.78
	1	3.4×10^{6}	1.3×10^{6}	7.6×10^{-12}	1.2
	2	1.3×10^{7}	7.0×10^{6}	2.8×10^{-11}	1.2
	3	1.2×10^{8}	2.7×10^{7}	9.0×10^{-11}	0.38
	4	4.9×10^{8}	7.5×10^{7}	3.3×10^{-10}	0.37
II	0	1.1×10^{6}		5.6×10^{-12}	2.8
	1	5.2×10^{6}		8.8×10^{-12}	0.94
	2	3.1×10^{7}		2.7×10^{-11}	0.48
	3	2.1×10^{8}		5.7×10^{-11}	0.15
	4	3.7×10^{9}		5.2×10^{-10}	0.078
III	0	5.4×10^{6}		2.2×10^{-11}	2.3
	1	3.9×10^{7}		8.6×10^{-11}	1.2
	2	1.2×10^{8}		1.4×10^{-10}	0.65
	3	1.9×10^{9}		6.0×10^{-10}	0.17

[a]Plated on Standard Methods Agar and incubated 4 days at 20°C.

[b]Plated on Standard Methods Agar and incubated 2 days at 35°C.

[c]Calculated using the 20°C plate counts.

Table 1.3. Plate counts and microbial ATP contents for retail ground beef (Store C) stored at 7°C.

Sample	Storage Time (Days)	Plate Counts (CFU/g) 20°C[a]	Plate Counts (CFU/g) 35°C[b]	Microbial ATP (Moles/g meat)	Microbial ATP (fg per CFU)[c]
I	0	1.4×10^{6}	2.2×10^{5}	3.0×10^{-12}	1.1
	1	2.4×10^{6}	3.8×10^{5}	4.4×10^{-12}	1.0
	2	1.7×10^{7}	1.9×10^{6}	4.0×10^{-11}	1.3
	3	1.8×10^{8}	1.2×10^{7}	1.2×10^{-10}	0.35
	4	1.3×10^{9}	8.3×10^{7}	8.7×10^{-10}	0.37
II	0	9.8×10^{6}		1.8×10^{-11}	1.0
	1	3.6×10^{7}		3.9×10^{-11}	0.60
	2	1.5×10^{8}		7.8×10^{-11}	0.29
	3	3.6×10^{9}		6.3×10^{-10}	0.096
III	0	2.3×10^{6}		1.0×10^{-11}	2.4
	1	5.2×10^{7}		3.9×10^{-11}	0.41
	2	1.0×10^{8}		1.3×10^{-10}	0.72
	3	4.6×10^{8}		1.4×10^{-10}	0.16
	4	9.8×10^{8}		3.6×10^{-10}	0.50

[a]Plated on Standard Methods Agar and incubated 4 days at 20°C.

[b]Plated on Standard Methods Agar and incubated 2 days at 35°C.

[c]Calculated using the 20°C plate counts.

A linear regression line derived from ATP values and 20°C plate count values was used to "predict" plate counts. These counts were compared with actual plate counts and are shown in Figure 1.2. Confidence limits (95%) for each "prediction" were ±0.7 log and limits for a population of "predictions" were ±0.2 log. Excellent correlation extended over the entire range of retail ground beef counts. These results indicate close agreement between "predicted" and actual counts, and demonstrate the potential value of luminometry for estimating unknown plate counts.

Stannard and Wood (1983b) stated that accuracy of the assay depends on low variation of ATP per cfu. However, from the results reported here, it appears that use of curve coefficients for predicting counts can correct for differences in bacterial ATP content as a function of cfu concentration in ground beef. Use of curve coefficients resulted in accurate plate count estimates even though the amount of ATP per cfu decreased about 10 fold during 7°C storage of both retail and laboratory-prepared ground beef. The assay is based on a predictable amount of ATP measured per cfu. Because a standard curve of microbial ATP vs plate count was used to correct for differences in ATP content per cfu, the assay was a good estimator of microbial numbers throughout the storage periods.

With laboratory-prepared ground beef, good agreement

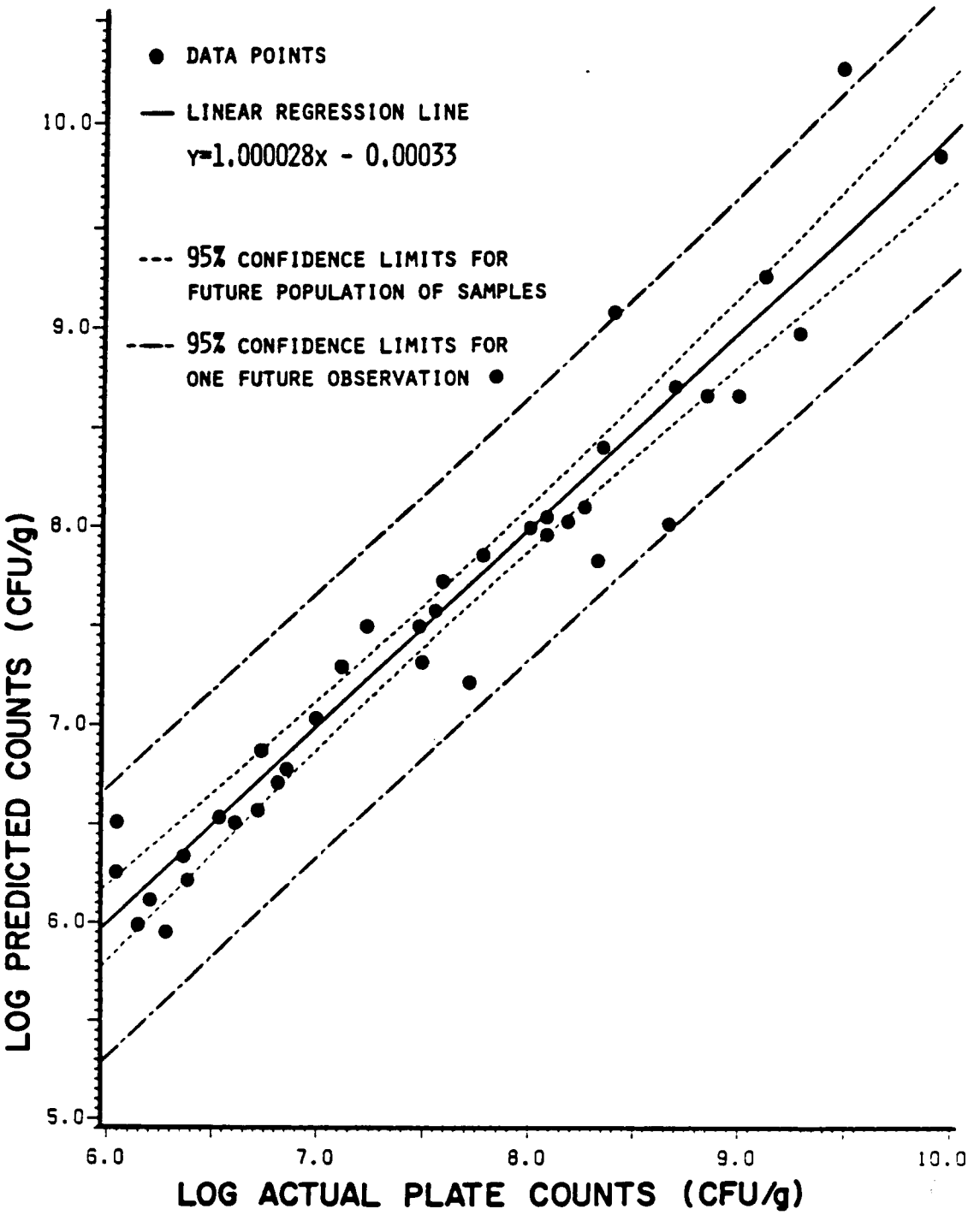

Figure 1.2. Relationship of predicted and actual plate counts for retail ground beef stored at 7°C.

between psychrotrophic counts and "predicted" counts was observed above 5×10^5 cfu/g (Tables 1.4-1.6). Below this value, actual plate counts of 10^3 to 10^5 cfu/g corresponded with apparent microbial ATP levels of 1.6×10^{-13} to 7.6×10^{-13} moles per gram. This small difference in ATP compared to the wide range of plate count values indicates that microbial ATP was not detectable in this range. Below 10^5 cfu/g the ATP/cfu values were calculated to be greater than 4 fg/CFU. Others (Karl, 1980; Sharpe et al.,1970) have reported that many bacteria contain a relatively constant amount of ATP. Published values average about 0.5 to 1.0 fg ATP per cell. Therefore, it is likely that at low bacterial levels there was interference by intrinsic ATP from the meat not completely removed by the filtration procedure. The lower detection limit of 10^6 cfu/g in beef for accurate plate count estimation is similar to that reported by Stannard and Wood (1983).

The relationship of microbial ATP and psychrotrophic plate counts for all experiments is shown in Figure 1.3. Above 5×10^5 cfu/g, log plate counts were roughly proportional to log ATP/g. Below this level, ATP concentrations determined were not dependent on bacterial concentrations in the ground beef. Therefore, microbial numbers could not be accurately predicted for samples with less than 10^6 cfu/g based on the amount of microbial ATP

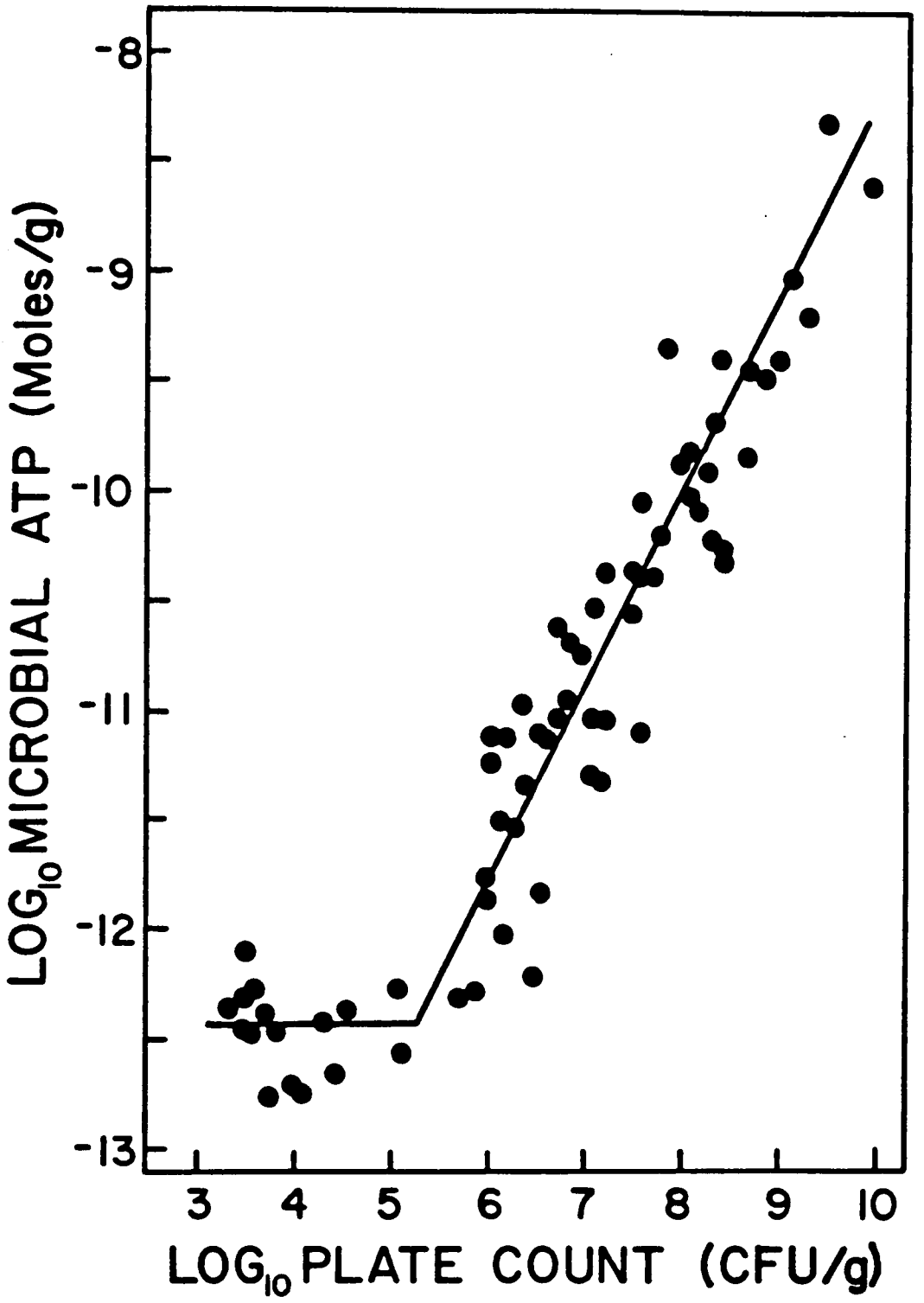

Figure 1.3. Microbial ATP concentrations and plate counts of retail and laboratory-prepared ground beef stored at 7°C.

Table 1.4. Plate counts, estimated plate counts, and microbial ATP contents for laboratory-prepared ground beef (from Store A) stored at 7°C. The dashed line represents the cutoff above which beef samples contained bacterial numbers too low to detect using luminometry.

Storage Time (Days)	Microbial ATP (Moles/g meat)	Predicted Counts (CFU/g)		Actual Counts (CFU/g)		Microbial ATP (fg per CFU)[e]
		Curve A[a]	Curve B[b]	20° C[c]	35° C[d]	
0	5.1×10^{-13}	3.0×10^{5}	3.2×10^{4}	3.9×10^{3}	--	72
1	4.0×10^{-13}	2.3×10^{5}	2.3×10^{4}	5.2×10^{3}	2.6×10^{3}	42
2	3.3×10^{-13}	1.9×10^{5}	1.7×10^{4}	3.6×10^{3}	8.0×10^{3}	50
3	4.2×10^{-13}	2.4×10^{5}	2.4×10^{4}	3.6×10^{4}	1.3×10^{4}	6.4
4	2.0×10^{-13}	1.0×10^{5}	7.9×10^{3}	1.0×10^{4}	5.6×10^{3}	11
5	5.0×10^{-13}	2.9×10^{5}	3.1×10^{4}	7.5×10^{5}	8.5×10^{4}	0.37
6	1.4×10^{-12}	9.0×10^{5}	1.4×10^{5}	3.6×10^{6}	3.8×10^{5}	0.21
7	5.8×10^{-13}	3.5×10^{5}	4.0×10^{4}	3.0×10^{6}	9.8×10^{4}	0.11
8	4.6×10^{-12}	3.4×10^{6}	1.1×10^{6}	1.4×10^{7}	4.8×10^{5}	0.18
9	8.7×10^{-12}	6.6×10^{6}	2.0×10^{6}	1.6×10^{7}	4.3×10^{5}	0.30

[a] Calculated using a curve supplied by Packard Instrument Company.

[b] Calculated using a curve derived from retail ground beef data.

[c] Incubated for 4 days at 20°C using Standard Methods Agar.

[d] Incubated for 2 days at 35°C using Standard Methods Agar.

[e] Calculated using 20° plate counts.

Table 1.5. Plate counts, estimated plate counts, and microbial ATP contents for laboratory-prepared ground beef (from Store B) stored at 7°C. The dashed line represents the cutoff above which beef samples contained bacterial numbers too low to detect using luminometry.

Storage Time (Days)	Microbial ATP (Moles/g meat)	Predicted Counts (CFU/g)		Actual Counts (CFU/g)		Microbial ATP (fg per CFU)[e]
		Curve A[a]	Curve B[b]	20° C[c]	35° C[d]	
0	4.5×10^{-13}	2.6×10^{5}	2.8×10^{4}	3.1×10^{3}	--	80
1	7.6×10^{-13}	4.6×10^{5}	5.8×10^{4}	3.2×10^{3}	2.2×10^{3}	130
2	1.8×10^{-13}	9.6×10^{4}	5.2×10^{3}	1.2×10^{4}	3.0×10^{3}	8.2
3	3.3×10^{-13}	1.9×10^{5}	1.8×10^{4}	6.7×10^{3}	6.7×10^{3}	27
4	2.1×10^{-13}	1.2×10^{5}	9.2×10^{3}	2.7×10^{4}	9.4×10^{3}	4.3
5	4.6×10^{-13}	2.7×10^{5}	2.9×10^{4}	5.3×10^{5}	8.6×10^{4}	0.48
6	1.6×10^{-12}	1.1×10^{6}	1.8×10^{5}	9.6×10^{5}	2.0×10^{5}	0.92
7	7.6×10^{-12}	5.8×10^{6}	1.7×10^{6}	3.8×10^{7}	3.9×10^{5}	0.11
8	8.8×10^{-12}	6.8×10^{6}	2.1×10^{6}	1.2×10^{7}	3.5×10^{5}	0.40
9	9.2×10^{-13}	5.7×10^{5}	2.6×10^{4}	1.5×10^{6}	9.6×10^{4}	0.34

[a] Calculated using a curve supplied by Packard Instrument Company.

[b] Calculated using a curve derived from retail ground beef data.

[c] Incubated for 4 days at 20°C using Standard Methods Agar.

[d] Incubated for 2 days at 35°C using Standard Methods Agar.

[e] Calculated using 20° plate counts.

Table 1.6. Plate counts, estimated plate counts, and microbial ATP contents for laboratory-prepared ground beef (from Store C) stored at 7°C. The dashed line represents the cutoff above which beef samples contained bacterial numbers too low to detect using luminometry.

Storage Time (Days)	Microbial ATP (Moles/g meat)	Predicted Counts (CFU/g)		Actual Counts (CFU/g)		Microbial ATP (fg per CFU)[e]
		Curve A[a]	Curve B[b]	20° C[c]	35° C[d]	
0	4.4×10^{-13}	2.5×10^{5}	2.6×10^{4}	2.4×10^{3}	--	100
1	3.4×10^{-13}	1.9×10^{5}	1.8×10^{4}	3.0×10^{3}	2.8×10^{3}	62
2	1.6×10^{-13}	9.1×10^{4}	6.6×10^{3}	5.6×10^{3}	2.6×10^{3}	16
3	3.6×10^{-13}	2.1×10^{5}	2.0×10^{4}	2.0×10^{4}	4.2×10^{3}	9.9
4	2.7×10^{-13}	1.4×10^{5}	1.2×10^{4}	1.3×10^{5}	2.6×10^{4}	1.1
5	5.2×10^{-13}	3.1×10^{5}	3.3×10^{4}	1.2×10^{5}	3.9×10^{4}	2.4
6	1.3×10^{-12}	8.5×10^{5}	1.3×10^{5}	9.8×10^{5}	1.7×10^{5}	0.73
7	4.6×10^{-12}	3.4×10^{6}	8.2×10^{5}	1.3×10^{7}	3.9×10^{5}	0.19
8	5.0×10^{-11}	4.5×10^{5}	2.5×10^{7}	2.6×10^{8}	5.0×10^{6}	0.11
9	9.3×10^{-11}	8.8×10^{7}	6.2×10^{7}	2.9×10^{8}	8.9×10^{5}	0.18

[a] Calculated using a curve supplied by Packard Instrument Company.

[b] Calculated using a curve derived from retail ground beef data.

[c] Incubated for 4 days at 20°C using Standard Methods Agar.

[d] Incubated for 2 days at 35°C using Standard Methods Agar.

[e] Calculated using 20° plate counts.

detectable.

Mean absolute residual values (error) for plate counts estimated using ATP luminometry are presented in Table 1.7. Retail beef samples from each of the 3 stores were estimated to within 1/3 log of the experimentally observed plate count values when a regression curve was used. When constant amounts of ATP per cell were assumed, counts for ground beef with $>5 \times 10^5$ cfu/g were estimated to within 0.44 log (Table 1.7). Estimates for beef with greater than 5×10^5 cfu/g were more accurate than those samples with less than 5×10^5 cfu/g. Counts predicted using regression coefficients derived from the data were different from those predicted using coefficients supplied by Packard Instrument Company. This observation indicates that it may be beneficial for more accurate plate count predictions to develop curve coefficients specifically for each storage condition. This would correct for differences in ATP per cfu which are influenced by storage condition.

Experimental results have shown that the double filtration procedure is useful for assaying microbial ATP in ground beef, and that accurate estimation ($\pm$ 1/2 log) of microbial numbers is possible over a wide range of contamination levels (10^6 and up). Since the assay takes less than 30 minutes (for single samples with everything set up in advance) luminometry has potential for helping with

Table 1.7. Accuracy (mean absolute residual) of 20°C plate count estimates using ATP-luminometry. For calculating accuracy all values were converted to $\log_{10}$.

Ground Beef	Curve A[a]	Curve B[b]	Curve C[c]
Store A (retail)	0.334	0.295	0.355
Store B (retail)	0.316	0.229	0.385
Store C (retail)	0.289	0.191	0.396
Lab-ground (>$5x10^5$ cfu/g)	0.591	0.448	0.621
Lab-ground (<$5x10^5$ cfu/g)	1.141	1.304	1.247
Combined (>$5x10^5$ cfu/g)	0.336	0.337	0.439
Combined (<$5x10^5$ cfu/g)	1.141	1.304	1.247

[a] Calculated using a curve supplied by Packard Instrument Company.

[b] Calculated using a curve derived from retail ground beef data.

[c] Calculated using constant amount of 1 fg ATP per cfu.

decisions concerning microbial quality of foods where an estimate of the microbial population is needed more quickly than would be possible using standard procedures. Multiple samples may be assayed concurrently so that assay time per sample may be as low as 5 minutes (not including set-up of materials, which can be done in advance). Although the assay involves much time and requires skilled technical ability, ATP luminometry may be useful for continually monitoring microbial quality of foods during production, for making rapid decisions of whether to accept shipments of raw materials, or for giving early approval of product release.

Additional research is needed to develop procedures for the application of luminometry to different types of foods. The procedure reported here has a lower predictive detection limit of about 10^6 cfu/g for ground beef. When counts were 5×10^5 cfu/g or below, ATP contributed by the meat interfered with the assay. Additional measures must be taken to further remove non-microbial ATP in order to improve the minimum level of detection. Stannard and Wood (1983) suggested destroying free ATP on the filter with an ATPase. Perhaps this step could be used to improve the procedure described in this chapter.

To further test the usefulness of the bioluminometric ATP assay for quality control in the food industry, research should also be done to determine the changes in ATP content

per cfu under various storage conditions and with several product formulations. It may be necessary to set up specific ATP-cfu curves for different situations in order to better predict microbial numbers.

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Chapter 2

Enhanced sensitivity of bioluminometry for the estimation of microbial numbers in ground beef.

ABSTRACT

Sample filtration and ATPase treatment designed to increase sensitivity of the ATP assay by reducing interference by non-microbial ATP were evaluated.

Filtration through 1um nylon mesh and 0.45 um membrane filters fitted in tandem effectively collected bacteria from ground beef homogenized to 1:20 or higher dilutions and retained their microbial ATP on the 0.45 um filter. ATPase treatment destroyed much of the remaining non-microbial ATP on the filter without reducing ATP from microorganisms.

Use of ATPase treatment increased sensitivity of ATP assay and APC estimation by about 1 log. Estimated APC values were generally accurate to within $\pm$0.50 log for ground beef samples above 5×10^4 cfu/g. Use of ATPase treatment increased assay time by 40 minutes, for a total of 60 minutes for 4 samples assayed in triplicate. Below 5×10^4 cfu/g, counts could not be estimated accurately due to remaining interference from non-microbial ATP.

INTRODUCTION

Early work by Sharpe et al. (1970) and Williams (1971) demonstrated the need to remove non-microbial ATP which interferes with microbial ATP determinations and reduces sensitivity. Sharpe et al. (1970) analyzed a wide variety of foods and found ATP levels ranging from 4×10^6 fg/g in bacon to over 10^9 fg/g in products such as fish and dried soup mix. Comminuted meat contained about 1×10^7 fg/g non-microbial ATP. Williams (1971) found 5×10^7 fg/g non-microbial ATP in milk. These researchers concluded that numbers of bacteria could not be estimated with sufficient sensitivity for most applications unless samples were treated to remove non-microbial ATP.

Since then, several methods have been used to reduce non-microbial ATP when analyzing foods. Vanstaen (1980) described a technique which reduced interfering ATP. With this method an extraction reagent specific for non-microbial cells was used to treat food samples, followed by treatment with an ATPase which hydrolyzed free ATP during a 45 min incubation period. Samples were then mixed with a bacterial extractant prior to firefly reagent addition and light output determination. Bossuyt (1982a; b), Waes and Bossuyt (1982), Waes and Bossuyt, (1981), Bossuyt,(1978), Bossuyt, (1981) and Waes et al. (1984) used this technique for

examining the microbial quality of milk and Graumlich (1985) used the technique for estimating microbial populations in orange juice. Baumgart et al. (1980b) and Kennedy and Oblinger (1985) used non-microbial extraction and ATPase treatment while determining microbial quality of beef.

Stannard and Wood (1983b) used physical separation methods to remove non-microbial ATP sources during microbial ATP determination in lamb and pork. They removed large particles by a centrifugation step, and then stirred the supernatant in a cation exchange resin to remove other non-microbial particles. This facilitated the third step of filtration through a 0.22 μm membrane filter. They achieved sensitivity of 10^5 cfu/g using this technique to prepare samples for ATP assay. However, the procedure is time consuming, uses much glassware and is labor intensive. A combination of enzymatic and physical separation steps was used by Stannard and Wood (1983a), who centrifuged and degraded soluble ATP in order to improve sensitivity of yeast ATP determination in fruit juice.

Use of a double filtration technique for reducing interference by non-microbial ATP in ground beef permitted sensitivity of ATP determination down to 5×10^5 cfu/g (Chapter 1). For prediction of total bacterial counts in fresh ground beef, improved sensitivity is needed since fresh retail ground beef may have counts as low as 10^5

cfu/g. This will allow ground beef with excellent microbial quality to be distinguished from lesser quality beef. The objectives of this study were to evaluate an ATPase treatment designed to increase sensitivity of the ATP assay when used in combination with the double filtration technique.

MATERIALS AND METHODS

Ground Beef

Bottom round was purchased from a local grocery store, and its exterior trimmed off with a sterile knife. Meat interior was cut roughly into 2 to 3 inch cubes and several pieces from the meat exterior were mixed in to provide a low initial inoculum of meat spoilage organisms. Cubes were then ground twice using a sterile grinder fitted with a 3/16 inch plate.

Ground beef was aseptically weighed and pressed into 35 g (patties 3" in diameter and 1/4" thick) using a custom made patty press. Patties were placed on 4-inch square styrofoam meat trays and wrapped with a single layer of PVC film (Reynold's Plastic Wrap). Packages were each numbered and stored at 10^{o}C in a single layer. Periodically, duplicate samples were pulled using randomly chosen package numbers and examined for bacterial ATP content and plate count. Styrofoam trays and PVC film were determined to be nearly sterile ($\leq$10 cfu/4in^{2}) by swabbing, and plating on Standard Methods Agar for 4 days at 20^{o}C.

Reagents

Luminometry-quality water (LQW) was made by autoclaving (20 min, 121^{o}C) and filtering (0.22μm, Nucleopore, Pleasanton, CA) distilled deionized water. Butterfield's

buffer (0.0003M KH_2PO_4) was made using LQW, filter sterilized (0.22μm, Nucleopore), and adjusted to pH 7.2 with 1N NaOH before autoclaving. Hank's Buffered Salt Solution (Sigma Chemical Co.) in 0.015M Trizma HCl (HT) was also made using LQW. The solution was filter sterilized (0.22μm, Nucleopore) and adjusted to pH 7.75 with 1N NaOH.

All reagents used for ATP determination were made using LQW. Bacterial extractant (PicoExB) was made by adding a proprietary compound (Packard Instrument Company, Downers Grove, IL) and 1.52g disodium EDTA to 1 liter HT. The solution was filter sterilized (0.22 μm, Nucleopore), adjusted to pH 7.75 with 1N NaOH or 1N HCl, and then stored at 2°C. Before use, PicoExB was swirled and warmed gently in a 44°C water bath until clarified. Luciferin-luciferase reagent (PicozymeF, Packard) was rehydrated with LQW, mixed gently by rotating, and allowed to stand at room temperature for 30 min before use. This manner of preparation resulted in more reproducible ATP readings and lower background levels.

ATP assay standard was made by rehydrating Picochec ATP (Packard) and diluting with HT to yield a final concentration of 3×10^{-7} M ATP. ATP assay standard was stored frozen (-20°C) until use. ATP standards used for ATP calibrations were solutions of Picochec ATP rehydrated with HT and serially diluted with PicoExB to yield solutions

ranging from 10^{-6} to 10^{-12} M ATP. ATP Calibration Standards were stored frozen (-20°C) and used immediately after thawing.

Somatic cell extractant (ExS) was made by adding apyrase (an ATPase), lipase and protease (Sigma Chemical Co.) to 0.03 M Hepes Buffer, pH 7.0. This mixture was stored frozen (-20°C) until diluting 1:100 in PicoExS (Packard) to yield 0.1 unit protease/ml, 0.1 unit lipase/ml and 0.02 unit apyrase/ml. ExS was stored at 2°C no longer than 1 month until use.

Sample Preparation

Ground beef patties were removed from storage and unwrapped. Sectors weighing 11 g were obtained from each patty and homogenized with 99 ml Butterfield's buffer for 90 sec using a Stomacher 400 (Tekmar Co., Cincinnati, OH). Sample homogenates were further diluted in Butterfield's buffer so that ATP concentration would be within the range of ATP standards used for calibration.

Diluted sample homogenates and blanks containing Butterfield's buffer only were assayed for ATP in triplicate and the results were averaged. For each assay, 10 ml of diluted homogenate in a sterile 10 ml syringe was filtered through two filters fitted in tandem. The first was a 1 μm nylon mesh filter (disposable unit) which retained relatively large meat particles. The second was a 0.45 μm

disposable filter (Acrodisk, Gelman, Ann Arbor, MI) which retained bacteria. Filtration was accomplished in approximately 100 sec using a syringe pump. Syringe tips were aimed upwards during filtration so as to avoid clogging of the filters.

After tandem filtration, two syringe volumes of air were passed through the 0.45 µm filter to remove as much of the meat sample as possible. This step did not reduce bacterial ATP recovered from the filterby extraction. Each filter with retained bacteria was then fitted to a sterile 5 ml syringe, saturated with 1 ml ExS, and incubated 30 min at 37^{o}C to extract and destroy non-microbial ATP. After incubation, two syringe volumes of air, 5 ml HT, and another two syringe volumes of air were passed through each filter to remove apyrase. Bacterial extraction was then accomplished by saturating the filter with 600 µl PicoExB at room temperature for about 30 sec. Extraction times between 15 sec and 2 min did not affect thoroughness of extraction. Ground beef samples were diluted so that no more than 5 x 10^{6} bacteria were extracted on the filter since extraction of greater numbers may result in decreased extraction efficiency (Packard Instruments Company, personal commumnication). After extraction, filtrate containing bacterial ATP was collected by passing three syringe volumes of air through the filter into a tube.

Tubes containing 300 µl of extract from each filtration (3 tubes per ground beef sample) were placed into a Picolite 6200 luminometer (Packard). The luminometer was programmed to automatically inject 200 µl PicozymeF and, after a 5 sec delay, record cumulative light output for 15 sec (count 1, or C1). After an additional injection of 100 µl ATP assay standard and 5 sec delay, a second 15 sec light output reading was recorded (count 2, or C2). Results were expressed as the ratio, C2 divided by C1 minus a blank [C2/(C1 - B)]. Ratios were converted to ATP concentrations using regression coefficients obtained during ATP calibration performed earlier the same day.

Other Extraction Procedures and Reagents

Several procedures were used to extract microbial ATP from ground beef and from a variety of meat spoilage bacteria in order to compare efficiency of extraction with PicoExB. ATP-Releasing Reagent (ATP-RR, Turner Designs, Mountain View, CA) and Extralight (Analytical Luminescence Laboratory, San Diego, CA) were commercial extractants available from luminometer distributors which were used according to instructions. ATP-RR was diluted 1:1 with HT buffer, and 200 µl of the mixture added to 100 µl of sample to be assayed for microbial ATP. For ATP extraction using Extralight, 200 µl was added to 100 µl sample in each tube. An extraction procedure using hot 20 mM Tris HCl buffer (pH

7.75, containing 2 mM EDTA) (T/E) was recommended by Lundin and Thore (1975) who found it to be one of the more thorough extraction methods among those convenient for routine use. In this procedure, 0.5 ml sample and 4.5 ml hot T/E were maintained in a boiling waterbath for 90 sec and then chilled rapidly using an ice water bath.

Each tube of extract (300 µl) prepared using PicoExB, ATP-RR, Extralight or hot T/E was placed into a chamber in the luminometer where 200 µl PicozymeF and 100 µl assay standard were added. Light output ratios were calculated as described previously. Separate ATP calibrations were done for each method using appropriate reagent and blanks. Extraction of microbial ATP on filters using each method was also done. Filter extraction procedures were similar to that described earlier for PicoExB.

Prediction of Microbial Numbers

Each ground beef sample was plated on Standard Methods Agar (BBL) and incubated for 4 days at 20°C. Previously, for ground beef stored at 10°C, plating at 20-25°C was determined to result in maximum counts.

For each sample, log plate count value was regressed linearly with log ATP concentration. A second set of ground beef samples prepared, packaged and stored in the same way as the set of samples described above was sampled for bacterial ATP content and plate count. Regression curve

coefficients derived from the first set of data were used to estimate plate counts of the second set based on the second set's microbial ATP concentrations. Estimated plate counts were then compared with plate counts experimentally determined in order to determine sensitivity and accuracy.

RESULTS AND DISCUSSION

The sensitivity for estimating microbial numbers by ATP content in ground beef has been reported to be approximately 10^6 per gram (Patel and Wood, 1983; Stannard and Smith, 1982; Stannard and Wood, 1983b; Kennedy and Oblinger, 1985; Chapter 1). Amounts of ATP detectable, however, are claimed by luminometer manufacturers to be much lower than would be present in 10^6 bacteria. Figure 2.1 depicts the theoretical sensitivity of the Picolite 6200 using reagents from Packard Instrument Company. Light output readings of ATP samples minus a blank (C1 - blank) were linear from 6.7 to 11.7 log fg ATP/liter in sample tubes. Since the minimum dilution of ground beef that was filterable was 1:20, the theoretical sensitivity of microbial ATP detection in ground beef would be equivalent to 6×10^3 fg ATP/g. Assuming 1 fg ATP average per bacterium, minimum sensitivity would be 6×10^3 cfu/g of ground beef. Maximum numbers of bacteria that can be estimated using luminometry are unlimited if samples are diluted so that microbial ATP levels are less than 3×10^6 cfu/ml in the homogenate. This is equivalent to 5×10^{10} log fg ATP/liter in the assay tube, the concentration at which sample ATP interferes with light output values (C2) of the assay standard (Figure 2.1).

To further test the theoretical minimum sensitivity of the ATP assay for determining microbial ATP in ground beef,

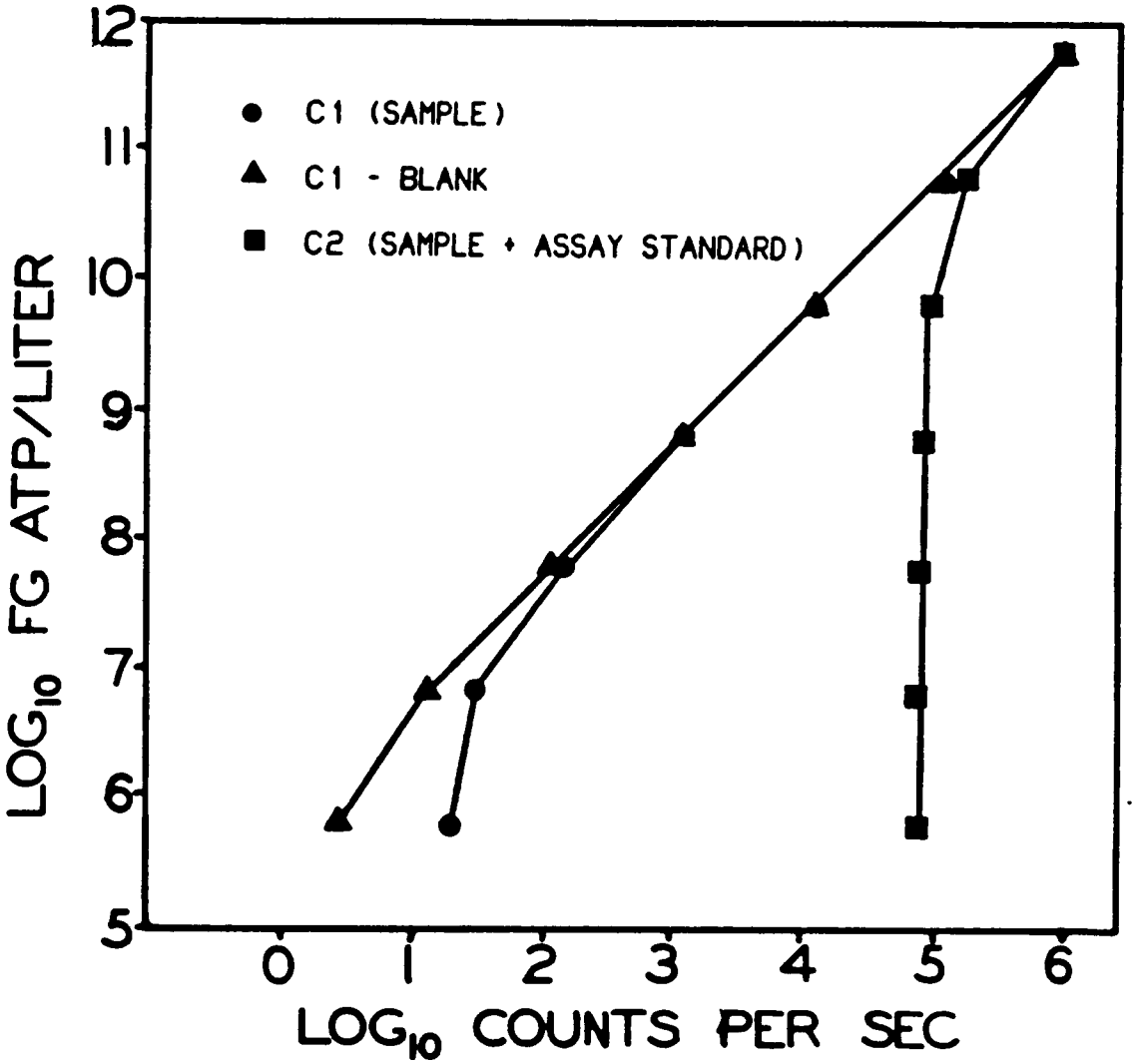

Figure 2.1. Light output measured for samples and assay standards over the range of measureable ATP concentrations.

a culture of Pseudomonas fluorescens ATCC 13525 was serially diluted and analyzed for ATP concentration. Results presented in Figure 2.2 illustrate that the lowest amount of ATP detected was 4.5×10^2 fg/ml in broth. If a 1:20 dilution had been required as in the case of bacteria in ground beef, the theoretical sensitivity limit would be 9.0×10^3 cfu/g of original sample. With theoretical sensitivity limits of 6×10^3 and 9×10^3 cfu/g, and experimentally observed sensitivity of 10^6 cfu/g for ground beef, it appears that further improvements may be possible to lower detection limits.

Bacterial ATP levels per cfu were similar to those reported by Baumgart et al. (1980b) and Chappelle and Levin (1968) who reported 0.96 fg and 0.31 fg per cell, respectively, for Pseudomonas fluorescens. Other researchers have reported bacterial ATP values as low as 0.01 or as high as 31 fg/cell (Kennedy and Oblinger, 1985; Karl, 1980). Extraction methods used, however, may cause differences in reported ATP content. In order to evaluate extraction efficiency of PicoExB, two other commercially available extractants and a boiling extraction were used for comparison. The boiling Tris with EDTA method was chosen since, in a study comparing ATP extraction methods, Lundin and Thore (1975) reported excellent extraction efficiency.

Each of the extractants contained EDTA, which is

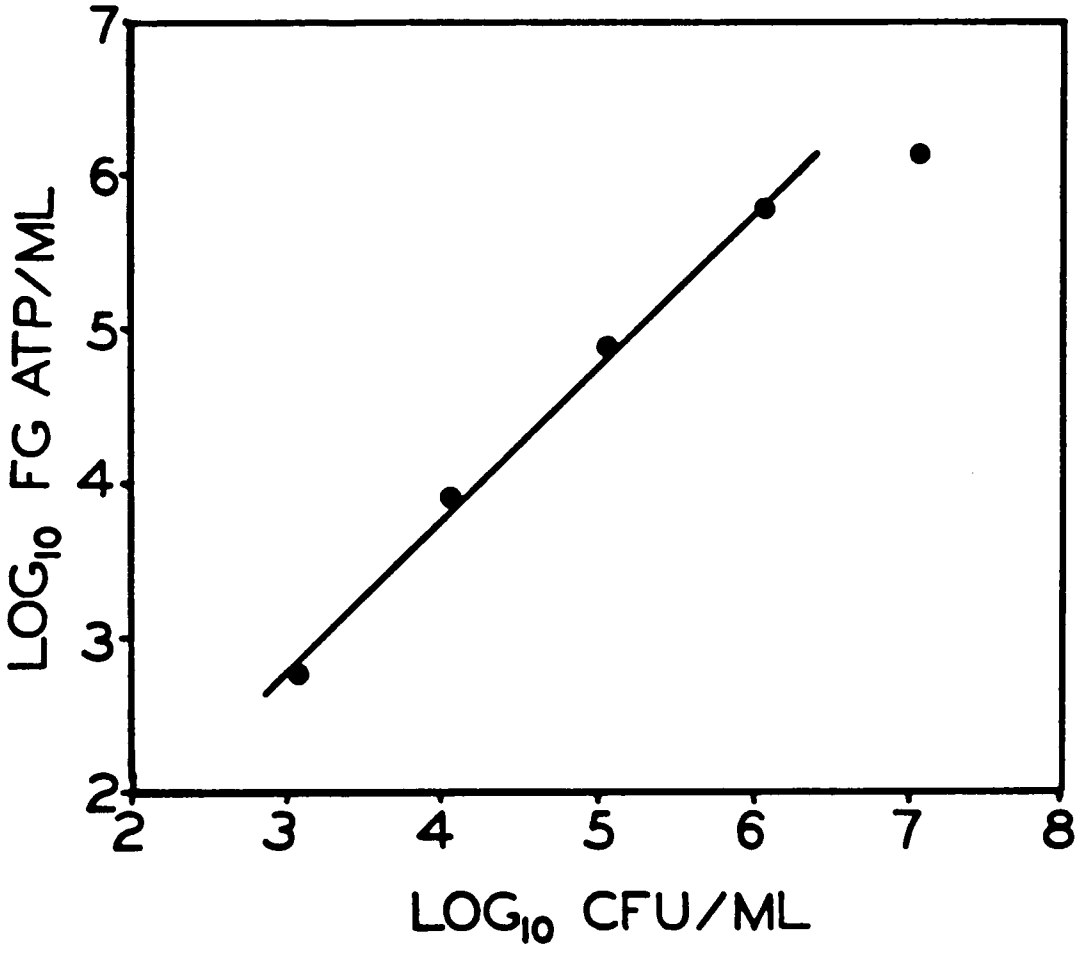

Figure 2.2. Quantitation of Pseudomonas fluorescens grown in TSB. Overnight culture was serially diluted, and 10 ml of each dilution was filtered and extracted with 600 ul PicoExB. For assay, 300 ul was collected.

necessary for inactivation of ATP-converting enzymes capable of interfering with the assay (Lundin and Thore, 1975). Presence of active ATP-converting enzymes would be indicated by a decrease in light-output readings when the assay was delayed. However, readings were stable for up to 30 min after extraction, indicating lack of this type of interference. With each extraction method light output during ATP assay reached a maximum value immediately after addition of the luciferin-luciferase reagent. After a few seconds light output dropped slightly and was stable for at least 60 seconds.

Using PicoExB resulted in ATP yields approximately the same or greater than the other extraction methods (Table 2.1). Use of ATP-RR and boiling Tris EDTA resulted in good extraction in most cases, but Extralight yielded low amounts of ATP, particularly with bacteria in ground beef. None of the extractants caused significant interference with the standard ATP solution.

Triplicate determinations were made within duplicate experiments. Results were similar for ATP extracted from bacteria on a filter, or directly in a tube. Samples were diluted 10 fold and re-extracted in order to determine if incomplete extraction was due to overloading with an excess number of bacteria. ATP levels determined were similar to those determined for undiluted samples when multiplied by a

Table 2.1. Comparison of extraction methods for recovery of microbial ATP. Results are expressed as percent ATP recovery relative to that using PicoExB extractant.

Sample	Commercial extractants			
	PicoExB	Extralight	ATP-RR	Boiling Tris EDTA
Lactobacillus plantarum ATCC 14917	100	69	100	75
Staphylococcus aureus[a]	100	45	86	67
Pseudomonas fluorescens ATCC 13525	100	34	106	95
Ground beef (1:100) (4.6×10^7 cfu/g)	100	18	78	78
ATP Standard	100	95	100	104

[a] Obtained from Dept. Food Science, Virginia Tech, Blacksburg, VA.

factor of 10, indicating no overloading.

The 1 um and 0.45 μm double fitration procedure was examined for efficiency of bacterial collection. Data presented in Table 2.2 indicate that for pure culture meat spoilage bacteria, as well as mixed retail flora in ground beef, the double filters allowed essentially all bacteria to pass through the 1 μm filter and none to pass through the 0.45 μm filter. This occured even though the bacterial types varied in size or were accompanied by meat particles. Essentially all bacteria were collected on the 0.45 μm filter.

Likewise, the double filtration system was found to be efficient for retaining bacterial ATP (Table 2.3). Microbial ATP from 10 pure cultures of meat spoilage bacteria was not retained by the 1 μm filter. Little microbial ATP passed through the filter tandem; therefore essentially all was retained by the 0.45 μm filter. Ground beef homogenates filtered through a 1 μm filter had ATP levels similar before and after filtering, although the filtrate was clearer than the unfiltered homogenate.

The use of a selective non-microbial ATP extraction and digestion with ATPase (ATPase treatment) was examined for its ability to degrade non-microbial ATP without reducing ATP present in bacteria (Table 2.4). Ten beef spoilage bacterial isolates were filtered with 0.45 μm filters and

Table 2.2. Efficiency of filtration for collecting bacteria.

Sample	CFU/ml[a] without filtering	after 1um filter	after 1um filter plus 0.45 um filter
Pure culture isolate:			
Pseudomonas R5[b]	3.2×10^{5}[c]	3.1×10^{5}[c]	<1[d]
Pseudomonas R19-1[b]	6.8×10^{5}[c]	6.4×10^{5}[c]	<1[d]
Pseudomonas ATCC 13525	8.4×10^{5}[c]	9.5×10^{5}[c]	2[d]
Aeromonas R24[b]	5.5×10^{4}[c]	5.4×10^{4}[c]	<1[d]
Enterobacter R25[b]	5.1×10^{5}[c]	6.2×10^{5}[c]	<1[d]
Lactobacillus R15[b]	7.2×10^{4}[c]	7.6×10^{4}[c]	<1[d]
Lactobacillus R17[b]	6.2×10^{4}[c]	6.0×10^{4}[c]	<1[d]
Lactobacillus ATCC 1497	8.1×10^{4}[c]	8.1×10^{4}[c]	<1[d]
Bacillus R7[b]	1.8×10^{5}[c]	2.2×10^{5}[c]	<1[d]
Brochothrix R13-1[b]	2.3×10^{5}[c]	2.1×10^{5}[c]	<1[d]
1:100 Ground beef homogenate	7.6×10^{4}[c]	7.4×10^{4}[c]	<1[d]

[a] Plate counting was done using Standard Methods agar and incubating at 20°C for 4 days Pure cultures were analyzed in duplicate, and 1:100 ground beef homogenate was analyzed in quadruplicate. CFU values were analyzed for statistical differences using ANOVA. Values in each row with different superscripts were significantly different ($p = 0.0001$).

[b] Ground beef spoilage isolates obtained from Dept. Food Science, Virginia Tech, Blacksburg, VA.

Table 2.3. Effect of filtration on ATP levels detected for ground beef isolates and homogenized ground beef samples.

Sample	ATP fg/ml[a] without filtering	ATP fg/ml[a] after 1um filter	ATP fg/ml[a] after 1um filter plus 0.45 um filter
Pure culture isolate:			
Pseudomonas R5[b]	4.1×10^{5}[c]	4.6×10^{5}[c]	4.5×10^{2}[d]
Pseudomonas R19-1[b]	8.8×10^{4}[c]	7.0×10^{4}[c]	4.3×10^{2}[d]
Pseudomonas ATCC 13525	2.3×10^{5}[c]	2.0×10^{5}[c]	5.1×10^{2}[d]
Aeromonas R24[b]	2.6×10^{5}[c]	1.8×10^{5}[c]	3.8×10^{2}[d]
Enterobacter R25[b]	1.8×10^{5}[c]	2.7×10^{5}[c]	5.3×10^{2}[d]
Lactobacillus R15[b]	2.6×10^{4}[c]	4.1×10^{4}[c]	1.2×10^{2}[d]
Lactobacillus R17[b]	9.2×10^{4}[c]	1.1×10^{5}[c]	2.8×10^{2}[d]
Lactobacillus ATCC 1497	7.0×10^{4}[c]	8.4×10^{4}[c]	6.3×10^{2}[d]
Bacillus R7[b]	3.6×10^{4}[c]	3.8×10^{4}[c]	4.9×10^{2}[d]
Brochothrix R13-1[b]	4.0×10^{4}[c]	4.3×10^{4}[c]	7.6×10^{2}[d]
1:100 Ground beef:			
homogenate #1 (3.5×10^{5} cfu/g)	8.0×10^{5}[c]	3.2×10^{5}[c]	-
homogenate #2 (6.6×10^{1} cfu/g)	8.5×10^{5}[c]	7.6×10^{5}[c]	-

[a] Analysis for ATP was done after 1:2 dilution in double strength PicoExB. ATP values were analyzed for statistical differences using ANOVA. Values in each row with different superscripts were significantly different ($p = 0.0001$).

[b] Ground beef spoilage isolates obtained from Dept. Food Science, Virginia Tech, Blacksburg, VA.

Table 2.4. ATPase treatment for the selective degradation of non-microbial ATP.

Sample	ATP (fg/filter)[a] No ATPase treatment	ATP (fg/filter)[a] ATPase treatment
Pure culture isolate:		
Pseudomonas R5[b]	2.1×10^{6c}	2.0×10^{6c}
Pseudomonas R19-1[b]	5.8×10^{5c}	5.5×10^{5c}
Pseudomonas ATCC 13525	1.3×10^{6c}	9.8×10^{5c}
Aeromonas R24[b]	1.1×10^{6c}	1.2×10^{6c}
Enterobacter R25[b]	3.2×10^{6c}	4.6×10^{6c}
Lactobacillus R15[b]	8.8×10^{5c}	9.0×10^{5c}
Lactobacillus R17[b]	9.1×10^{5c}	4.0×10^{5c}
Lactobacillus ATCC 1497	7.5×10^{5c}	8.0×10^{5c}
Bacillus R7[b]	9.0×10^{5c}	7.1×10^{5c}
Brochothrix R13-1[b]	8.5×10^{5c}	7.3×10^{5c}
1:100 Ground beef:		
homogenate #1 (3.5×10^{5} cfu/g)	4.1×10^{6c}	4.8×10^{6c}
homogenate #2 (6.6×10^{1} cfu/g)	5.0×10^{4c}	5.6×10^{3d}

[a] Bacteria or beef homogenate on filters were extracted with 600ul PicoExB. For analysis, 300 ul of the extract was collected. ATP values were analyzed for statistical differences using ANOVA. Values in each row with different superscripts were significantly different ($p = 0.0001$).

[b] Ground beef spoilage isolates obtained from Dept. Food Science, Virginia Tech, Blacksburg, VA.

subjected to the ATPase treatment. Their ATP contents were not significantly different following extraction with PicoExB. Ten ml of 1:100 ground beef homogenates were also filtered through 1 μm and 0.45 μm filters and given ATPase treatment before ATP extraction. Amounts of ATP determined after PicoExB extraction were the same with or without ATPase treatment for homogenate from beef containing 3.5×10^5 cfu/g. ATP levels for low count beef with 6.6×10^1 cfu/g decreased about 90% as a result of ATPase treatment. This indicates digestion of non-microbial ATP. The 5.6×10^3 fg ATP left on the filter after ATPase treatment would be equivalent to 5.6×10^4 cfu/g of ground beef, assuming 1 fg per cfu. This value, although not as low as the theoretical sensitivity limit of 6×10^3 cfu/g for ATP alone or 9×10^3 cfu/g for a pure culture of bacteria, is free of enough interference by non-microbial ATP to allow for improvement of the sensitivity limit of 10^6/g previously obtained.

The improved sample preparation procedure was tested for sensitivity of APC estimation by preparing low-count laboratory-ground beef with mixed ground beef spoilage flora, storing at 10°C and sampling periodically for microbial ATP content and APC. This storage provided a wide range of contamination levels for examination. The relationship of microbial ATP and APC values is illustrated

in Figure 2.3. Minimum sensitivity was approximately 5 x 10^4 cfu/g for ground beef. APC values falling below the sensitivity limit had variable non-correlation when compared with ATP concentrations determined for each ground beef patty. Excellent correlation (r^2 = 0.97) was obtained when APC values greater than 5 x 10^4 cfu/g were compared with microbial ATP values.

Regression coefficients obtained from the data for the above set of ground beef patties were used to estimate APC values for a second set of patties prepared and stored in the same way as the first set. Accuracy of APC estimates was within 1/2 log for nearly all samples above 5 x 10^4 cfu/g (See Figure 2.4). Below this level APC values could not be estimated due to remaining interference from non-microbial ATP.

The sensitivity of the assay can theoretically be improved since the lower limit of microbial ATP detection in pure culture was 9 x 10^3 fg/ml and the lower limit of microbial ATP in this study was 5 x 10^4 fg/g. Methods could be developed for use in addition to ATPase treatment and syringe filtration in an attempt to further remove non-microbial ATP and improve sensitivity to microbial ATP in food homogenates. Centrifugation steps to differentially separate bacteria from food particles and development of ways to filter lower dilutions or greater amounts of sample

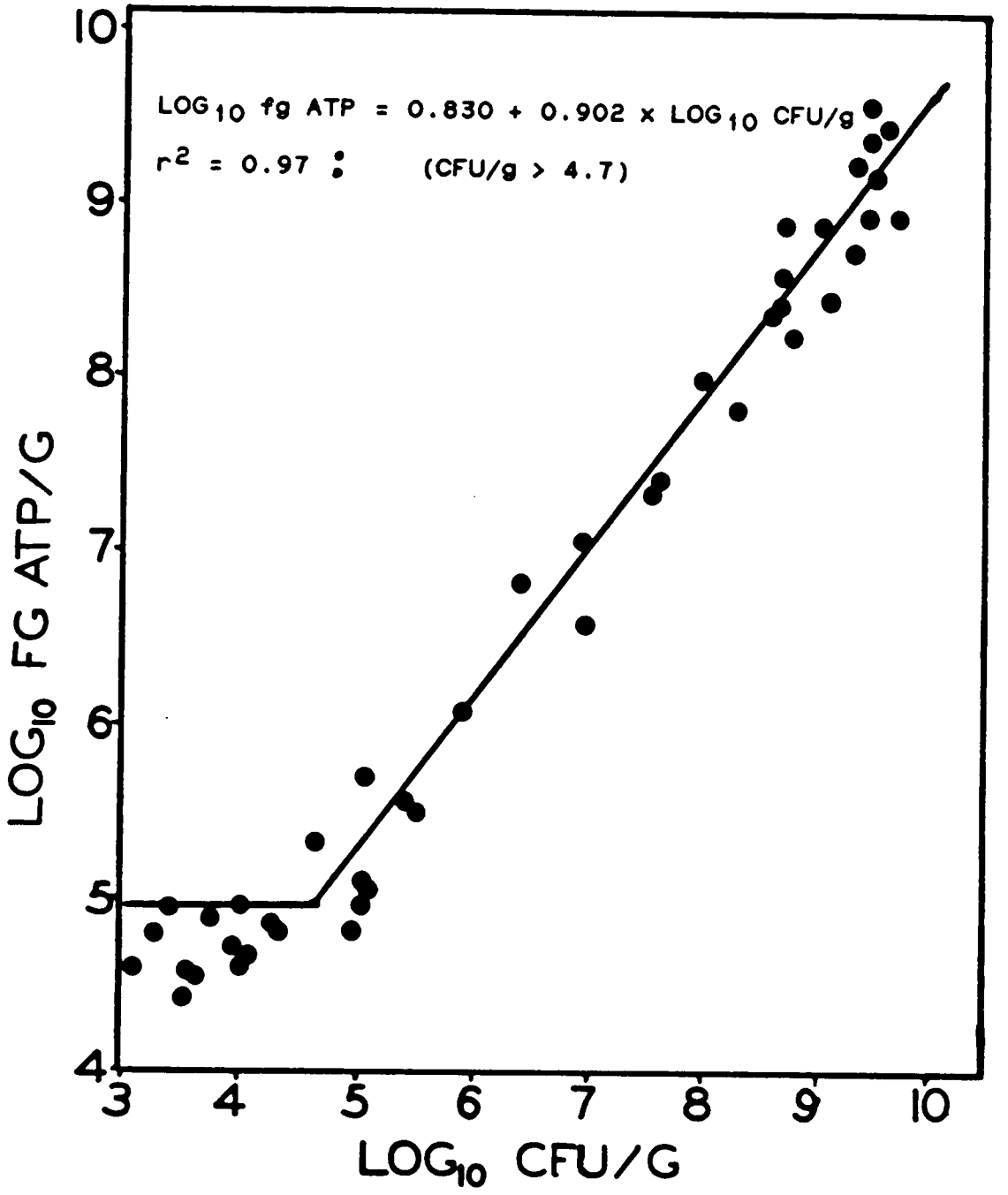

Figure 2.3. Sensitivity of microbial ATP determination in ground beef.

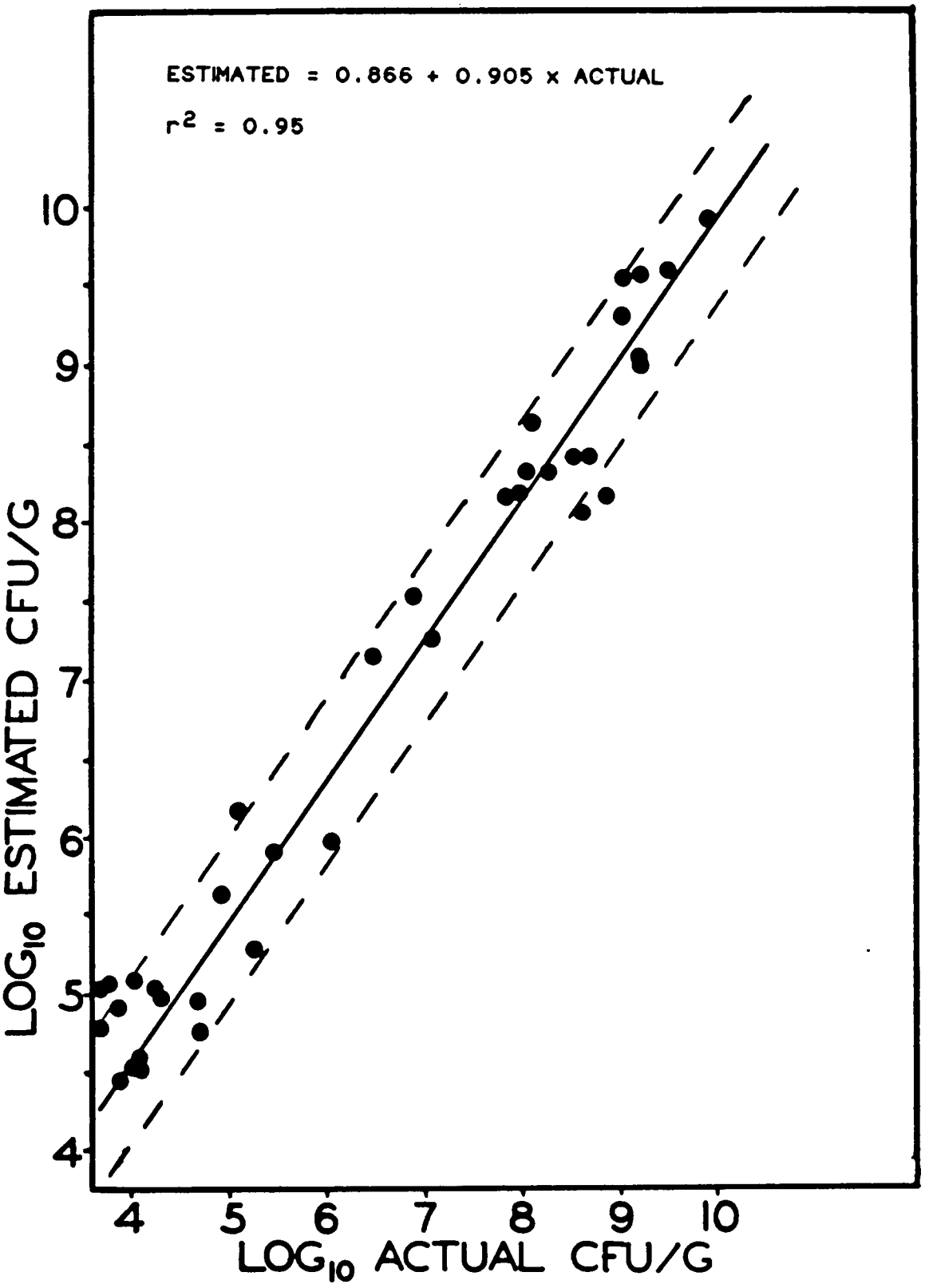

Figure 2.4. Accuracy of APC estimations using the ATP assay. Dashed lines indicate the boundaries for estimations accurate to within 1/2 log.

will lower the microbial ATP detection limit. However, added steps would not be worth the small amount of sensitivity improvement. Low, consistent blanks are essential for maximum sensitivity, since samples with low ATP concentrations must be detected above the highest levels of blank readings. Improvements in purity of luciferin-luciferase enzyme reagent and improved ability of luminometers to detect lower levels of light will also contribute to enhanced sensitivity of luminometry.

Chapter 3

Influence of storage condition and microbial flora on the bioluminescent assay of microorganisms in ground beef

ABSTRACT

The ATP assay was used to estimate plate counts of ground beef patties inoculated with a mixed ground beef spoilage flora, Pseudomonas or Lactobacillus, packaged aerobically or anaerobically, and stored at 2^{o} or $10^{o}C$. Regression curves were developed and used to estimate microbial numbers per g in additional beef samples, given their concentrations of microbial ATP. For beef inoculated with mixed ground beef spoilage flora correlations (r^2) of 0.94, 0.92 and 0.90 were obtained when microbial ATP values were compared with aerobic counts (APC), anaerobic counts and Lactobacillus counts, respectively. Close agreement was obtained for each inoculum and storage method over the range of 5×10^4 to 10^9 cfu/g, although estimated counts were more accurate for beef with $>10^5$ cfu/g. APC estimates were accurate to within $\pm$0.50 log units for 86% of all samples with $>5 \times 10^4$ cfu/g. Results indicate the bioluminescent ATP assay can be used to rapidly and accurately estimate microbial concentrations using a single regression equation for a variety of storage conditions and types of bacteria.

INTRODUCTION

In order for the ATP assay to be used for accurately estimating microbial numbers, it is necessary that microbial cells have a consistent ATP content for a given cfu concentration in the sample. Many studies have shown that microbial ATP content of microorganisms found in meat averages about 1 fentogram ATP per cfu (1 fg = 10^{-15}g) and that quantities range from 0.1 to 4.0 fg for most bacteria (Karl, 1980; Theron et al., 1983; Lee et al., 1971; Hysert et al., 1976; Chappelle and Levin, 1968; Levin et al., 1964, 1975; Thore et al., 1975; Karl and LaRock, 1975; Hamilton and Holm-Hansen, 1967). However, bacterial ATP content has been shown to be dependent on growth conditions such as temperature, availability of oxygen and nutritional quality of substrate (Karl, 1980; Chapman and Atkinson, 1977). Knowles and Smith (1970) reported that ATP levels of Azotobacter vinlandii are four times as high when cells are incubated aerobically than when incubated anaerobically. Others have observed this phenomenon with other bacteria (Strange et al., 1963; Cole et al., 1967). Availability of nutrients has also been shown to affect bacterial ATP levels. ATP levels decreased with glucose limitation for Acetobacter aceti and E. coli, and could be raised by restoring glucose to high levels (Chapman et al., 1971; Bachi and Ettlinger, 1973). Strange et al. (1963) observed

a decreased ATP content in Aerobacter when it was grown in a carbon limiting medium. Theron et al. (1983) reported that placing cold-stored bacteria at a warmer temperature caused their ATP levels to rise. Strange et al. (1963) also observed variation in bacterial ATP levels as influenced by holding temperature.

In Chapter 1 it was shown that cfu values could be accurately estimated using the ATP assay for ground beef held at 7°C. Since bacterial ATP levels can vary due to environmental factors, the objective of this study was to examine the relationship between microbial ATP levels and bacterial plate counts for ground beef containing different microflora and stored under different conditions of packaging and temperature. Since the history of a ground beef sample may not be known at the time of sampling, the applicability of a single regression curve for estimating plate counts was examined.

MATERIALS AND METHODS

Ground Beef

Beef bottom round purchased from a local store was surface-flamed and its exterior trimmed off using a sterile knife. The interior was cut into 2 to 3 inch cubes and ground twice through a 3/16 inch plate using a sterile grinder. Beef ground in this manner was inoculated with Lactobacillus plantarum ATCC 14917 or Pseudomonas fluorescens ATCC 13525 culture grown overnight. Inoculation was accomplished by evenly distributing culture over thin flattened layers of ground beef (1 ml culture per 1000g). Mixing was accomplished by kneading the inoculated meat in a plastic bag. Ground beef inoculated with mixed ground beef spoilage flora was prepared by adding unflamed surface trimmings to interior cubes prior to grinding.

Storage Conditions

The inoculated ground beef was aseptically weighed and pressed into 35 g patties, 3 inches in diameter and 1/4 inch thick using a custom made patty press. Patties to be packaged aerobically were placed onto 4 inch x 4 inch styrofoam trays and overwrapped with PVC film (Reynold's plastic wrap; O_2 transmission rate of 300 cc / 100 in^2 / 24 hr). Anaerobic packaging was done by placing patties into a Saran-nylon-polyethylene laminated pouch (Cryovac P850-S, W.

R. Grace, Simpsonville, SC; O_2 transmission rate of <0.016 cc/O_2 / 100 in^2 / 24 hr at 72°F) and vacuumed sealing. Packaged patties were stored at 2°C or 10°C until sampled and examined for microbial ATP content, pH and plate counts. Aerobically packaged patties were stored in single layers so that each was fully exposed to air. Styrofoam trays, PVC film and Saran pouches were determined to be free of microbial contamination (<5 cfu / 4 in^2) by swabbing and plating on Standard Methods Agar for 4 days at 20°C.

Sample Preparation and ATP Assay

At each sampling time, duplicate packages were chosen using random package numbers. For each package microbial ATP was determined in triplicate using the methods described in Chapter 2 and the results averaged.

Plate Counts

For each ground beef sample, total aerobic (APC), total anaerobic and Lactobacillus counts were determined. Total counts were determined by plating samples with Standard Methods Agar and incubating plates for 4 days at 20°C. Anaerobic counts done similarly to total counts except that an anaerobe jar (BBL) was used during incubation. Lactobacillus was enumerated by plating samples on MRS agar and incubating plates for 2 days at 35°C in an anaerobe jar.

The logarithm of each plate count value was regressed

linearly with log ATP concentration for ground beef spoilage flora-inoculated ground beef stored under each of the packaging and temperature conditions. Regression curves generated from these data were then used to estimate plate count values for a second set of ground beef patties prepared packaged and stored in the same way as the first set, as well as for Pseudomonas- and Lactobacillus-inoculated patties packaged and stored under each of the described conditions. Estimated plate counts for each inoculum and packaging condition and storage temperature were compared with actual plate counts in order to determine accuracy.

RESULTS AND DISCUSSION

Plate counts for ground beef patties stored under each of the packaging and storage temperatures are shown in Figures 3.1 through 3.4. For patties stored at $2^{o}C$ under aerobic conditions (Figure 3.1) total aerobic counts (APC) reached 10^{9} cfu/g within 14 days while total anaerobic (NPC) and Lactobacillus (MRS) counts reached 2×10^{8} and 3×10^{7}, cfu/g respectively (Figure 3.1). At all sampling times up to 14 days, APC values were higher than NPC or MRS values. The patties increased in pH during storage and developed off-odor by day 10.

Beef packaged aerobically and stored at $10^{o}C$ (Figure 3.2) had a similar type of spoilage as beef stored aerobically at $2^{o}C$, except that each type of plate count as well as pH value increased at a much faster rate. By day 4 off-odor developed, at which time total bacterial numbers were approximately 5×10^{8} cfu/g.

Patties stored in anaerobic (vacuum) packaging at $2^{o}C$ (Figure 3.3) or $10^{o}C$ (Figure 3.4) developed slightly noticable off-odor by 14 or 7 days of storage, respectively (Figures 3.3 and 3.4). APC, NPC and MRS plate counts increased, with APC values reaching a maximum of about 10^{8} cfu/g. This was accompanied by a decrease in pH. APC values were at all times equal to or higher than NPC and MRS values, with NPC and APC values very similar.

All data are in agreement with observations made by

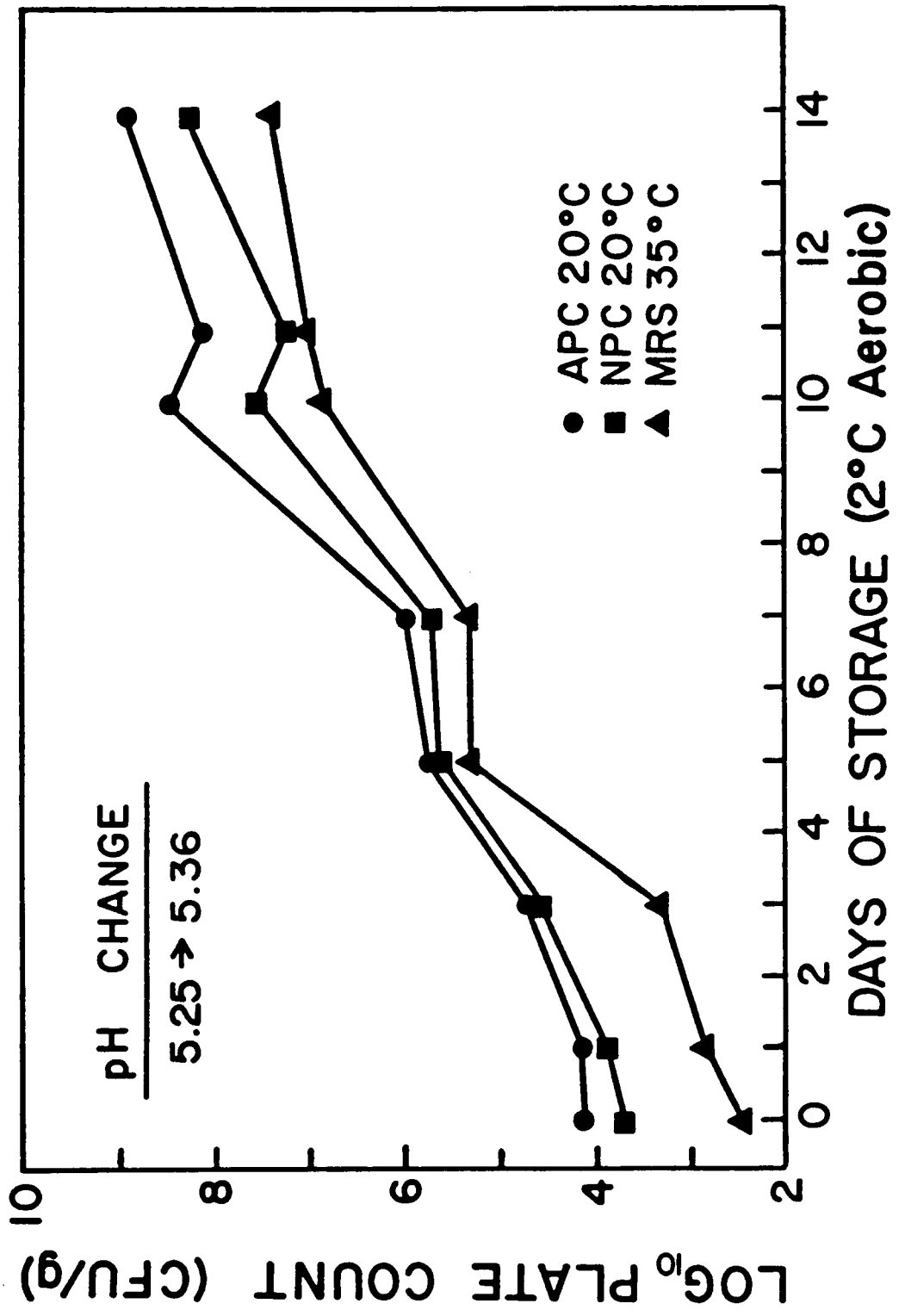

Figure 3.1. Plate counts of ground beef stored aerobically at 2°C. APC, NPC and MRS stand for total aerobic count, total anaerobic count and Lactobacillus count, respectively.

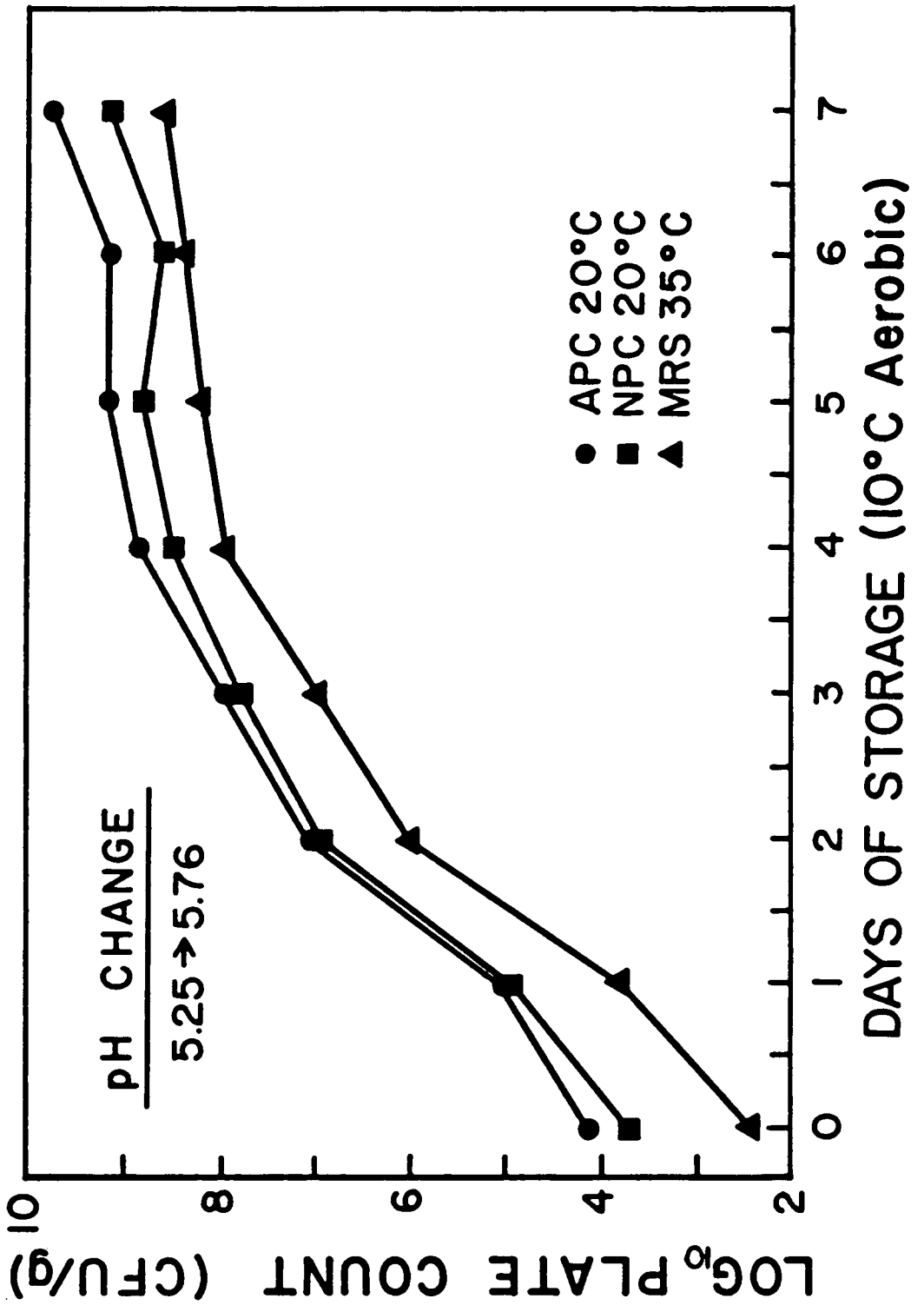

Figure 3.2. Plate counts of ground beef stored aerobically at 10°C. APC, NPC and MRS stand for total aerobic count, total anaerobic count and Lactobacillus count, respectively.

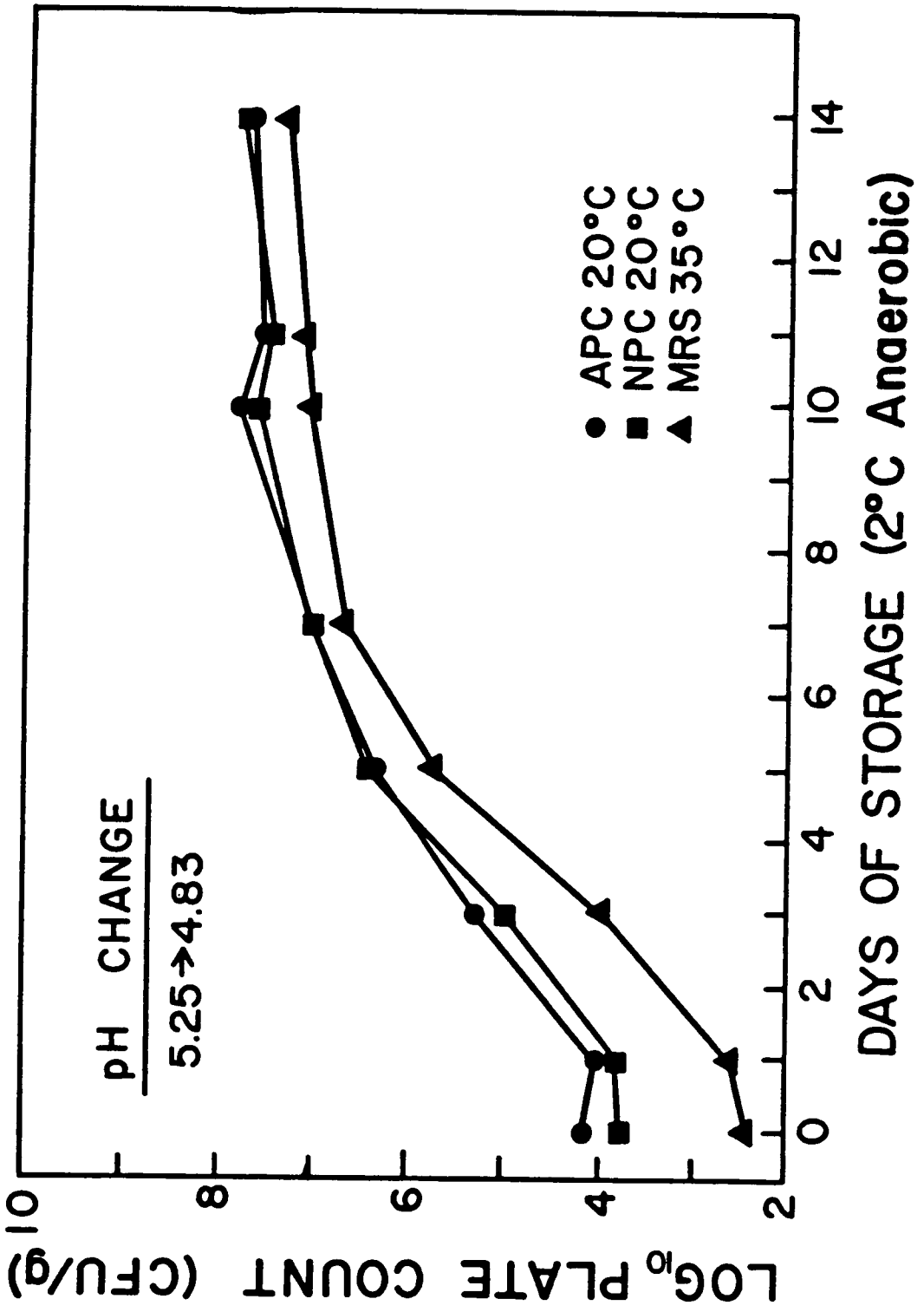

Figure 3.3. Plate counts of ground beef stored anaerobically at 2°C. APC, NPC and MRS stand for total aerobic count, total anaerobic count and Lactobacillus count, respectively.

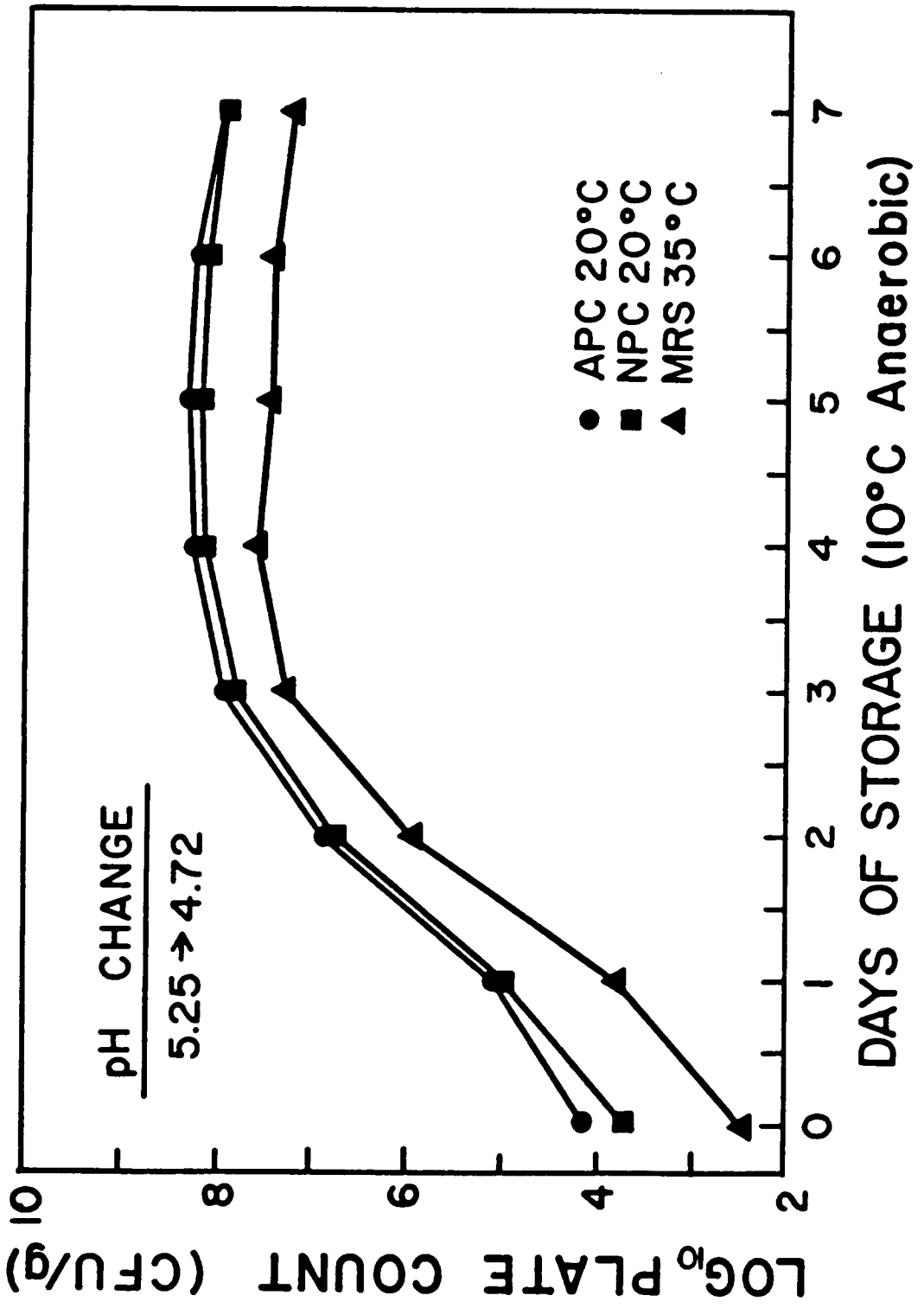

Figure 3.4. Plate counts of ground beef stored anaerobically at 10°C. APC, NPC and MRS stand for total aerobic count, total anaerobic count and Lactobacillus count, respectively.

many others who have studied or reviewed spoilage of aerobically- or anaerobically-packaged beef (McMeekin, 1982; Newton and Rigg, 1979; Gill, 1983; Gill and Newton, 1980; Brown and Baird-Parker, 1982; Pierson et al., 1970). Aerobic packaging favors growth of Pseudomonas species, with accompanying proteolysis and breakdown of amines, producing off-odors, rise in pH, and slime. Anaerobic packaging prevents growth of Pseudomonas and favors Lactobacillus and Brochothrix thermosphacta with accompanying acid production and pH drop. The growth of B. thermosphacta would be unlikely in these studies since its minimum pH for aerobic or anaerobic growth is 5.5 (Campbell et al., 1979). Therefore, Lactobacillus was the dominant organism in the anaerobically-stored ground beef. Plate counts for each storage temperature - packaging condition indicated likely dominance of strict aerobes (such as Pseudomonas) during aerobic storage and dominance of facultatively aerobic fermenters (such as Lactobacillus) during anaerobic storage. Data presented in Table 3.1 confirm the types of bacteria that could be included in each of the plate counts.

The above results indicated that with each storage temperature and packaging condition there was a different type of microbial activity, as indicated by pH increase or decrease, rate of microbial growth, similarity of APC values and NPC values, and maximum microbial concentrations obtained. Each of the ground beef samples was analyzed for

Table 3.1. Recovery of meat spoilage isolates with various plating conditions. Pour plate technique was used.

Bacterial Isolate	Plating medium A20[a]	N20[b]	MRS[c]
Pseudomonas R5[e]	+	-	-
Pseudomonas R19-1[e]	+	-	-
Pseudomonas ATCC 13525	+	-	-
Aeromonas R24[e]	+	+	-
Enterobacter R25[e]	+	+	+
Lactobacillus R15[e]	+[d]	+[d]	+
Lactobacillus R17[e]	+[d]	+[d]	+
Lactobacillus ATCC 14917	+	+	+
Bacillus R7[e]	+	-	+
Brochothrix R13-1[e]	+	+[d]	-

[a] A20 counts were done using Standard Methods Agar and incubating at 20°C for 4 days

[b] N20 counts were done using Standard Methods Agar and incubating at 20°C for 4 days in an anaerobic jar using the GasPak system

[c] MRS counts were done using MRS agar and incubating at 35°C for 2 days in an anaerobic jar using the GasPak system

[d] Colonies were very small even when incubation time was tripled. Some colonies may be overlooked when performing plate counting

[e] Ground beef spoilage isolates were obtained from Dept. Food Science, Virginia Tech, Blacksburg, VA.

microbial ATP content and compared with plate count results. Patties stored aerobically or anaerobically at $2^{o}C$ or $10^{o}C$ exhibited a close linear relationship over the entire range of 10^{4} to 10^{10} cfu/g (Figures 3.5 - 3.8) when log APC values were compared to log microbial ATP values. Most APC values were within 1/2 log of the regression lines, with exceptions observed for several samples which had been incubated at $2^{o}C$ aerobically or anaerobically. Comparison of correlations (r^{2}) between microbial ATP values and each plate count type revealed best correlation with APC, followed in decreasing order by NPC and MRS counts. In general, better correlation was obtained with $10^{o}C$ - stored patties than $2^{o}C$ - stored patties (Table 3.2).

Average ATP content per cell about 1 fg for aerobically packaged patties and about 1.5 fg for anaerobically packaged patties. These values are similar to those determined for a wide variety of bacterial genera isolated from beef by Baumgart et al. (1980b) who reported ATP contents between 0.026 and 2.2 fg per cell. Other researchers have also reported bacterial ATP concentrations in this range (Lee et al., 1971; Hysert et al., 1976; Chappelle and Levin, 1968; Levin et al., 1964; Levin et al., 1975; Thore et al., 1975; Karl and LaRock, 1975; Hamilton and Holm-Hansen, 1967). Some researchers have reported ATP levels of 5 fg per bacterium or higher (Kennedy and Oblinger, 1985; Ng et al., 1985); however, bacterial ATP levels have generally been

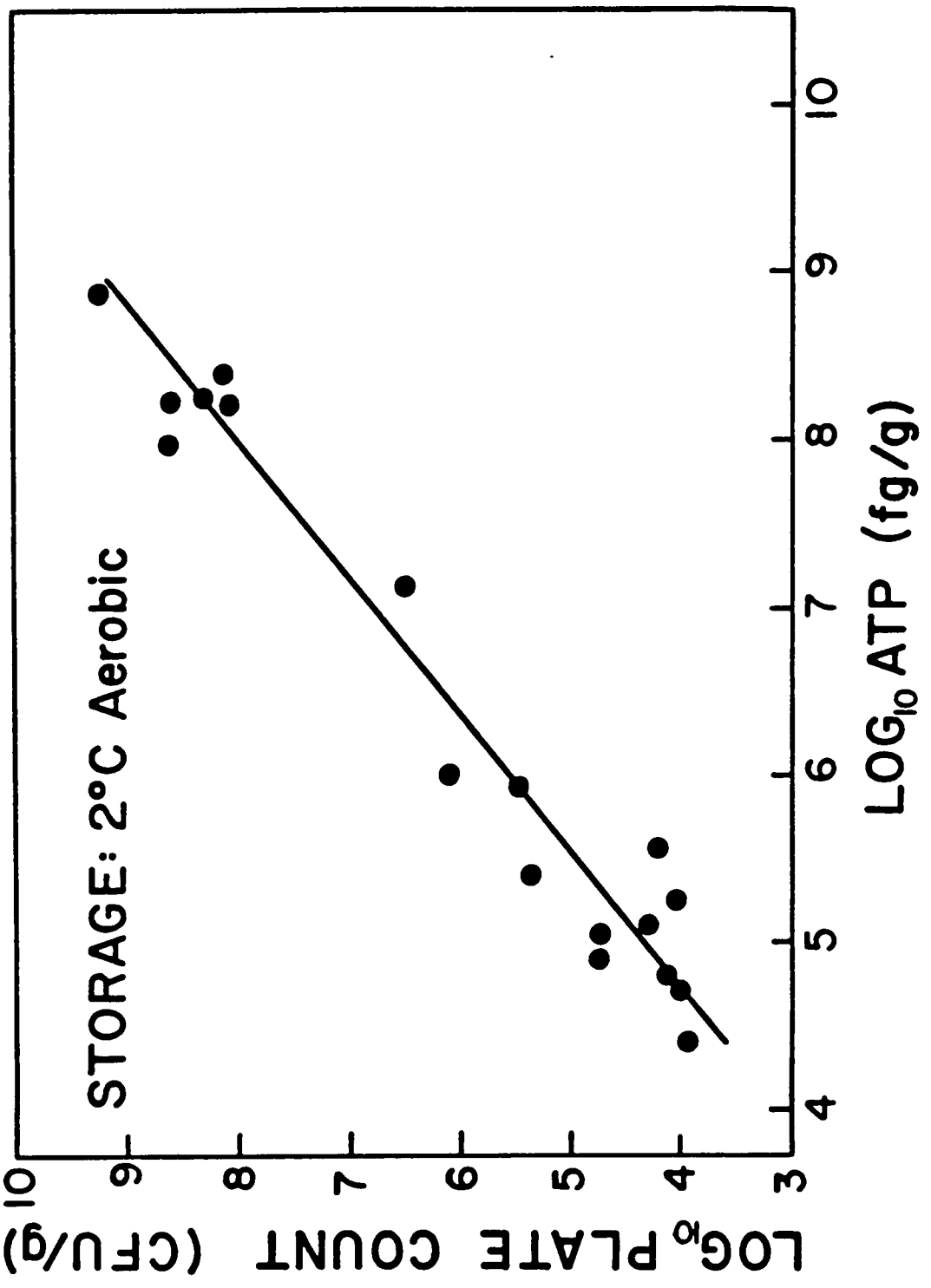

Figure 3.5. Relationship of microbial ATP and plate count for ground beef stored aerobically at 2°C.

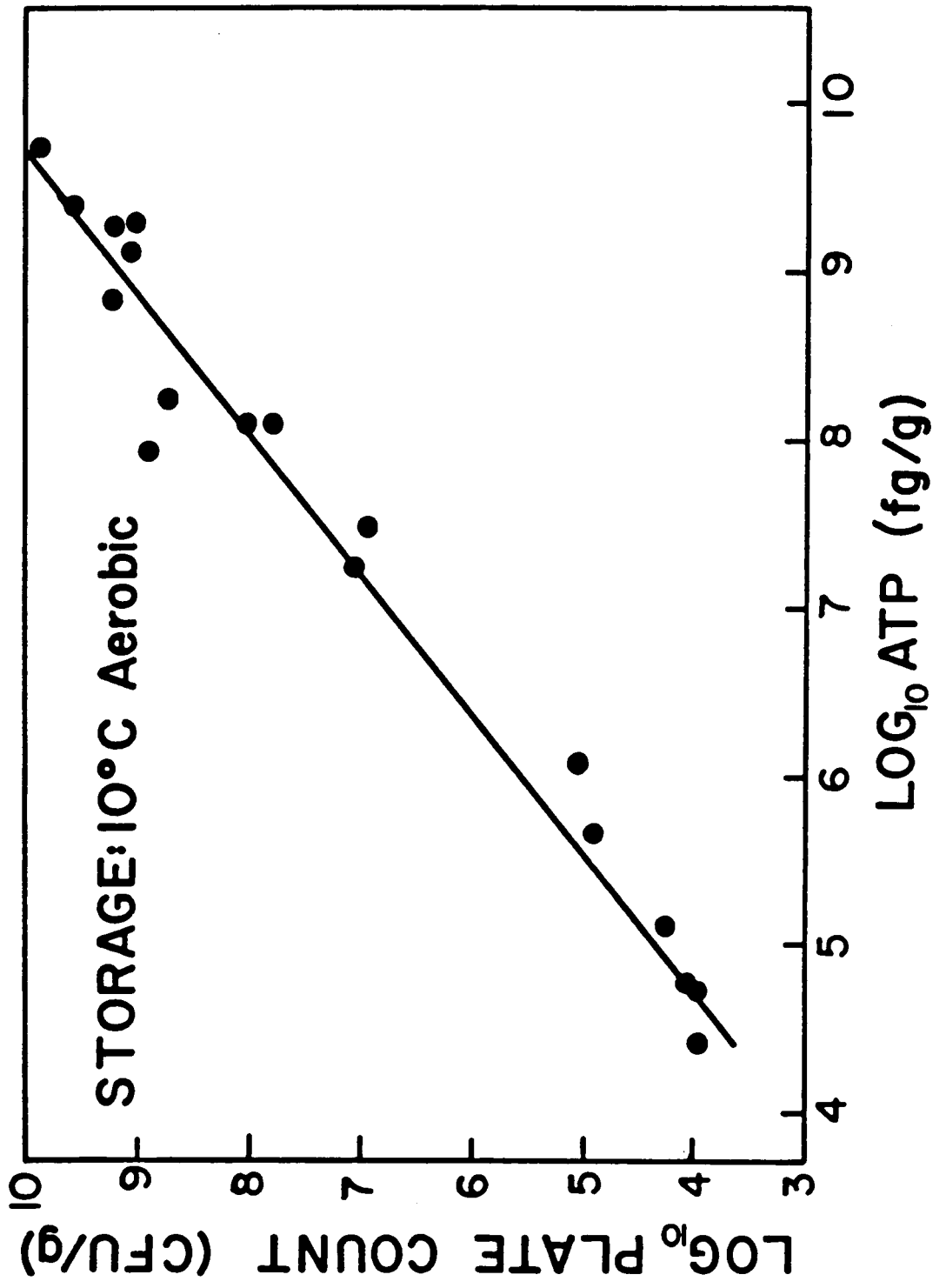

Figure 3.6. Relationship of microbial ATP and plate count for ground beef stored aerobically at 10°C.

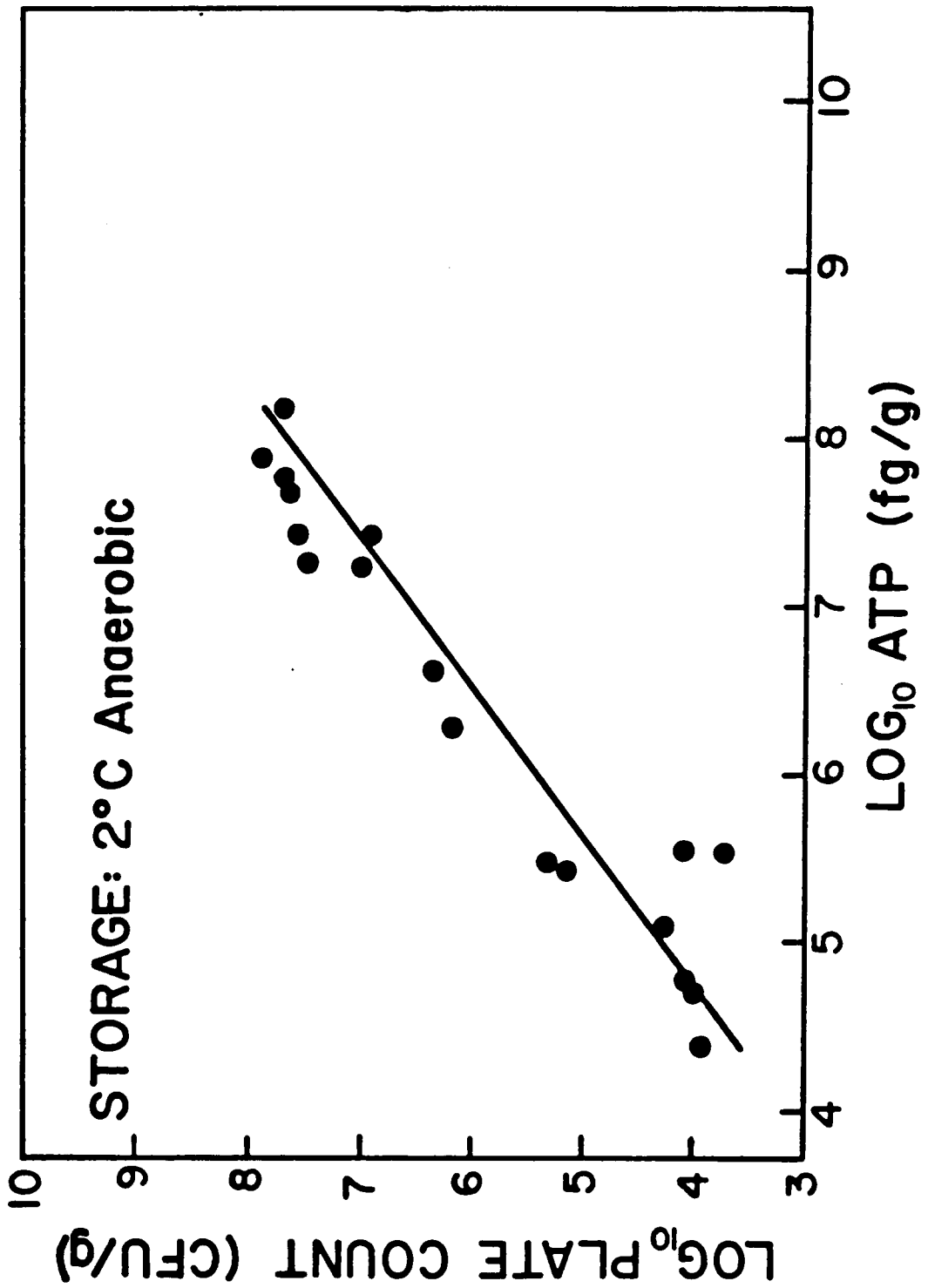

Figure 3.7. Relationship of microbial ATP and plate count for ground beef stored anaerobically at 2°C.

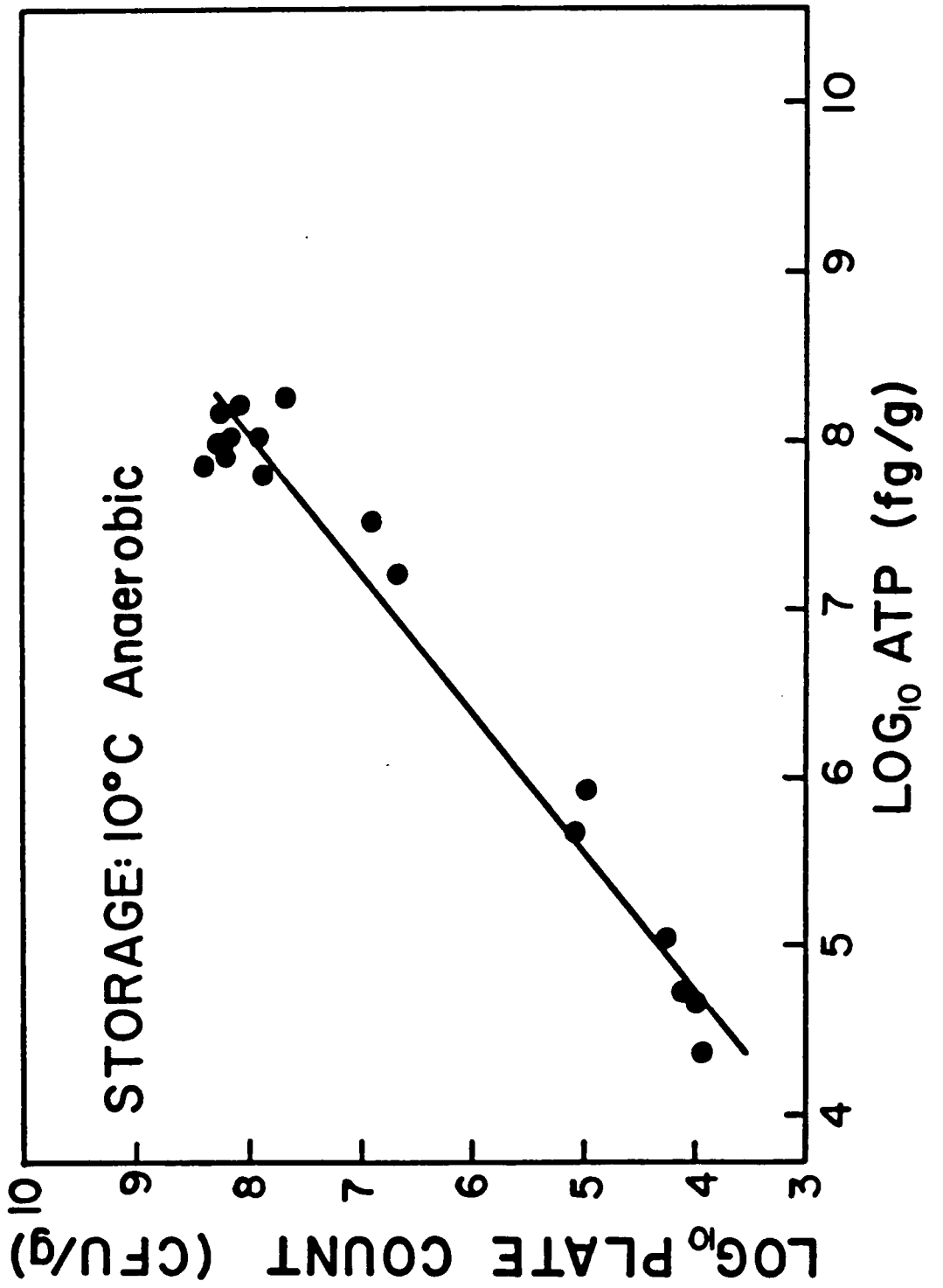

Figure 3.8. Relationship of microbial ATP and plate count for ground beef stored anaerobically at 10°C.

Table 3.2. Correlation (r^2) of microbial ATP with plate counts.

Storage Condition	Aerobic Plate Count[a]	Anaerobic Plate Count[b]	*Lactobacillus* Plate Count[c]
2°C aerobic	0.96	0.93	0.90
2°C anaerobic	0.92	0.93	0.92
10°C aerobic	0.97	0.97	0.96
10°C anaerobic	0.97	0.98	0.98
combined	0.94	0.92	0.90

[a] Plated on Standard Methods Agar and incubated 20°C for 4 days

[b] Plated on Standard Methods Agar and incubated 20°C for 4 days using GasPak system

[c] Plated on acidified MRS agar and incubated at 35°C for 2 days using the GasPak system

found to range from 0.1 to 4.0 fg/cfu (LaRocco et al., 1986). Bacterial ATP levels (up to 31 fg ATP/cfu) reported by Kennedy and Oblinger (1985) may have been artificially high due to interference by non-microbial ATP. Decrease in ATP content per cfu was observed throughout storage for each packaging and temperature condition, which was similar to that observed for ground beef in Chapter 1.

A second set of ground beef patties was prepared and stored under each of the packaging and temperature conditions described above. These samples were examined for APC, NPC and MRS counts as well as pH in order to confirm presence of spoilage activity similar to the first trial. Regression curves derived from the first set of data, using total bacterial count (APC) and microbial ATP values, were used to predict APC values for each microbial ATP value obtained for the second set of samples. Results are presented in Figures 3.9 - 3.12. A direct relationship was observed between predicted and actual APC values over the level of 10^4 cfu/g. Microbial ATP values less than 10^4 fg/g showed no correlation with APC values and therefore are excluded. Of the 64 samples examined with greater than 10^4 cfu/g, 48 estimated APC values were accurate to within 1/2 log, 52 to within 3/4 and 58 accurate to within 1 log of actual APC values. APC values for patties with higher bacterial numbers were estimated more closely than for patties with lower numbers (Table 3.3).

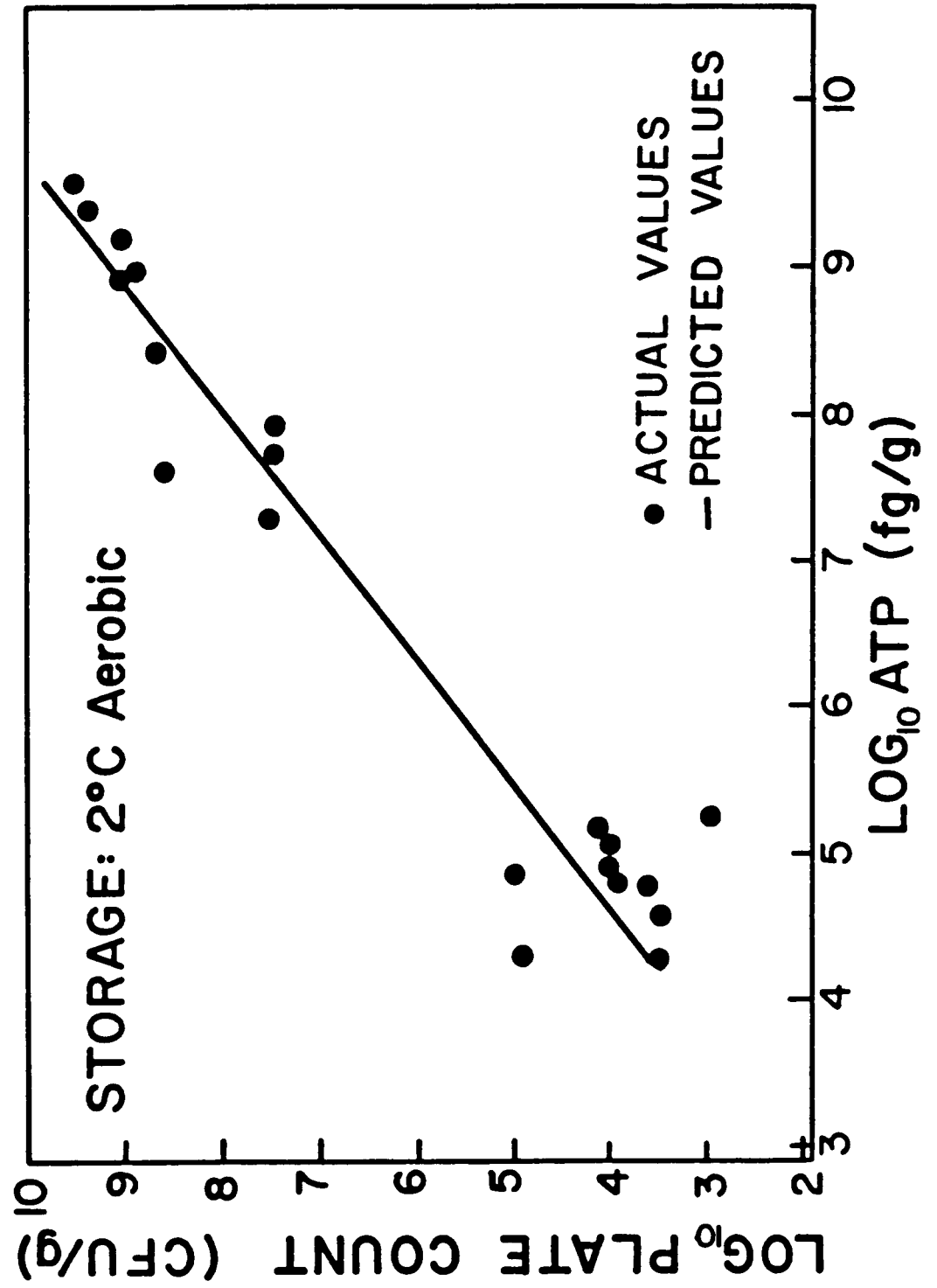

Figure 3.9. Predicted and actual plate count values for ground beef stored aerobically at 2°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

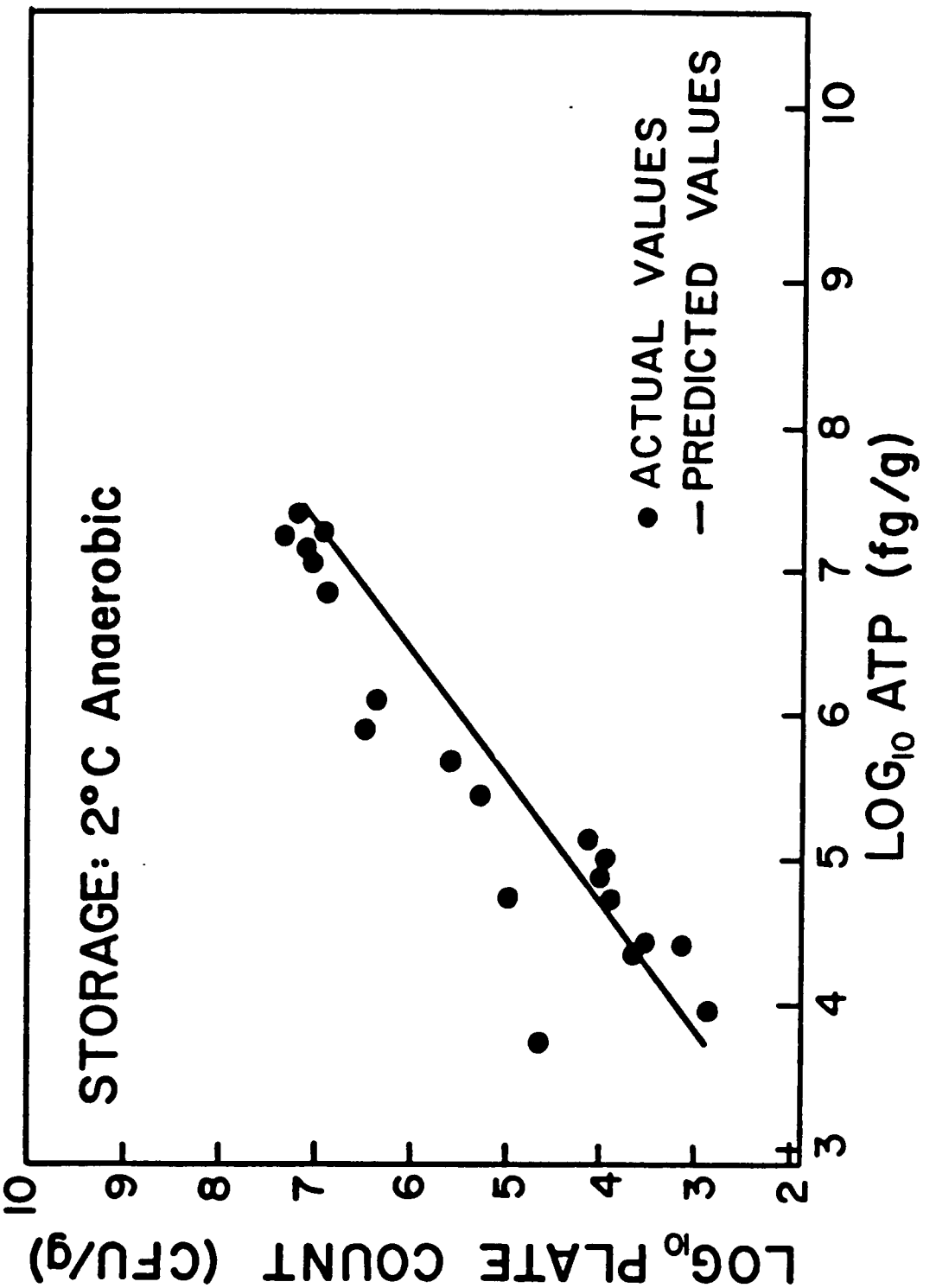

Figure 3.10. Predicted and actual plate count values for ground beef stored anaerobically at 2°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

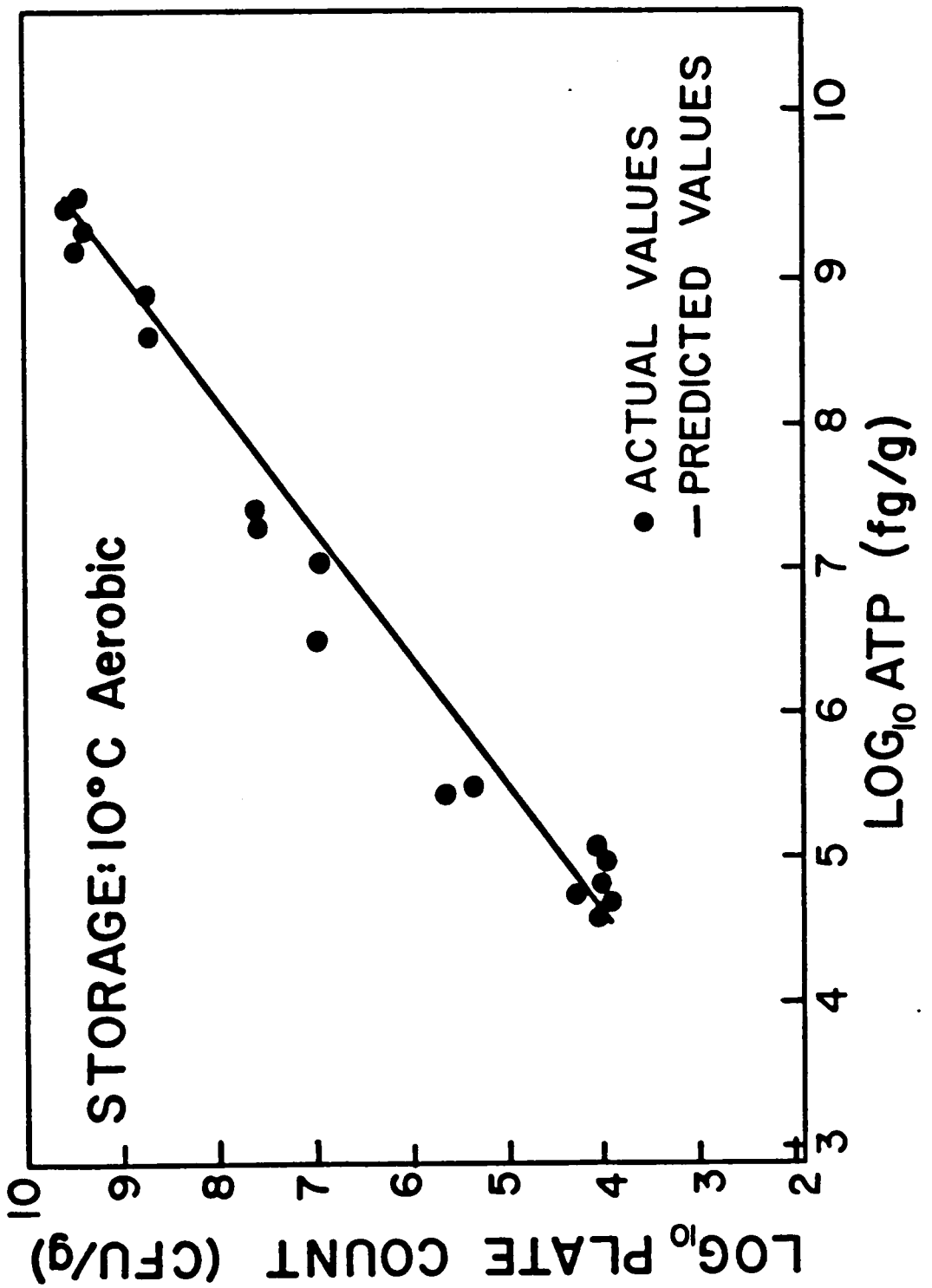

Figure 3.11. Predicted and actual plate count values for ground beef stored aerobically at 10°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

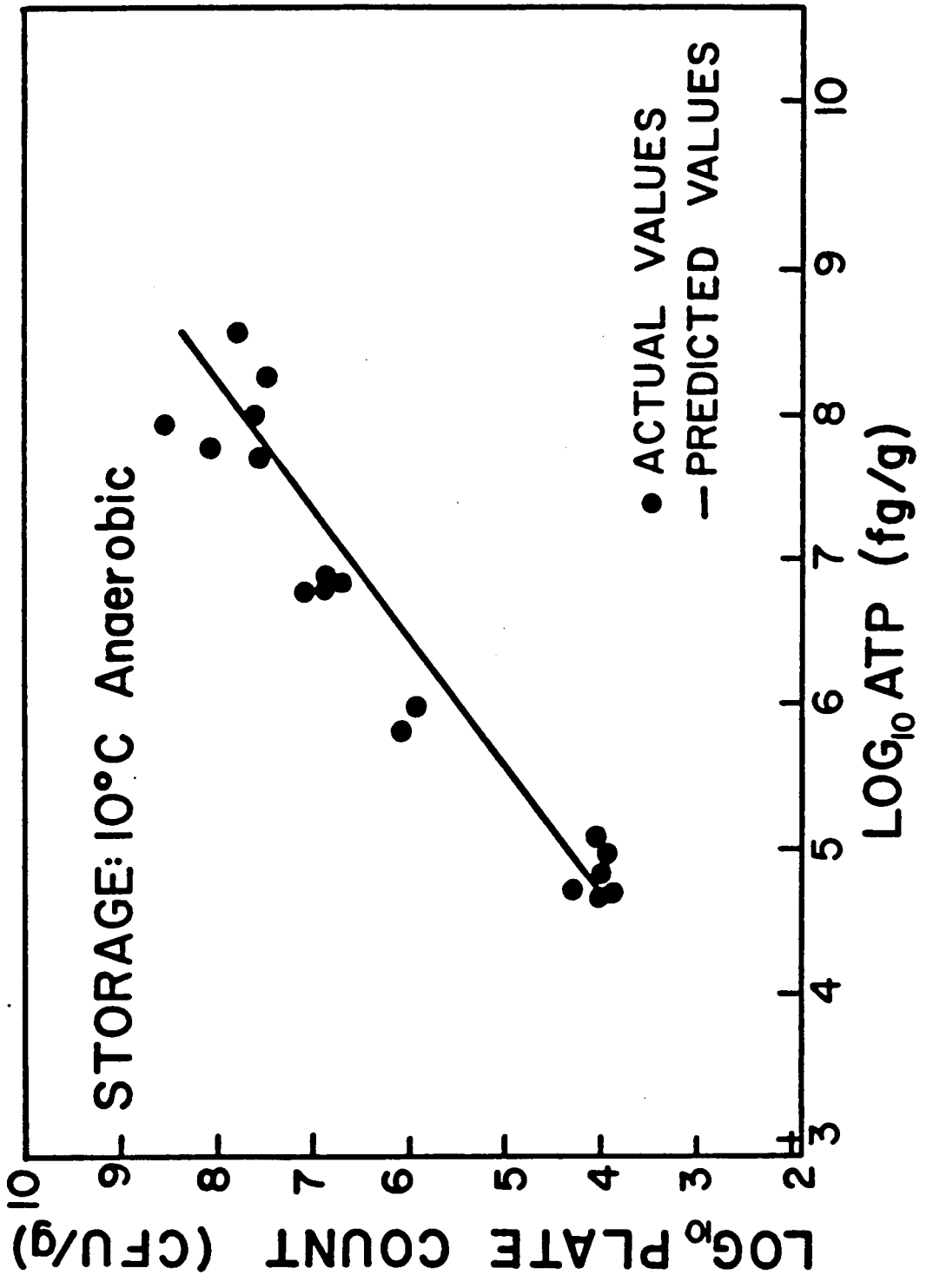

Figure 3.12. Predicted and actual plate count values for ground beef stored anaerobically at 10°C. Errors of predictions are represented by the actual values and the prediction curve.

Table 3.3. Accuracy of aerobic plate count estimates using ATP-luminometry

Actual Plate Count range	Error of plate count estimates		
	<0.50 log[a]	<0.75 log	<1.00 log
$<10^5$	15/19[b]	15/19	15/19
10^5	2/5	3/5	5/5
10^6	7/10	7/10	9/10
10^7	11/15	13/15	15/15
10^8	5/7	6/7	6/7
$>10^8$	8/8	8/8	8/8
Total	48/64	52/64	58/64

[a] $\log_{10}$ of plate count estimates were less than 0.50 different from $\log_{10}$ of actual plate counts

[b] number of ground beef samples within error range per number of samples in plate count range

Additional ground beef samples were prepared with Pseudomonas fluorescens and Lactobacillus plantarum. These organisms were chosen since they can be are dominant spoilage organisms in meat. Pseudomonas and Lactobacillus are different in several ways. Pseudomonas is Gram negative, catalase positive, and an obligate aerobe. Lactobacillus is Gram positive, catalase negative and a facultatively anaerobic obligate fermenter. Pseudomonas is generally smaller than Lactobacillus, Pseudomonas being 0.7 - 0.8 μm x 2.3 - 2.8 μm and Lactobacillus 0.9 - 1.2 μm x 3 - 8 μm.

Each set of patties inoculated with a culture of one of these organisms was packaged and stored under each of the same conditions as the mixed ground beef spoilage flora-inoculated patties. During storage, inoculated samples as well as uninoculated controls were removed at random and examined for APC, NPC and MRS counts as well as for pH in order to confirm that the inoculum was the predominant organism in the ground beef. The Pseudomonas strain grew at 20^{o}C but not 35^{o}C, aerobically but not anaerobically, and on SMA but not on MRS agar. The Lactobacillus strain grew well at 20^{o}C and 35^{o}C, aerobically as well as anaerobically, and on SMA or MRS agar although colonies were larger on MRS agar. Therefore, Pseudomonas was detected with APC counts but not NPC or MRS counts, and Lactobacillus was detected with all three types of counts.

For beef inoculated with Pseudomonas fluorescens, the initial inoculum was 3.7 log cfu/g. Uninoculated beef had less than 1.0 log cfu/g. During anaerobic storage at 2°C, numbers of Pseudomonas did not increase to levels detectable by the ATP assay. Anaerobic storage at 10°C allowed bacterial growth, but APC, NPC and MRS counts increased to the same degree, indicating dominance of non-Pseudomonas, which is not included in NPC or MRS counts (Table 3.4). Counts of Pseudomonas-inoculated beef stored aerobically at 2°C or 10°C indicated pure culture dominance, since NPC and MRS counts were low and uninoculated controls showed no interfering growth from bacteria originally in the meat.

Aerobic storage of Lactobacillus-inoculated beef at 2°C and 10°C resulted in overgrowth by strictly aerobic bacteria originally present in the meat (Table 3.5). Dominance by strict aerobes is indicated by high APC counts and low NPC and MRS counts. Although initial Lactobacillus counts were 400/g and initial numbers of aerobic bacteria in the meat were 12/g, the patties spoiled due to growth of strict aerobes before Lactobacillus counts became high enough to be detected using luminometry. Under anaerobic conditions, Lactobacillus did not grow at 2°C (nor did any other bacteria present in the meat). At 10°C, however, Lactobacillus dominated, reducing the pH and increasing to detectable numbers. The bacteria in uninoculated meat did not increase to significant numbers when stored under these

Table 3.4. Bacterial ATP concentrations and plate counts for ground beef inoculated with Pseudomonas fluorescens ATCC 13525 and incubated under various conditions.

Incubation Condition	ATP (log fg/g)	Plate count (log cfu/g) APC	NPC	MRS	pH
Initial inoculum	5.52	3.74	0.00	0.72	5.3
Initial uninoculated	5.49	0.82	0.18	0.35	5.3
A2[a]-inoculated	9.24	8.92	0.70	0.50	5.8
A2[a]-uninoculated	4.94	4.04	1.60	1.18	5.5
N2[b]-inoculated	4.98	3.30	1.71	0.00	5.4
N2[b]-uninoculated	5.02	0.70	0.00	0.70	5.4
A10[c]-inoculated	9.61	9.52	4.16	4.70	6.0
A10[c]-uninoculated	6.90	6.23	3.40	5.00	5.6
N10[d]-inoculated	6.33	5.92	5.62	5.92	5.4
N10[d]-uninoculated	4.77	1.18	0.70	0.00	5.4

[a] A2: Ground beef was wrapped in PVC film (aerobic condition) and stored at 2°C until visible spoilage occured (10 days)

[b] N2: Ground beef was vacuum packaged (anaerobic condition) and stored at 2°C for 10 days

[c] A10: Ground beef was wrapped in PVC film (aerobic condition) and stored at 10°C until visible spoilage occured (8 days)

[d] N10: Ground beef was vacuum packaged (anaerobic condition) and stored at 10°C for 10 days

Table 3.5. Determinations at the end of storage for ground beef inoculated with Lactobacillus plantarium ATCC 14917 and incubated under various conditions.

Incubation Condition	ATP (log fg/g)	Plate count (log cfu/g) APC	NPC	MRS	pH
Initial inoculum	5.42	2.68	2.62	2.56	5.3
Initial uninoculated	5.36	1.09	0.00	0.35	5.3
A2[a]-inoculated	9.41	9.40	2.38	5.27	5.8
A2[a]-uninoculated	9.33	9.30	4.58	5.26	5.8
N2[b]-inoculated	4.88	1.82	1.82	1.61	5.3
N2[b]-uninoculated	4.83	0.00	0.00	0.00	5.3
A10[c]-inoculated	8.78	8.74	4.96	4.95	5.8
A10[c]-uninoculated	8.98	9.00	-	4.08	5.7
N10[d]-inoculated	6.78	6.33	6.21	5.92	5.0
N10[d]-uninoculated	4.72	2.89	0.00	0.00	5.3

[a] A2: Ground beef was wraped in PVC film (aerobic condition) and stored at 2°C until visible spoilage occured (16 days)

[b] N2: Ground beef was vacuum packaged (anaerobic condition) and stored at 2°C for 19 days

[c] A10: Ground beef was wrapped in PVC film (aerobic condition) and stored at 10°C until visible spoilage occured (7 days)

[d] N10: Ground beef was vacuum packaged (anaerobic condition) and stored at 10°C for 19 days

conditions.

Each of the 3 pure culture inoculation groups resulting in dominance by the inoculum was examined for correlation of APC values with microbial ATP content. Pseudomonas-inoculated beef incubated aerobically at 2°C or 10°C and Lactobacillus-inoculated beef incubated anaerobically at 10°C had high linear correlation (r^2) values of 0.97, 0.96, and 0.95, respectively (Table 3.6). Average ATP per cell for Pseudomonas was about 1 fg and average ATP per cell for Lactobacillus was 1.8 fg. This agrees well with values reported by Baumgart et al. (1980b) who found 0.96 fg ATP per cell for Pseudomonas and 2.0 fg ATP per cell for Lactobacillus. Other studies have shown ATP contents to be 0.3 - 1.5 fg ATP per cell for Pseudomonas (Chappelle and Levin, 1968) and 1.1 - 1.3 fg ATP per cell for 3 Lactobacillus species (Hysert et al., 1976).

The regression curve derived from experiments with mixed groung beef spoilage flora-inoculated ground beef was used to estimate APC values for each of the pure-culture ground beef sets. Predicted APC values were compared to actual counts and found to correlate well (Figures 3.13 - 3.15, Table 3.6). Predicted APC values for Pseudomonas-inoculated beef stored aerobically at 2°C or 10°C had mean-squared errors of 0.20 for the APC range of 4.7 to 9.5 log cfu/g. Mean squared error of APC estimates for Lactobacillus-inoculated beef stored at 10°C

Table 3.6. Comparison of inoculum type for accuracy of plate count estimates of ground beef with greater than 5×10^4 cfu/g.

Inoculum	Storage Condition	Correlation (r^2) CFU vs ATP	Accuracy[a] of CFU estimates
Ground beef spoilage flora	combined	0.94	0.30
Pseudomonas	2°C aerobic	0.97	0.20
Pseudomonas	10°C aerobic	0.96	0.20
Lactobacillus	10°C anaerobic	0.95	0.071

[a] mean squared error

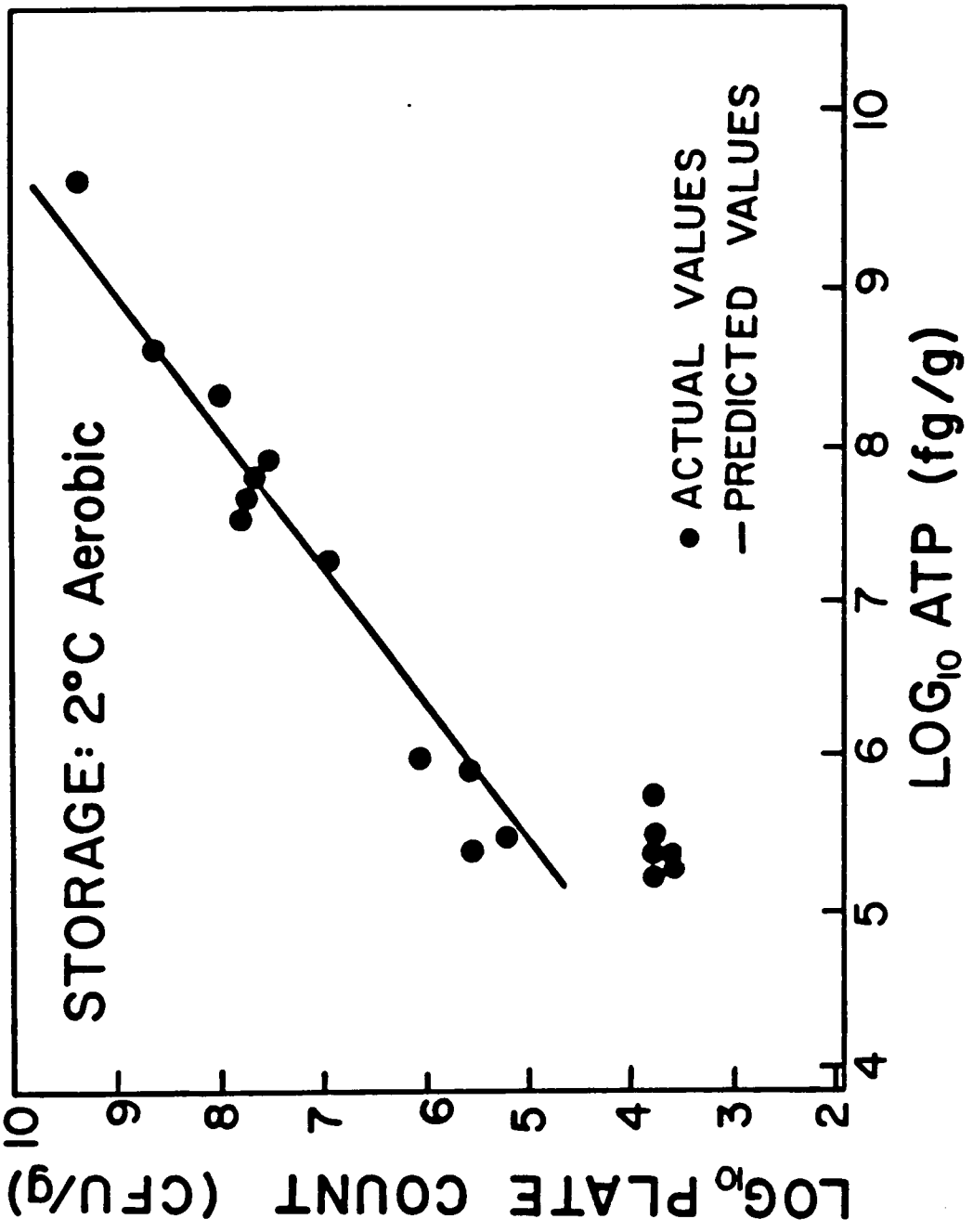

Figure 3.13. Predicted and actual plate count values for low-count ground beef inoculated with Pseudomonas fluorescens and incubated aerobically at 2°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

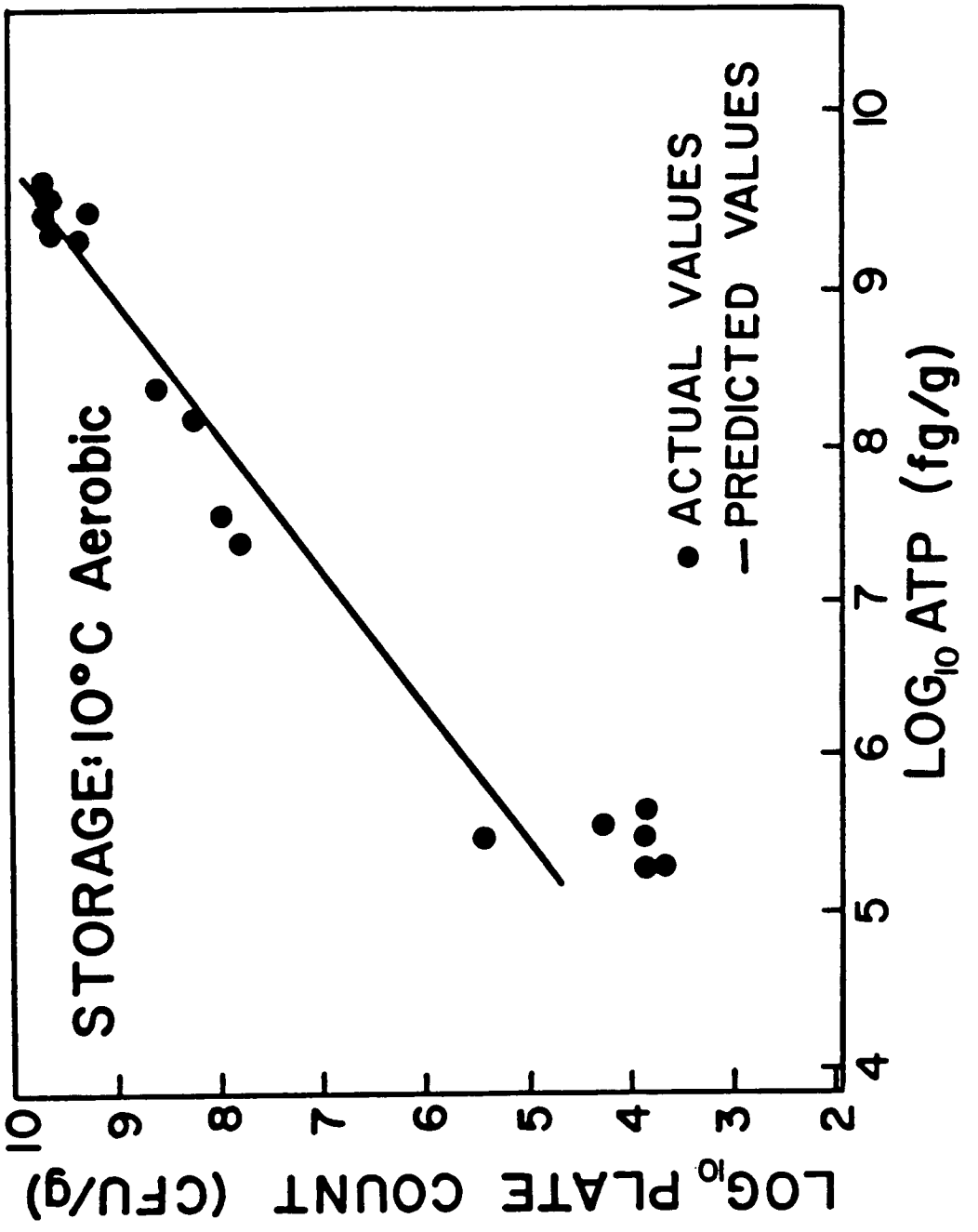

Figure 3.14. Predicted and actual plate count values for low-count ground beef inoculated with Pseudomonas fluorescens and incubated aerobically at 10°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

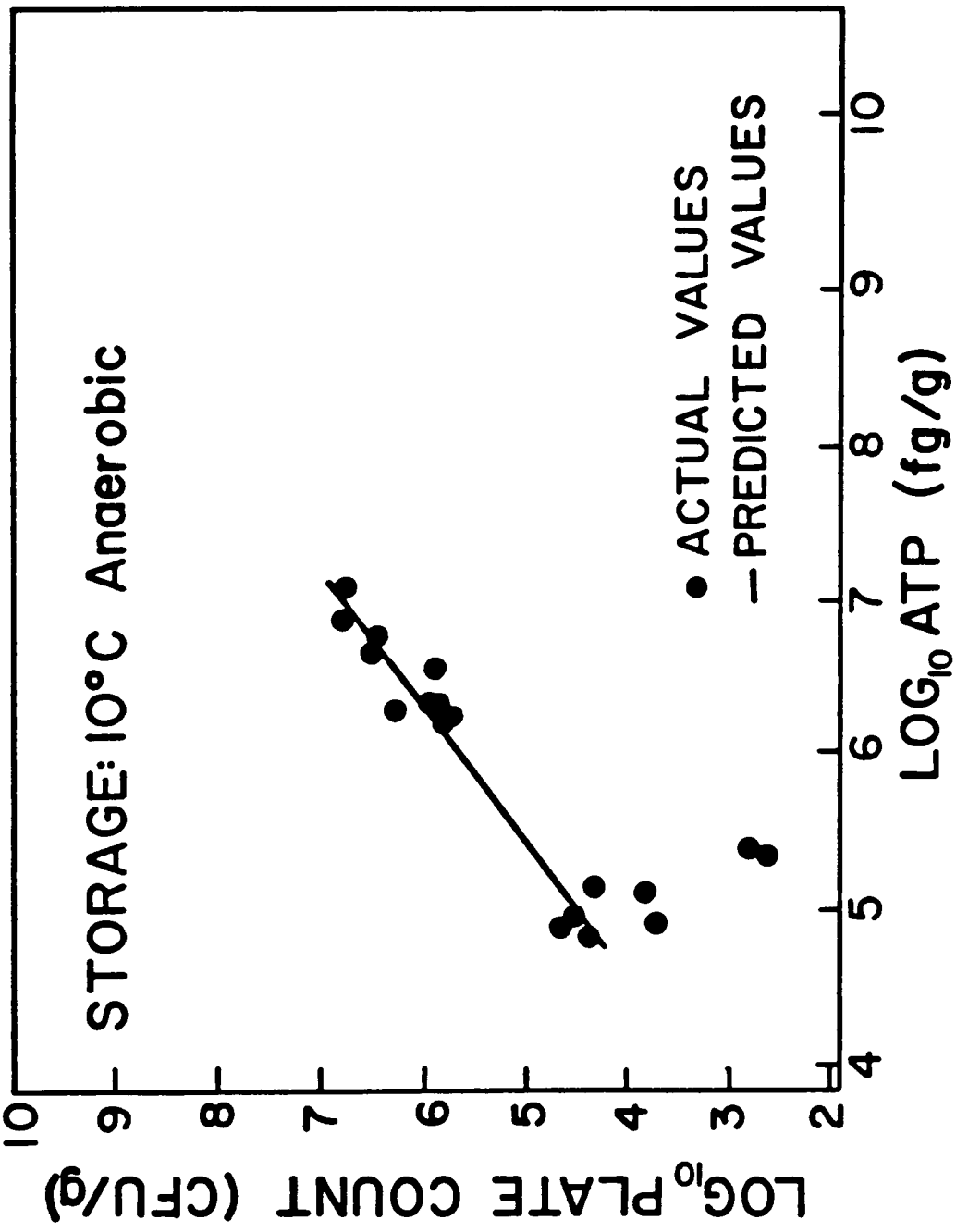

Figure 3.15. Predicted and actual plate count values for low-count ground beef inoculated with Lactobacillus plantarum and incubated anaerobically at 10°C. Errors of predictions are represented by the vertical distances between actual values and the prediction curve.

anaerobically was 0.071 over the range of 4.7 to 6.8 log cfu/g.

The lower sensitivity limit using ATP luminometry was 5 x 10^4 cfu/g (4.7 log cfu/g) for each inoculum, packaging and storage temperature condition used in this study (Figures 3.13 - 3.15). ATP values were not dependent on plate count values when plate counts were less than 4.7 log cfu/g. ATP values correlated well with the highest plate count value for each sample, except when total plate counts were less than about 5 log cfu/g (Tables 3.4 and 3.5). Luminometric data correctly determined which beef patties contained less than 10^5 bacteria/g, and was quantitative for those samples with 10^5 or more bacteria/g.

Data from each set of ground beef patties was recalculated to determine if plate count estimates would be more accurate if separate curves were used for each storage condition than if one curve were used to estimate counts for samples stored under any of the conditions. Results presented in Table 3.7 illustrate the lack of differences between estimates made with each of the curves. Ground beef APC values were estimated equally well whether one curve derived using data from all storage conditions was used, or if storage condition-specific curves were used. Therefore only one regression equation is required for estimation of plate counts of ground beef. For the storage and packaging conditions used in this study, knowledge of product history

Table 3.7. Comparison of universal curve (derived using data from all storage conditions) with storage condition-specific curves for accuracy of aerobic plate count estimates of ground beef.

Storage Condition	Mean squared error of plate count estimates	
	Universal curve	Storage-specific curves
2°C aerobic	0.40	0.40
2°C anaerobic	0.40	0.41
10°C aerobic	0.14	0.13
10°C anaerobic	0.21	0.21

was not needed to to rapidly and accurately estimate microbial numbers using the bioluminometric ATP assay.

ACKNOWLEDGMENTS

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Thanks are extended to for supplying ground beef spoilage isolates used in determining the types of bacteria each plate count detects. Appreciation is given to , and for helping with beef patty preparation, and to and for their assistance in the meat lab.

Chapter 4

Determination of bacterial counts in frozen ground beef using the bioluminescent ATP assay

ABSTRACT

Usefulness of the bioluminometric ATP assay for estimating total plate counts of frozen ground beef was evaluated. Laboratory-ground beef inoculated with *Lactobacillus plantarum* or *Pseudomonas fluorescens*, and retail ground beef were made into patties, vacuum-packaged, and frozen and thawed at different rates. Results indicated that plate counts and microbial ATP levels correlated closely before and after freezing and thawing for each ground beef patty, with the exception of increased microbial ATP levels for ground beef inoculated with *Lactobacillus*, frozen slowly and thawed slowly. Plate count estimates were within about 1/2 log of actual plate counts for all variables except for the *Lactobacillus*-inoculated beef noted above, which was accurate to 1 1/2 log. Thus, use of the ATP assay accurately estimated total plate counts of frozen ground beef.

INTRODUCTION

The ATP assay has been proposed as a method for estimating numbers of microorganisms in ground beef. Microbial ATP levels correlate well with ground beef containing mixed flora, Pseudomonas or Lactobacillus and stored aerobically or anaerobically at 2°C or 10°C (Chapter 3).

With frozen foods, bacterial injury or loss of viability may occur as a consequence of freezing and/or thawing stress. Causes of damage to bacteria during freezing and/or thawing may be intracellular ice crystal formation, extracellular ice crystal formation, cellular dehydration or exposure to concentrated solutes in the food (Lowry and Gill, 1985). Effects of injury due to freezing include increased nutritional requirements, alteration of membrane permeability and reduced tolerance to various compounds (Moss and Speck, 1966a; 1966b; Warseck et al., 1973). Leakage of bacterial cell components as a result of freezing include cellular proteins such as various active enzymes, RNA, DNA and amino acids (Davies and Obafemi, 1985). Gritsavage (1965) showed correlation between extent of death of frozen E. coli and leakage of adenosine monophosphate.

Accuracy of estimations of plate counts using the ATP assay is dependent on consistent microbial ATP contents per

cell at given cell concentrations. With frozen ground beef, damage to bacteria during freezing or thawing may result in loss of ATP or permeability to intracellular ATP-converting enzymes. This could affect the ATP content per cell which, in turn, may affect estimation of microbial numbers. The objectives of this study were to measure changes in bacterial ATP content in ground beef containing mixed flora, Pseudomonas or Lactobacillus frozen and thawed at different rates, and to determine the effect of freezing and thawing on accuracy of plate count estimation.

MATERIALS AND METHODS

Ground beef patties

Lean retail beef bottom round was purchased locally, the surfaces flamed, and the exterior aseptically trimmed off. The interior was aseptically cut into 2 to 3 inch cubes and ground twice through a 3/16 plate using a sterile grinder. One half of this ground beef was inoculated with Pseudomonas fluorescens ATCC 13525 grown at 20°C overnight in Trypticase Soy Broth. The other half was inoculated with an overnight culture of Lactobacillus plantarum ATCC 14917 grown at 35°C in MRS broth. Culture was concentrated by centrifugation and evenly distributed over a thin flattened layer of ground beef (5 ml culture per 1000g). Ground beef inoculated in this manner was then mixed in a bag to achieve uniform distribution.

Lean retail ground beef was purchased locally on the day of the pull date as indicated on the product label. This retail ground beef and the Pseudomonas-inoculated and Lactobacillus-inoculated ground beef were each aseptically weighed and pressed into 35 g patties 3 inches in diameter and 1/4 inch thick using a custom-made patty press. Patties were each placed into an oxygen-impermeable saran pouch, vacuum-packaged and heat-sealed.

Freezing and thawing

Each of the packaged patties were placed into plastic

pin-type test tube racks in preparation for freezing. Every other patty had a copper-constantin thermocouple wire in the center of the patty, which had been inserted just prior to heat sealing the packages. Silicone glue was used to seal the packages around the thermocouple wires in order to assure no leakage and proper thermocouple wire placement. Each rack of packaged patties was immersed in a plastic container full of 1:1 solution of automotive antifreeze and water. For rapid freezing, racks of patties were immersed in cold (-16^{o}C) antifreeze solution and placed into a freezer (-16^{o}C). For slow freezing, racks of patties were immersed in room temperature (23^{o}C) antifreeze solution and placed into a freezer (-16^{o}C). Temperatures of patties during freezing and thawing were monitored using a data logger.

Patties were maintained frozen for 48 to 72 hours, and then thawed. For rapid thawing, racks of patties were immersed in room temperature (23^{o}C) antifreeze solution and placed into a refrigerator (4^{o}C). Racks of patties thawed slowly were placed into a refrigerator (4^{o}C) while still immersed in cold (-16^{o}C) antifreeze solution. Temperature during thawing was monitored using a data logger.

Reagents and ATP assay

Reagents and ground beef samples were prepared as described in Chapter 2. For ATP assay, 4 ground beef samples for each inoculum-freezing rate-thawing rate

combination were analyzed in triplicate using a Picolite 6200 luminometer (Packard Instument Company, Downers Grove, IL). Operation of the luminometer is described in Chapter 2.

Plate counts and their estimation using the ATP assay

Microbial plate counts were determined for each ground beef patty by plating on Standard Methods Agar and incubating for 4 days at 20°C. These values were compared with plate count values estimated using the microbial ATP concentrations determined for each sample and regression coefficients developed from data reported in Chapter 3.

RESULTS AND DISCUSSION

The total aerobic plate count for ground beef prior to inoculation was 65 per g using Standard Methods Agar incubated for 4 days at 20°C. Total anaerobic count (plated on Standard Methods Agar and incubated anaerobically for 4 days at 20°C) was 8 per g and Lactobacillus count was 25/g (plated on MRS agar and incubated anaerobically for 2 days at 35°C).

Freezing rates for fast- and slow- frozen patties are shown in Figure 4.1. Placement of packaged patties in test tube racks assured equal spacing and allowed antifreeze solution to flow around and contact all surfaces of each patty. In addition, uniform weight, shape and 1/4-inch thickness of each patty assured uniform rate of cooling and heating among patties. Variation in temperature among patties was less than 2°C at any time (data not shown). The time to reach 0°C was 4 hr 15 min for slow rate and 5 min for fast rate. Drop in temperature from 0 to -5°C occured in 3 hr 15 min for slow rate and 32 min for the fast rate. Inflection in the slow-freezing curve at -4°C may indicate the temperature at which the ground beef froze. Between -1°C and -5°C, 74% of total muscle water freezes (Lowry and Gill, 1985). Exposure of bacteria to temperatures in this range is extended due to demand of the meat for latent heat during freezing. Haines (1938)

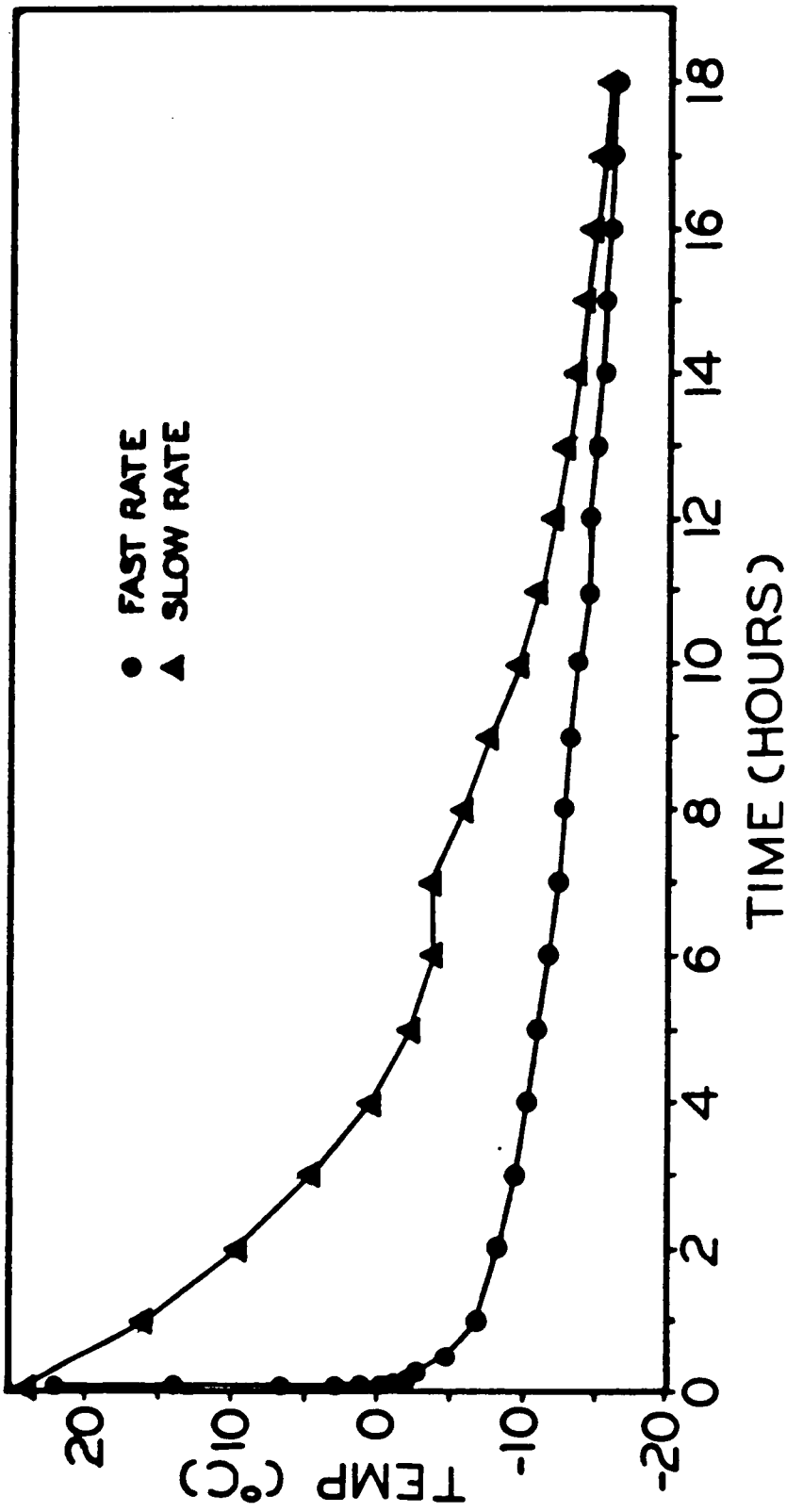

Figure 4.1. Freezing rates for vacuum-packaged ground beef patties immersed in -16° and 23°C antifreeze solution and stored in a -16°C freezer.

reported that destruction of bacteria is most rapid in this range, which may be due to intracellular or extracellular ice crystal formation (Davies and Obafemi, 1985). However, Lowry and Gill (1985) stated that cooling rates slower than 1°C per min are too slow for intracellular ice formation. The fast rate reported here is 0.16°C per min; therefore intracellular ice formation is unlikely to have occured.

Thawing rate data is shown in Figure 4.2. Fast thawing ground beef rose from -15°C to 0°C in less than 9 min. Slow thawing ground beef took 5 hr 30 min to rise to -5°C and then 13 hr 30 min to approach 0°C. Again, an inflection in the thawing curve at about -4°C may indicate thawing of ground beef.

Following freezing and thawing, Pseudomonas plate counts were approximately 1 log lower than counts before freezing (Table 4.1). Counts for mixed ground beef spoilage flora and Lactobacillus, in contrast, were about the same before and after freezing and thawing. Since intracellular ice formation is unlikely, damage to Pseudomonas could have been due to extracellular ice formation. This may have caused physical damage to cell walls or membranes by ice crystals, damage to membrane selective permeability function by concentrated solutes or damage of intracellular functions as a result of dehydration. Lowry and Gill (1985) postulated that cooling would be more detrimental to psychrotrophs than mesophiles since the latter would have

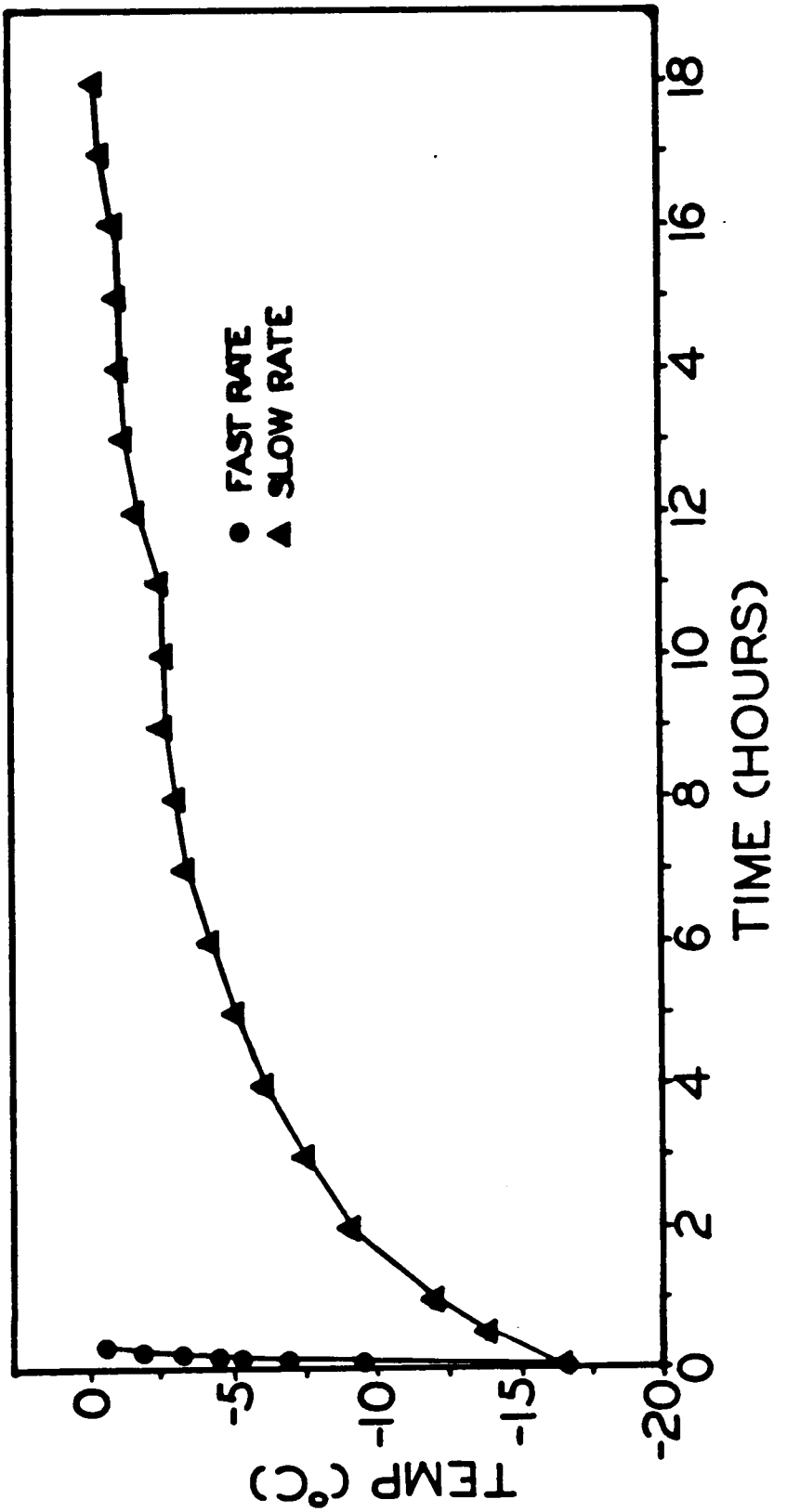

Figure 4.2. Thawing rates for vacuum-packaged ground beef patties immersed in -16° and 23°C antifreeze solution and stored in a 4°C refrigerator.

Table 4.1. Prediction of plate counts for frozen and thawed ground beef with inoculation of mixed retail flora, *Lactobacillus plantarum* and *Pseudomonas fluorescens*.[c]

Inoculum	Freezing rate-Thawing rate	log APC (cfu/g)	log ATP (fg/g)	APC estimate (log cfu/g)	Mean Abs Residual
Mixed Ground Beef Flora	-Unfrozen-	7.9[a]	8.04[a]	8.0	0.16
	Fast - Fast	7.8[a]	8.06[a]	8.0	0.23
	Fast - Slow	7.7[a]	8.32[a]	8.3	0.62
	Slow - Fast	7.7[a]	8.26[a]	8.2	0.56
	Slow - Slow	7.8[a]	8.30[a]	8.4	0.61
Lactobacillus	-Unfrozen-	6.8[a]	7.11[a]	6.9	0.12
	Fast - Fast	6.7[a]	7.02[a]	6.7	0.12
	Fast - Slow	6.9[a]	7.31[a]	7.1	0.29
	Slow - Fast	6.6[a]	6.98[a]	6.6	0.17
	Slow - Slow	6.8[a]	8.36[b]	8.4	1.57
Pseudomonas	-Unfrozen-	8.9[a]	8.54[a]	8.6[a]	0.25
	Fast - Fast	8.0[b]	7.92[b]	7.9[b]	0.14
	Fast - Slow	7.6[b]	7.50[b]	7.1[b]	0.39
	Slow - Fast	8.0[b]	7.79[b]	7.7[b]	0.24
	Slow - Slow	7.8[b]	7.51[b]	7.3[b]	0.41

[c] APC and ATP values were analyzed for statistical differences using ANOVA. Values in each column for each inoculum with different superscripts were significantly different ($p=0.001$).

stopped growing at a higher temperature (earlier), and growing cells are more susceptible to freeze-injury than non-growing cells. Furthermore, rate of cell dehydration during freezing is dependent on cell size, with dehydration more severe for smaller cells (Lowry and Gill, 1985). Pseudomonas is a psychrotroph and smaller than Lactobacillus, which is a mesophile. This may account for greater damage of Pseudomonas due to freezing.

Damage to bacteria is thought to be dependent on both the rate of freezing and of thawing. Quick passage through this range during freezing or thawing will result in less cell damage (Boegh-Soerensen and Jul, 1985). Slow freezing causes larger ice crystals to form in meat tissue, and results in areas of increased solute concentration around the crystals. This exposes bacteria to higher solute concentrations and may result in more extensive dehydration. Survival of bacteria may also be dependent on rate of thawing, with lower survival rate associated with slower thawing rate. Gebre-Egziabher et al., (1982) observed that slower thawing of skim milk resulted in greater destruction of Pseudomonas, E. coli and S. aureus. Ingram and Mackey (1976) attributed this phenomenon to cell-damaging ice crystal growth which may occur to a larger degree during slower thawing.

Generally, plate counts and microbial ATP levels were consistent for all ground beef samples no matter what the

freezing rate or thawing rate. The only exception was an increased microbial ATP level with Lactobacillus - inoculated beef frozen slowly and thawed slowly. The reason for this is unknown, but may be due to enhanced ATP extraction due to cell membrane damage or accumulation of ATP in the absence of cell division. Microscopic examination revealed no unusual amount of clumping or chain formation. Reports of freezing studies, unfortunately, often do not indicate rates of freezing, or express them as cm per min rather than ^{o}C per min. Therefore, comparison of bacterial effects due to freezing is difficult.

Estimates of APC values were close to actual APC values (Table 4.1), with mean residuals generally within 1/2 log for all variables with the exception of Lactobacillus - inoculated ground beef, which was accurate to 1 1/2 log. The ATP assay is a good estimator of APC values in part because it does not detect dead cells. Viable cell number as well as microbial ATP concentration of Pseudomonas decreased when ground beef was frozen and thawed. Freezing rates used in this study resemble those that may be used commercially. The faster rate for freezing in the range of 0^{o}C to -5^{o}C reported here was 0.16^{o}C per min, and the slower rate was 0.026^{o}C per min. Meat blast-frozen at -30^{o}C at an air speed of 1 meter per sec would have a surface cooling rate of 0.2^{o}C per min (James and Bailey, 1982). Slower freezing and thawing rates can increase the amount of drip

for beef (Jul, 1984). It is possible that other effects such as ATP release or leakage of ATP - converting enzymes may also occur. Therefore, it appears that procedures used in this study were adequate for removing non-microbial ATP in possible commercial freezing and thawing situations, and that the ATP assay was able to be used for estimating microbial plate counts with reasonable accuracy.

ACKNOWLEDGMENTS

This work was included in a presentation given at the Annual Meeting of the Institute of Food Technologists, June 15-18, 1986 in Dallas TX.

Appreciation is given to and for their help in preparing and packaging ground beef patties. Thanks are extended to for his help in using the data logger.

Chapter 5

Study of methods for enhancing accuracy of microbial numbers estimated using ATP luminometry

ABSTRACT

Calculations were done to determine optimum ways of evaluating luminometry data for enhancement of sensitivity and accuracy of microbial plate count estimates. Little difference was seen between variability of daily and weekly calibrations of ATP standards; therefore performing weekly calibrations may save work without sacrificing accuracy.

Consideration of ways to use an assay standard for calculation of light-output values led to an improved ratio formula which was better able to compensate for quenching ions; however, use of the improved formula did not enhance accuracy of ground beef plate count estimates over those obtained using the ratio formula used previously. Comparison of various linear regression methods did not lead to a more accurate way for estimating plate count values. The value of using an assay standard for correcting light output values altered by variable enzyme activity or reduced by various ions was demonstrated and its use is recommended.

INTRODUCTION

Use of the ATP assay for estimating microbial numbers depends on the relationship between ATP concentration and light output during the assay. The use of an assay standard of consistent ATP concentration is included in the analysis to provide stable light readings, that is, free of alteration from light output-altering factors. Some of these factors are enzyme stability, reaction temperature, pH of reaction mixture and presence of ions which can quench the light-producing reaction. The Packard luminometer used in this study closely controls temperature, and pH is controlled by use of a buffer in the reaction mixture. The effectiveness of an assay standard was investigated for its effect on mitigating the effect of enzyme instability and quenching due to the presence of various ions. Alternate ways of using the assay standard for reducing error due to light output-altering factors were examined.

Much time and effort is being spent to develop and evaluate rapid methods for determining total numbers of microorganisms in foods. Although it is possible to determine the precision of any method to evaluate reproducibility of measurement, accuracy is difficult to determine since there is no error-free reference method with which to compare. For determining numbers of microorganisms

in foods the plate count is the standard reference method. However, plate count values may not represent the true microbial concentration due to factors such as clumping, inability of cells to grow in the agar medium, or lack of colony formation due to environmental factors. For a more detailed consideration of errors associated with plate counting and bioluminometric determination of microbial numbers, the reader is referred to the Summary section.

Use of luminometry for the determination of microbial numbers requires the establishment of a relationship between microbial ATP levels and plate counts. Classical linear regression, which assumes one of these variables to be without error, is used to define this relationship and is used for estimating plate counts given microbial ATP concentrations. Unfortunately, neither ATP concentration nor plate count is a direct measurement of the other. Instead, both are dependent on the concentration of bacteria in the food sample and both are subject to error. One of the objectives of research in this chapter was to evaluate several regression methods aimed at minimizing this problem in order to make more accurate plate count estimates using the ATP assay.

MATERIALS AND METHODS

Effectiveness of assay standard

An experiment was done to evaluate the effectiveness of the assay standard in correcting for variation in light output not due to sample ATP concentration differences. Since accurate ATP calibration is critical for determining microbial ATP concentration, correction for variation of enzyme activity was studied. Luciferin-luciferase enzyme reagent (PicozymeF, Packard Instrument Company, Downers Grove IL) was placed at room temperature for 10 hours (25 - 28^{o}C) and periodically used to perform ATP calibration. Light output of the 10^{-8}M ATP sample and the assay standard were monitored and compared with changes in their ratio calculated using the following formula: ratio = C2 / (C1 - B1). B1 was light output counts per second (CPS) due to extractant (PicoExB, Packard) plus PicozymeF. C1 was CPS due to sample plus PicozymeF. C2 was CPS due to sample plus PicozymeF plus assay standard.

Evaluation of ratios

Limitations of the above ratio (ratio A) became apparent in the upper ATP concentration range. Therefore, other ratios were developed and used to determine CPS values. Ratios used were Ratio B = (C1 - B1) / (C2 - C1), Ratio C = (C1 - B1) / (C2 - 5/6 x C1) and Ratio D = (C1 -

B1) x B2 / (1.2 x C2 - 0.2 x B2) where B2 was CPS due to PicoExB plus PicozymeF plus assay standard. Each ratio was compared with sample CPS (C1 - B1) to determine the degree to which it corrected for quenching due to concentrations of NaCl, KCl, Na_2HPO_4 and K_2HPO_4 ranging from 0.20 to 0.01 Molar.

Frequency of ATP calibration

A comparison was made to determine whether less frequent ATP calibrations could be done without causing unacceptable loss of accuracy. The range for each ATP calibration curve coefficient was determined for each week, month, and over a 4-month period. Error of plate count estimation for each of these ranges was calculated.

Linear regression methods

Four regression methods were used to predict microbial numbers, given the microbial ATP concentration. The classical method, which has been used throughout this study, was set up so that plate count value was the independent variable and microbial ATP concentration was the dependent variable. Another classical approach was used so as to treat plate count as the dependent variable and microbial ATP concentration as the independent variable. With each of these methods the independent variable is assumed to be free of random error. The third linear method used gave equal weight to both variables, and therefore both plate count and

ATP concentrations were treated as dependent variables and subject to error. The fourth method, proposed by Deming (1943) and described fully and applied by Wakkers et al (1975) also assumed both methods to be associated with error, but gave relative weight to each method based on their individual variances. An IBM PC was programed using BASIC in order to perform the four linear regression methods. A description of how each regression line was calculated is given in Table 8.

RESULTS AND DISCUSSION

Instrument and reagent performance checks were used extensively during the course of this study. Reagent pumping volumes, empty chamber background readings, tritium source chamber readings and reagent background readings were routinely measured in order to check on pump volume calibration, chamber and reflector cleanliness, pump system cleanliness and instrument operation. In addition, 10^{-8} Molar ATP standards were included with samples during assay, and ATP calibration using 3 ATP concentrations were done each day. To reduce the amount of work, the feasibility of less frequent ATP calibration was investigated. Results shown in Table 5.1 indicate that the ranges of values for regression coefficients used for ATP calibration were much higher during a 4 month period than during a single day. This would result in much larger possible error of APC estimation than if daily calibration were done. There was little difference between weekly and daily calibration; therefore, time could be saved by performing weekly instead of daily ATP calibrations without adding a large amount of potential error.

Throughout this study, an assay standard has been used during all ATP concentration determinations in order to obtain accurate results. Ideally, the assay standard should

Table 5.1. Effect of less frequent ATP calibration on prediction accuracy of the ATP assay.

Frequency of calibration	n[a]	Average range for coefficients		Maximum possible error of APC estimation due to inaccurate ATP calibration	
		Coef. A	Coef. B	5×10^4 fg/g	5×10^9 fg/g
Daily[b]	2	0.0551	0.0421	0.10 log	0.26 log
Weekly[c]	8	0.0698	0.0395	0.11 log	0.28 log
Monthly[d]	4	0.0953	0.0605	0.17 log	0.41 log
4 Month[e]	1	0.1864	0.1002	0.30 log	0.70 log

[a] n = number of periods considered

[b] each daily period included 8 ATP calibrations

[c] each weekly period included 3 to 7 ATP calibrations

[d] each monthly period included 7 to 14 ATP calibration

[e] the 4-month period included 49 ATP calibrations

have high ATP concentration with lowest possible volume. The principle is that light output due to the assay standard is influenced by the same factors as light output due to ATP in samples. Therefore, their ratios should mitigate light output-altering factors which can alter accuracy of ATP determination. Results of an evaluation of the effectiveness of using an assay standard are presented in Table 5.2. Although light output (CPS) of 10^{-8}M ATP sample decreased as enzyme activity decreased over time, the assay standard CPS also decreased. Their ratio (ratio A) was therefore more consistent than sample CPS for a given ATP concentration with varying PicozymeF activity. With a 15% decrease in sample CPS (C1 - B1), and a 16% decrease in assay standard CPS (C2) there was only a 2% difference in the ratio and the ATP calibration coefficients. Use of the assay standard, therefore, greatly added to the reliability of the assay.

Ratio A was the standard ratio recommended by Packard Instrument Company [Ratio A = C2 / (C1 - B1)]. C2 is the numerator and (C1-B1) the denominator instead of the reverse so that during calculation of the power regression used by Packard software installed with the luminometer, no logarithms of negative numbers are calculated (which cannot be done). Power regression [$y = (a)x^b$] is equivalent to linear regression with log values of x and y variables

Table 5.2. Variation of enzyme activity and its effect on ratio values and calibration curve coefficients.

Storage Time (hrs)	Light output (CPS)		Ratio	Curve Coefficients	
	10^{-8}M ATP	Assay Standard		a	b
1	11630	79535	6.86	-7.17	-1.004
2	11571	78519	6.80	-7.17	-1.007
3	11063	75145	6.81	-7.18	-1.002
4	11188	75622	6.78	-7.17	-1.006
6	10358	69608	6.74	-7.17	-1.007
7	10261	69381	6.78	-7.17	-1.006
9	10207	69513	6.83	-7.18	-0.998
10	9936	66886	6.75	-7.18	-1.004

calculated [log(y) = a + (b)log(x)] except that coefficient a with power regression is the log of coefficient a with linear regression. Both methods are identical for establishing curves used for estimating microbial plate counts, which was confirmed using data from this study (data not shown).

When higher concentrations of ATP were assayed ratio A resulted in less accurate determinations. Therefore, other ratios designed to compensate for high sample ATP concentrations and better adjust for light output-altering factors were developed. When log C1 was below 0.8 (ATP concentration about 9.7 $\log_{10}$ fg/liter in assay tube) the relationship between CPS and ATP concentration for Ratio A deviated sharply from linear. (See Figure 5.1). This was due to influence of C1 on C2. To correct for this, Ratio B was calculated by subtracting C1 from C2. Ratio B was closer to linearity than Ratio A for ATP concentrations greater than 8.7 $\log_{10}$ fg/liter. Since the concentration of ATP in the sample is diluted to 5/6 its concentration when C2 recorded, theoretically only 5/6 of the value of C1 should be subtracted. Ratio C was calculated to reflect this. When CPS values of Ratio C were plotted against ATP concentration, the curve appeared identical to the reciprocal of Ratio A (Figure 5.1). Therefore, practical differences between these two ratios were insignificant.

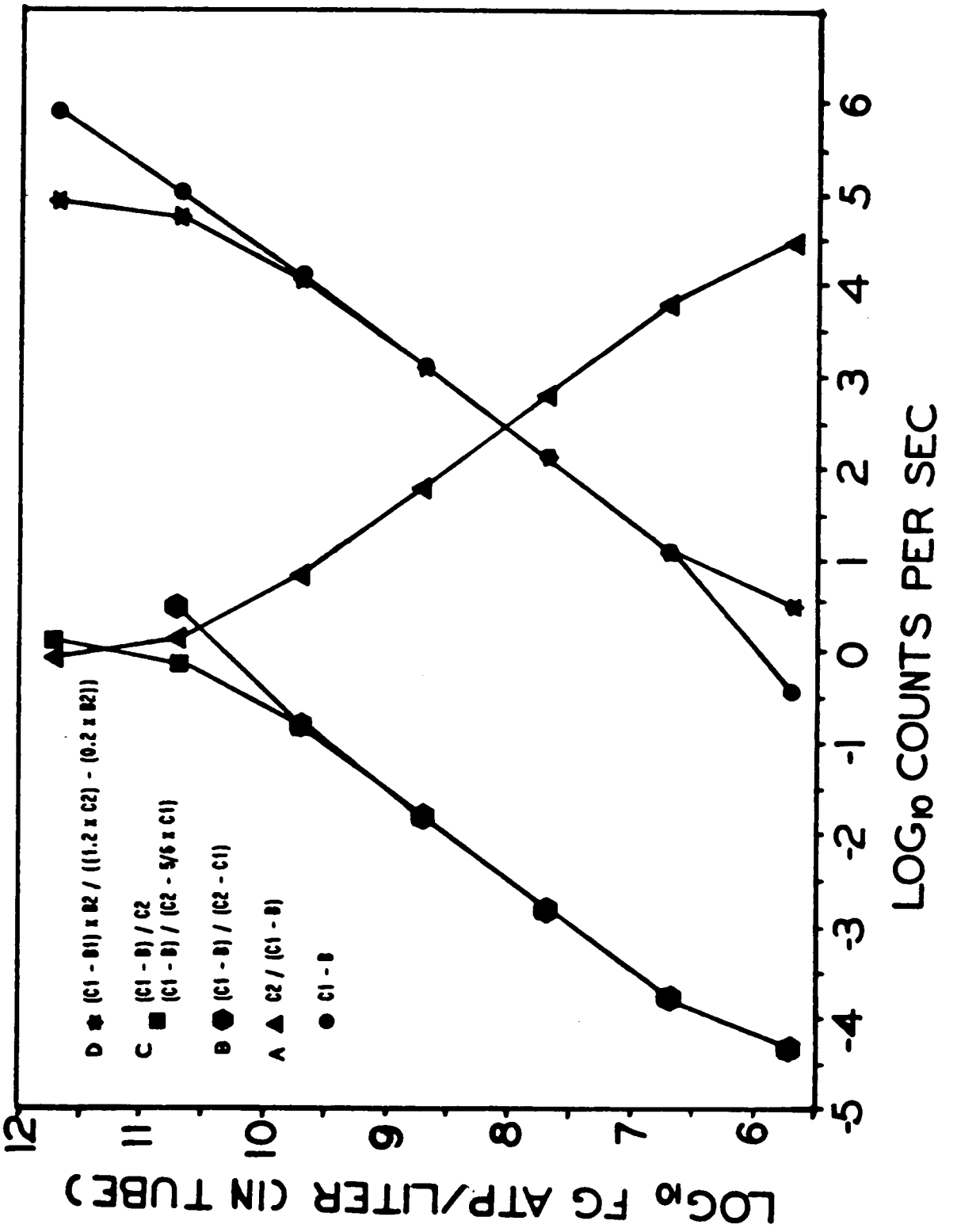

Figure 5.1. Linearity of various ratios and the relationship between ATP concentration and light output.

Since during the C1 count any possible quenching factors are 1.2 times as concentrated as they are for the C2 count, sample light output (C1 - B) should be corrected 1.2 times the amount of quenching observed during light output of the assay standard (C2). Using this idea, Ratio D was calculated. It was linear over the same range of ATP concentrations as Ratio A.

Experiments designed to determine which ratio provides the best correction for light output-altering factors were done. The effects of various concentrations of NaCl, KCl, Na_2HPO_4 and K_2HPO_4 on each of the ratios are shown in Tables 5.3-5.6.

Quenching effect was greatest with Na_2HPO_4, followed in order by K_2HPO_4, KCl and NaCl (on a molar basis). With increasing concentration of each ion, a 10^{-9} Molar ATP sample decreased in light output following addition of PicozymeF. Light outputs of assay standards also decreased, causing stabilization of light output ratio values shown in the 4 columns on the right of Tables 5.3-5.6. For nearly all concentrations of all ions studied, ratio D provided the best correction for quenching. The exceptions were 0.20 Molar Na_2HPO_4 and 0.20 Molar K_2HPO_4 where quenching of the 10^{-9} Molar ATP sample was in excess of 85%.

Since some of the ratios provided better correction for quenching than Ratio A used throughout this study, data from

Table 5.3. Quenching by NaCl and effect of various ratios for adjusting light output values.

NaCl (Moles/L)	% quench (light reduction) Sample[a] (10^{-7} M ATP)	Assay Standard[b]	% change in ratio A[c]	B[d]	C[e]	D[f]
0.20	71.0	62.4	29.0	22.7	22.7	16.4
0.10	53.3	42.8	22.2	18.3	18.3	3.6
0.05	34.2	23.3	16.2	14.1	14.1	8.3
0.01	13.7	7.3	7.3	6.9	6.9	5.3
0.001	2.9	1.4	1.5	1.5	1.5	1.2

[a] C1-B1

[b] C2-C1

[c] C2 / (C1-B1)

[d] (C1-B1) / (C2-C1)

[e] (C1-B1) / (C2-5/6*C1)

[f] (C1-B1)*B2 / (1.2*C2-0.2*B2)

Table 5.4. Quenching by KCl and effect of various ratios for adjusting light output values.

KCl (Moles/L)	% quench (light reduction) Sample[a] (10^{-9} M ATP)	Assay Standard[b]	% change in ratio A[c]	B[d]	C[e]	D[f]
0.20	74.5	64.7	38.2	27.9	27.9	14.4
0.10	55.4	44.3	24.5	19.9	19.9	4.4
0.05	36.8	26.5	16.2	14.1	14.1	7.2
0.01	11.0	4.5	7.1	6.8	6.7	5.8
0.001	8.9	1.3	2.2	2.2	2.2	2.4

[a] C1-B1

[b] C2-C1

[c] C2 / (C1-B1)

[d] (C1-B1) / (C2-C1)

[e] (C1-B1) / (C2-5/6*C1)

[f] (C1-B1)*B2 / (1.2*C2-0.2*B2)

Table 5.5. Quenching by Na_2HPO_4 and effect of various ratios for adjusting light output values.

Na_2HPO_4 (Moles/L)	% quench (light reduction) Sample[a] (10^{-9} M ATP)	Assay Standard[b]	% change in ratio A[c]	B[d]	C[e]	D[f]
0.20	89.6	80.7	83.9	46.0	45.9	250.2
0.10	72.2	58.8	47.5	32.5	32.5	4.8
0.05	48.9	34.2	28.2	22.3	22.2	12.9
0.01	13.0	4.4	9.7	9.0	9.0	8.0
0.001	1.1	2.1	1.0	1.0	1.0	1.4

[a] C1-B1

[b] C2-C1

[c] C2 / (C1-B1)

[d] (C1-B1) / (C2-C1)

[e] (C1-B1) / (C2-5/6*C1)

[f] (C1-B1)*B2 / (1.2*C2-0.2*B2)

Table 5.6. Quenching by K_2HPO_4 and effect of various ratios for adjusting light output values.

K_2HPO_4 (Moles/L)	% quench (light reduction)		% change in ratio			
	Sample[a] (10^{-9} M ATP)	Assay Standard[b]	A[c]	B[d]	C[e]	D[f]
0.20	86.7	76.6	74.9	43.2	43.1	68.3
0.10	70.2	56.4	45.8	31.7	31.6	7.3
0.05	48.6	34.9	26.3	21.1	21.1	11.3
0.01	13.4	4.1	10.6	9.7	9.7	8.8
0.001	1.5	1.0	2.1	2.1	2.1	2.1

[a] C1-B1

[b] C2-C1

[c] C2 / (C1-B1)

[d] (C1-B1) / (C2-C1)

[e] (C1-B1) / (C2-5/6*C1)

[f] (C1-B1)*B2 / (1.2*C2-0.2*B2)

experiments described in Chapter 3 were recalculated with each ratio to determine if better plate count accuracy could be obtained. Results presented in Table 5.7 depict how none of the "improved" ratios showed significantly better accuracy than Ratio A for ground beef patties stored at $2^{o}C$ or $10^{o}C$. Although all ratios had much lower mean squared error than when no assay standard was used (C1 - B1), there was no practical difference between the ratios. This may be because variability of the ATP assay or plate count determination were high enough to mask small differences between the ratios or because differences in quenching among samples were minimal. The type of ratio used may be important, however, for direct ATP assay of samples where filtration is not used to remove interfering substances.

Alternative regression methods were used in order to determine if another method could be used to obtain more accurate plate count estimates using the ATP assay. Three regression methods were compared with the standard method for estimating plate counts (Figure 5.2).

With plate count as the independent (x) variable and microbial ATP concentration as the dependent (y) variable, the classical linear regression method (used throughout this study) fitted curves by minimizing the squared values of the vertical distances between data points and the curve (Figure 5.2, Curve A). In this way all adjustments were made to

Table 5.7. Effect of various ratios on accuracy of predictions of microbial numbers in ground beef patties.

Ratio	Accuracy (MSE) of APC estimates	
	2°C storage	10°C storage
C1-B1	0.4070	0.8621
C2 / (C1-B1)	0.0950	0.1386
(C1-B1) / (C2-C1)	0.0946	0.1312
(C1-B1) / (C2-5/6*C1)	0.0952	0.1369
(C1-B1)*B2 / (1.2*C2-0.2*B2)	0.0942	0.1394

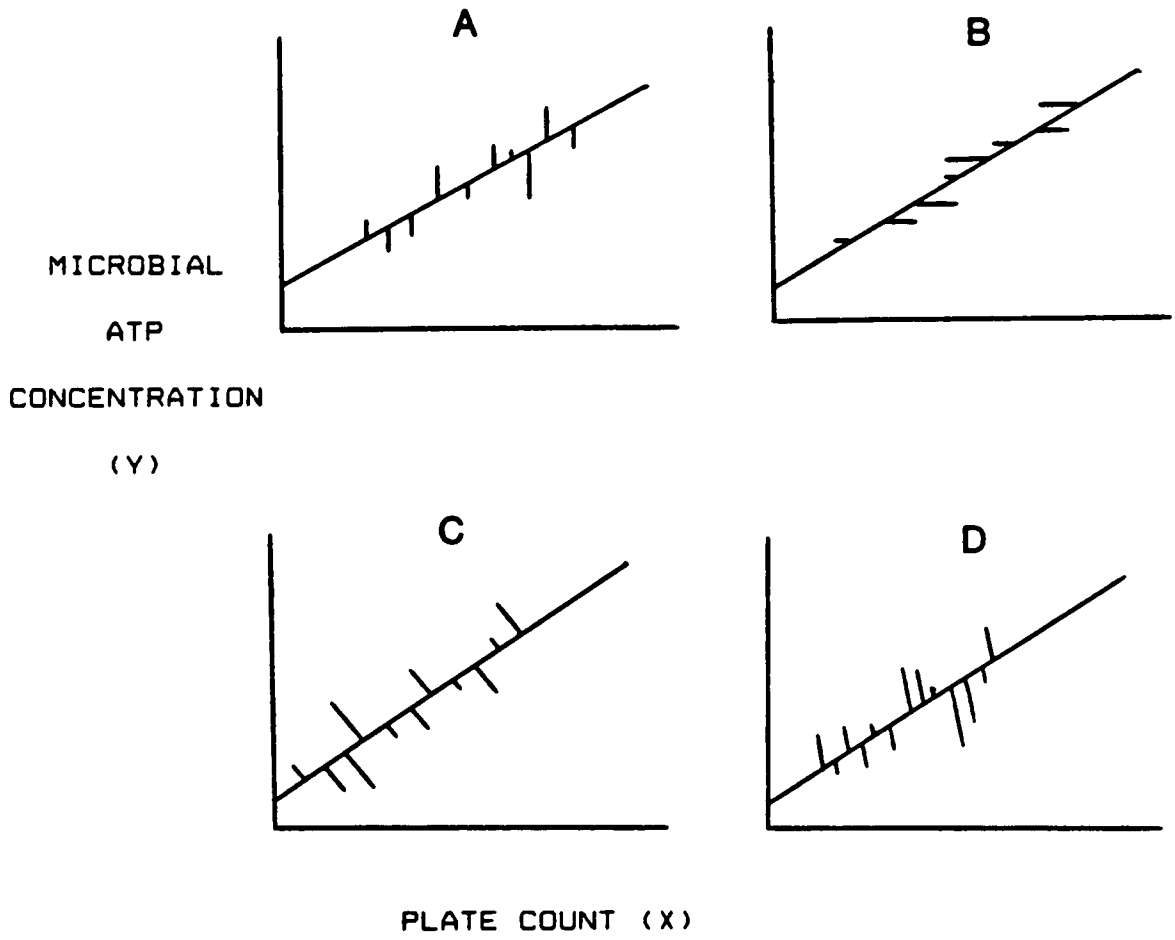

Figure 5.2. Illustrations of regression methods evaluated for use with luminometry data. A: Classical regression with (x) as the independent (error free) variable and (y) as the dependent (error assumed) variable. B: Classical regression with (x) dependent variable and (y) independent variable. C: Variables (x) and (y) considered to have equal error. D: Deming's method (Wakkers et al, 1975) which assumes unequal error of (x) and (y) variables.

microbial ATP values and plate count values were assumed to be free of error.

Data was also regressed by treating plate count as the dependent (x) variable and microbial ATP as the independent (y) variable (Figure 5.2, Curve B). This should form more accurate curves if there is more error associated with plate count than microbial ATP concentration. With this method, the regression curve was fitted by minimizing the squared values of the horizontal distances between data points and the curve. Adjustments were made to plate count and ATP concentration was assumed to be free of error.

Neither of the above methods assume error of both plate count and microbial ATP concentration. Therefore, another regression which made adjustments to values of both variables was used. With this method, equal adjustments were made in the x and y directions in order to minimize the squared distances between data points and the regression curve (Figure 5.2, Curve C).

In the above method equal error of both methods was assumed. Deming's method (Wakkers et al, 1975) was also done which gave unequal weight to plate count relative to microbial ATP concentration based on their variances. Variance for plate counts was calculated to be 0.002431 and variance for ATP concentration was calculated to be 0.009832. These calculations were based on 18 replicate

determinations of ground beef and ATP standards, respectively, for 3 log dilutions of each, which were within the range of ATP detection. This value is less than the value of 0.01045 reported by Peeler et al. for standard plate counts of milk analysed by different laboratories. Ground beef was plated at 20°C for 4 days as previously described. Thus, the variance of ATP determination was found to be about 4 times that of plate count. Regression lines using Deming's method were calculated by minimizing squared differences along a slope determined by the ratio of variances (Figure 5.2, Curve D).

Formulas used to calculate each of the regression types are shown in Table 5.8. Wakkers et al (1975) were able to show better correlation between two error-associated analytical methods when Deming's method was used than when classical regression was used. It was expected that the methods assuming error in both variables would provide greater accuracy with ground beef; however this was not the case. Results of the use of four different regression methods for estimating plate count values is presented in Table 5.9.

MSE values using each regression method were no better than values obtained using the original Packard method in which error is assumed to be associated with ATP determination but not with the plate count. Deming's

Table 5.8. Calculation of linear regression curves using four methods.

$y = bx + a$, x = independent, y = dependent

$$\text{coefficient } b = \frac{Qxy}{Qx} \qquad \text{coefficient } a = \frac{\sum y_i - b\sum x_i}{n}$$

$y = bx + a$, x = dependent, y = independent

$$\text{coefficient } b = \frac{Qy}{Qxy} \qquad \text{coefficient } a = \frac{\sum y_i - b\sum x_i}{n}$$

$y = bx + a$, both variables with error

$$\text{coefficient } b = \frac{Qy - Qx + \sqrt{(Qx - Qy)^2 + 4Qxy^2}}{2Qxy}$$

$$\text{coefficient } a = \frac{\sum y_i - b\sum x_i}{n}$$

$y = bx + a$, Deming's relative variance method

$$\text{coefficient } b = \frac{\lambda Qy - Qx + \sqrt{(Qx - \lambda Qy)^2 + 4\lambda Qxy^2}}{2\lambda Qxy}$$

$$\text{coefficient } a = \frac{\sum y_i - b\sum x_i}{n}$$

	Textbook method	Calculator method (faster)
$\bar{x} = \frac{x_i}{n}$	$Qx = \sum (x_i - \bar{x})^2$	$Qx = \sum x_i^2 - \frac{(\sum x_i)^2}{n}$
$\bar{y} = \frac{y_i}{n}$	$Qy = \sum (y_i - \bar{y})^2$	$Qy = \sum y_i^2 - \frac{(\sum y_i)^2}{n}$
	$Qxy = (x_i - \bar{x})(y_i - \bar{y})$	$Qxy = \sum x_i y_i - \frac{\sum x_i \sum y_i}{n}$

Table 5.9. Comparison of alternate regression methods for estimating microbial numbers in ground beef patties.

Regression method	MSE of estimates	MSE of estimates for cfu/g $>10^5$/g	
		2^{o}C storage	10^{o}C storage
Plate count independent, ATP/g dependent (Packard's method)	0.2803	0.0950	0.1386
ATP/g independent, Plate count dependent	0.3023	0.1018	0.1539
Equal weight for ATP/g and plate count	0.2875	0.0969	0.1430
Relative weights for ATP/g and plate count (Deming's method)	0.9432	0.3946	0.4472

regression method resulted in much worse plate count estimates than the Packard method. Optimization of Deming's method for obtaining the lowest MSE values was calculated and found to require an ATP per plate count variance ratio of 0.93 for beef stored at $2^{o}C$ and 0.89 for beef stored at $10^{o}C$. When these variance ratios were used MSE values were identical to those obtained using the Packard (classical) regression method. Therefore, there was no advantage to using a non-classical ratio.

The assay standard and ratio calculation, together with controlled temperature and pH, help to provide precision and accuracy for the ATP assay. Other methods may be developed in attempts to further improve the assay.

Each sample could have its own blank - one to be extracted and one not to be extracted. However, this would not work if the extracting reagent has a significant effect on light output.

Lyophilized standard cultures could be used as internal standards to be assayed along with other samples. However, lyophilized cultures may not be perfect standards since they may have different ATP extraction properties than bacteria in a food homogenate.

Another method that could be tried would be to filter a food homogenate into an assay tube, add somatic cell ATP extractant and measure light output. After this bacterial

cell ATP extractant could be added and light output again measured. Subtraction of the first measurement from the second could provide a measurement of ATP present in the bacterial cells. This method would depend on bacterial ATP being concentrated enough to be detected amid the non-bacterial ATP, and the lack of excess ATP-converting enzymes from the food.

ATP standard concentrations could be included along with samples during an assay. To avoid errors due to performing ATP calibration and sample assays at different times (with different assay conditions not completely corrected by use of assay standard). ATP calibration would be performed at the same time under very similar conditions as sample readings and would use the same blank.

The ATP assay may be used in many ways for estimating plate count values. Work reported here led to no "improved" light output ratio formula or regression method. However, time may be saved by performing less frequent ATP calibrations. Value of continued use of an assay standard was demonstrated.

Summary and Conclusions

In this study the bioluminescent ATP assay was found to be useful for estimating bacterial plate counts of ground beef. Use of a double filtration procedure for removing non-microbial ATP resulted in lower sensitivity of 10^6 cfu/g. Use of selective non-microbial ATP extraction and digestion by ATPase in conjunction with double filtration resulted in an additional log sensitivity, so that total plate count values could be estimated within 1/2 log over the range of 10^5 to 10^9 cfu/g.

The assay worked well for ground beef patties containing mixed ground beef spoilage flora, pure Lactobacillus or pure Pseudomonas inocula whether the product was packaged aerobically or anaerobically, stored at 2^o or 10^oC, or frozen and thawed at different rates. Generally, correlations (r^2) of approximately 0.95 were obtained when estimated plate counts were compared to experimentally observed plate counts. Only one regression curve was needed for estimating plate counts no matter how the beef was packaged or stored.

Procedures to standardize and control variation of light output added to the amount of time and effort needed to perform the ATP assay. Less frequent ATP calibration or

elimination of the assay standard could be done, however, if the resulting decrease in accuracy were acceptable. If sensitivity of 10^6 cfu/g of ground beef were desired, the assay would take less than 30 minutes to complete. If, however, greater sensitivity were needed, then measures such as ATPase digestion of non-microbial ATP would be needed, which would extend assay time by 40 minutes.

At this time, the agar plate count is the most widely accepted method for determining total microbial concentrations in foods. The use and accuracy of plating, however, is dependent on several assumptions which may not hold true:

1. Each colony arises from one cell: bacteria often grow in chains or clusters, or adhere to food particles, causing plate counts to underestimate actual numbers. Mixing, shaking and blending do not guarantee separation of cells.

2. All viable cells plated will produce a visible colony: No one environmental condition will support the growth of all types of microbial cells. Specific growth requirements may not be met such as incubation temperature, oxygen concentration or specific nutrients. Cells may be prevented from forming colonies by antimicrobial substances introduced with the sample or by inhibitory growth of nearby colonies. Cells injured by sublethal heating, freezing or

irradiation may not form visible colonies due to inadequate time or nutrients required for repair.

3. The number of viable organisms is an indicator of future shelf life, past handling sanitation, and potential health hazard: Total numbers do not necessarily represent organisms capable of spoiling the food being sampled or indicate the rate at which growth and subsequent spoilage will occur. Total numbers do not necessarily indicate the presence of pathogens.

4. Technical errors: Accuracy of diluting and pipetting, and contamination of agar, petri dishes, or the sample are sources of error.

Main advantages: Plate counts are sensitive, require little skill, and can be used to determine the types of bacteria present based on growth temperature, nutrients utilized or required, susceptibility to inhibitors, oxygen requirements, dye reactions, acid production or enzyme activity.

Main disadvantages: A long time (48 to 96 hrs) is required for cells to grow into visible colonies. Preparation time is expensive as is equipment required for preparing agar plates.

The use of ATP luminometry for determining microbial concentrations in foods also involves certain assumptions which influence the value of results:

1. Consistent recovery of organisms from food particles is assumed. This includes lack of adsorption of organisms to food and filterability of food.

2. Little interference by ATP sources other than live bacteria is required for accurate results. Food particles and dead bacteria should not contribute ATP which can worsen sensitivity to microbial ATP.

3. Consistent amount of ATP extracted from bacteria is needed. Bacteria grown in the food under various conditions, or different types of bacteria must have predictable ATP contents.

4. Little interference by factors which alter light output such as variability of pH, temperature, enzyme activity, quenching factors or other factors are assumed.

5. Numbers of bacteria estimated using luminometry represent spoilage potential, past sanitation and health hazard potential. Total numbers of bacteria may not be a reliable indicator of these.

6. Technical errors: Cleanliness of glassware, stomacher bags, diluent, filter apparatus and tubes can cause error, since ATP sources or reaction quenchers such as salts are a problem. Accuracy of diluting and pipetting, attention to stability of reagents and proper calibration of the luminometer are also subject to error.

Main advantages: Luminometry in rapid. The method

requires no agar preparation.

Main disadvantages: Much time is required for preparation of reagents, equipment and ATP calibration. Technician expertise is required for proper operation of luminometry including recognition and correction of factors which cause invalid results. Equipment and reagents are expensive. Sample preparation is tedious. Total counts only can be determined; no differentiation is possible at this time. Luminometry is not as sensitive as the plate count. For maximum accuracy, much preliminary work is required for each food product in order to be confident of accuracy.

Consideration of using luminometry in place of the plate count is warranted when plate count results take too long and when technical expertise is available to perform the ATP assay. Trials must be done for each type of food, to determine whether microbial ATP levels can be determined with sufficient sensitivity and accuracy and whether the microbial ATP- microbial numbers relationship is predictable under a variety of conditions.

Further improvements of the ATP assay for estimating microbial plate counts may include enhanced sensitivity, better accuracy, and applicability to a wider variety of foods. Development of more sensitive photomultiplier tubes or other light-detecting devices for luminometers will

improve lower ATP detection limits. Although commercial reagents available now are highly purified, further purification to eliminate traces of ATP will result in lower backgrounds and better sensitivity. Improvements in stability of luciferin/luciferase reagents and better pH or temperature control during ATP assay would improve accuracy. Applicability of luminometry to different types of foods depends on the ability to suspend foodborne micoorganisms in water and to remove interference by non-microbial ATP. Improvements in these areas would increase sensitivity.In addition, some foods may contain light output-quenching substances or opacity which might need to be corrected in order to prevent hampering of light output readings. Applicability of luminometry for some foods may depend on the ability to concentrate foodborne organisms in order to obtain the required sensitivity. Further developments in these areas will permit more widespread use of luminometry. The use of dye reduction testing by the dairy industry shows that non-colony counting methods may be used for assessing microbial quality of foods. The basis of dye reduction testing of milk is microbial metabolic activity which can be correlated with shelf life potential. Measurement of microbial activity rather than microbial numbers may be a better indicator of food quality. Similarly, measurement of certain microbial components such as ATP may be more

indicative of shelf life potential or microbial deterioration of a food than plate counts, especially since the latter are subject to errors outlined previously. Sharpe (1979) expressed criticism of the plate count, stating that microbiologists are too dependent on numbers of microorganisms and that acceptability of foods should involve measurement of microbial effects which can be physical, chemical, biochemical or immunological in nature.

Among the "rapid" microbiological methods available, only a few are able to determine microbial contamination in 60 minutes or less. These include the Limulus Lysate assay which measures Gram negative endotoxin (including that contributed by non-viable cells) and catalimetry which determines amounts of catalase activity. Only ATP luminometry or microscopic counting techniques such as the Direct Epifluorescent Filter Technique (DEFT) are capable of estimating total numbers of microorganisms within one hour. DEFT has the disadvantages of requiring high efficiency of sample filtration as well as subjectivity of determining live cells from dead ones. Because of its rapidity and potential for automation, luminometry is useful for making rapid decisions regarding microbial quality of foods. The method has potential for use in monitoring microbial quality during production since, with results available in less than 30 minutes, corrective actions can be made before problems

develop. Rapid indication of cleanup efficiency using luminometry would provide needed information before production start-up. Use of a rapid indicator of ingredient or product quality would permit decisions such as whether to accept or reject incoming raw material and whether to ship or withhold finished product. Early knowledge of microbial levels will help to reduce waste and provide confidence with decisions regarding microbial quality.

References

Afghan, B. K., Tobin, R. S. and Ryan, J. F. 1977. An improved method for quantitative measurement of ATP and its application to measure microbial activity in natural waters, activated sludges and sediments. In "2nd Biannual ATP Methodology Symposium", (ed.) Borun, G. A. p. 349. SAI Technology Co., San Diego, CA.

Alexander, D. N., Ederer, G. M. and Matsen, J. N. 1976. Evaluation of an adenosine 5'-triphosphate assay as a screening method to detect significant bacteriuria. J. Clin. Microbiol. 3:42.

Azam, F. and Hodson, R. E. 1977. Use of ATP as an indicator of bacterial biomass in seawater. In "2nd Biannual ATP Methodology Symposium", (ed.) Borun, G. A. p. 109. SAI Technology Co., San Diego, CA.

Bachi, B. and Ettlinger, L. 1973. Influence of glucose on adenine nucleotide levels and energy charge in Acetobacter aceti. Arch. Mikrobiol. 93:155.

Baumgart, J., Fricke, K. and Huy, C. 1980. Schnellnachweis von mikroorganismen in naturlichen mineralwassern durch bestimmung von adenosintriphosphat mit einem bioluminizenz-verfahren. Alimenta 19:37.

Baumgart, J., Fricke, K. and Huy, C. 1980. Quick determination of surface bacterial content of fresh meat using a bioluminescence method to determine adenosine triphosphate (ATP). Fleischwirtschaft 60:266.

Beckers, B. and Lang, H. R. M. 1983. Rapid diagnosis of bacteremia by measuring adenosine triphosphate in blood culture bottles using biolumniescence. Med. Micriobiol. Immunol. 172:117.

Bishop, J. R., White, C. H. and Firstenberg-Eden, R. 1984. Rapid impedimetric method for determing the potential shelf-life of pasteurized whole milk. J. Food Prot. 47(6):471.

Boegh-Soerensen, L. and Jul, M. 1985. Effects of Freezing/thawing on foods. In "Microbiology of Frozen Foods", (ed.) Robinson, R. K. Elsevier Applied Science, New York.

Bossuyt, R. 1978. Usefulness of an ATP assay technique in evaluating the somatic cell content of milk. Milchwissenschaft 33:11.

Bossuyt, R. 1981. Determination of bacteriological quality of raw milk by an ATP assay technique. Milchwissebschaft 36:257.

Bossuyt, R. 1982. A 5-minute ATP platform test for judging the bacteriological quality of raw milk. Neth. Milk Dairy J. 36:355.

Bossuyt, R. 1982. Usefulness of an ATP assay technique in evaluating the bacteriological quality of raw milk. Milchwissenschaft 34:129.

Bossuyt, R. G. and Waes, G. M. 1983. Impedance Measurements to detect post-pasteurization contamination of pasteurized milk. J. Food Prot. 46(7): 622.

Britz, T. J., Bezuidenhout, J. J., Dreyer, J. M. and Steyn, P. L. 1980. Use of adenosine triphosphate as an indicator of the microbial counts in milk. South African J. Dairy Technol. 12:89.

Brown, M. H. and Baird-Parker, A. C. 1982. The microbiological examination of meat. In "Meat Microbiology", (ed.) Brown, M. H. p. 423. Applied Sci. Pub, New York

Bulleid, N. C. 1978. An improved method for the extraction of adenosine triphosphate from marine sediment and seawater. Limnol. Oceanogr. 23:174.

Campbell, R. J., Egam, A. F., Grau, F. H. and Shay, B. J. 1979. The growth of Microbacterium thermosphactum on beef. J. Appl. Bacteriol. 47:505.

Caslow, M., Ellnrr, P. D. and Kiehn, T. E. 1974. Comparison of the BACTEC system with blind subculture for the detection of bacteriuria. Appl. Microbiol. 28:435.

Chapman, A. G. and Atkinson, D. E. 1977. Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast. In "Advances in Microbial Phisiology, Vol. 15." (eds.) Rose, A. H. and Tempest, D. W. p. 253. Academic Press, New York, NY.

Chapman, A. G., Fall, L. and Atkinson, D. E. 1971. Adenylate energy charge in Escherichia coli during growth and starvation. J. Bact. 108:1072.

Chappelle, E. W. and Levin, G. V. 1968. Use of the firefly bioluminescent reaction for rapid detection and counting of bacteria. Biochem. Med. 2:41.

Charbonneau, R., Therrien, I. and Gagnon, M. 1975. Detection and measurement of bacterial catalase by the disc-flotation method using the catalasemeter. Can. J. Microbiol. 21:580.

Christian, R. R., Bancroft, K. and Wiebe, W. J. 1975. Distribution of microbial adenosine triphosphate in salt marsh sediments at Sapelo Island Georgia. Soil Sci. 119:89.

Cole, H. A., Wimpenny, J. W. T. and Hughes, D. E. 1967. The ATP pool of Escherichia coli: measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta 143:445.

Conn, R. B., Charache, P. and Chappelle, E. W. 1975. Limits of applicability of the firefly luminescence ATP assay for the detection of bacteria in clinical specimens. Am. J. Clin. Pathol. 63:493.

Curtis, G. D. W., Nichols, W. W. and Johnston, H. H. 1981. Studies on the effect of urine on the firefly luciferase screening technique for bacteriuria. In "Abstracts 3rd International Symposium on Rapid Methods and Automation in Microbiology" Washington: Amer. Soc. Microbiol.

Davies, R. and Obafemi, A. 1985. Response of microorganisms to freeze - thaw stress. In "Microbiology of Frozen Foods", (ed.) Robinson, R. K. p. 83. Elsevier Applied Acience, New York.

DeLuca, M. A. and McElroy, W. D., Eds. 1981. Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications. Academic Press, New York, NY.

DeLuca, M. 1976. Firefly luciferase. In, "Advances in Enzymology, Vol. 44", (ed.) Meister, A. p. 37. John Wiley and Sons, New York, NY.

DeLuca, M. A., Ed. 1978. Methods of Enzymology, Vol. LVII, Academic Press, New York, NY.

Deming, W. E. 1943. Statistical Adjustment of Data. Wiley, New York, NY.

Dodds, K.L., Holley, R.A. and Kempton, A.G. 1983. Evaluation of the catalase and Limulus amoebocyte lysate tests for rapid determination of microbial quality of vacuum-packed cooked turkey. Can. Inst. Food Sci. Technol. 16:167.

Doxtader, K. G. 1969. Estimation of microbial biomass in soil on the basis of adenosine triphosphate measurements. Bacterial. Proc. 69:14.

Firstenberg-Eden, R. 1983. Rapid estimation of the number of microorganisms in raw meat by impedance measurement. Food Technol. 37:64.

Gagnon, M., Hunting, W.M. and Esselen, W.B., 1959. New method for catalase determination. Anal. Chem. 31:144.

Galligan, P., LaRocco, K., Tsai, T. and Spurgash, A. 1984. A rapid bioluminescent screening method for determining yeast contamination in a carbonated beverage. Presented at 44th Ann. Meeting Inst. Food Technologists, Anaheim, CA, June 10-13.

Gebre-Egziabher, A., Thompson, B. and Blankenagel, G. 1982. Destruction of microorganisms during thawing of skim milk. J. Food Prot. 45:125.

Gill, C. O. 1983. Meat spoilage and evaluation of the potential storage life of fresh meat. J. Food Prot. 46:444.

Gill, C. O. and Newton, K. G. 1980. Growth of bacteria on meat at room temperatures. J. Appl. Bacteriol. 49:315.

Gnau, S. and Leudecke,L.O. 1982. Impedance measurements in raw milk as an alternative to the standard plate count. J. Food Protec. 45:4.

Gram, L. and Sogaard, H. 1985. Microcalorimetry as a rapid method for estimation of bacterial levels in ground meat. J. Food Prot. 48:341.

Graumlich, T. R. 1985. Estimation of microbial populations in orange juice by bioluminescence. J. Food Sci. 50:116.

Gritsavage, E. F. 1965. The cytochemical effect of freezing on Escherichia coli. PhD Thesis, Michigan State University. Cited in Davies and Obafemi, 1985.

Guinn, G. and Eidenbock, M. P. 1972. Extraction, purification and estimation of ATP from leaves, floral buds and immature fruits of cotton. Anal. Biochem. 50:89.

Gutekunst, R. R., Deming, J. W., Jaffe, B. D., Marshall, K., Picciolo, G. L., Chappelle, E. W. and Schrock, C. F. 1977. Luminescence assays for the determination of microbial levels. Clinical results. Detection of bacteria in urine and blood. In "2nd Biannual ATP - Methodology symposium: Proceedings", (ed.) Borun, G. A. p. 491. SAI Technology Co., San Diego, CA.

Haines, R. B. 1938. The effect of freezing bacteria. Roy. Soc. (London) Proc. B. 124:451.

Hamilton, R. D. and Holm-Hansen, O. 1967. Adenosine triphosphate content of marine bacteria. Limnol. Oceanogr. 12:319.

Hansen, K., Mikkelsen, T. and Moller-Madsen, A. 1982. Use of the Limulus test to determine the hygienic status of milk products as characterized by levels of Gram-negative bacterial lipopolysaccharide present. J. Dairy Research 49:323.

Hardy, D., Kraeger, S. J., Dufour, S. W. and Cady, P. 1977. Rapid detection of microbial contamination in frozen vegetables by automated impedance measurements. Appl. Environ. Microbiol. 34:14.

Herring, P.J., ed. 1978. Bioluminescence in Action. Academic Press, New York, NY.

Holm-Hansen, O. 1970. ATP levels in algal cells as influenced by environmental conditions. Plant Cell Physiol. 11:689.

Hysert, D. W., Kovecses, F. and Morrisson, N. M. 1976. A firefly bioluminescence ATP assay method for rapid detection and enumeration of brewery microorganisms. J. Am. Soc. Brew. Chem. 34:145.

Ingram, M. and Macky, B. m. 1976. In "Inhibition and Inactivation of Vegetative Microbes", (eds.) Skinner, F. A. and Hugo, W. B. Academic Press, London.

Issen, R. M. and Tsai, T. 1985. Rapid screening of somatic and bacterial cells in milk using fitration and bioluminescent techniques. Presented at 45th Ann. Meeting Inst. Food Technol., Atlanta, GA, June 9-12.

James, S.J. and Bailey, C. 1982. Institute of Refrigeration proceedings 78:33. Cited in Lowry and Gill, 1985.

Jay, J. M. 1977. The Limulus lysate endotoxin assay as a test of microbial quality of ground beef. J. Appl. Bacteriol. 43:99.

Jay, J. M., Margitic, S., Shereds, A. L. and Covington, H. V. 1979. Determining endotoxin content of ground beef by the Limulus amoebocyte lysate test as a rapid indicator of microbial quality. Appl. Environ. Microbiol. 38:885.

Johnston, H. H., Mitchell, C. J. and Curtis, G. D. W. 1976. An automated test for the detection of significant bacteriuria. Lancet ii:400.

Johnston, H. H., Mitchell, C. J. and Curtis, G. D. W. 1976. Automation in clinical microbiology; a system for urine specimens. In, "2nd International Symposium on Rapid Methods and Automation in Microbiology," (eds.) Johnston, H. H. and Newson, S. W. B. p. 210. Learned Information, NY.

Jul, M. 1984. The Quality of Frozen Foods. Academic Press, New York.

Kaneko, T., Yokoyama, H. and Takahashi, T. 1984. A rapid determination of microbial counts in raw milk and yoghurt by an ATP assay technique. J. Food Hyg. Soc. Japan 25:193.

Karl, D. M. 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbial Rev. 44:739.

Karl, D. M. and Bossard, P. 1985. Measurement and significance of ATP and adenine nucleotide pool turnover in microbial cells and environmental samples. J. Microbiolog. Meth. 3:125.

Karl, D. M. and LaRock, P. A. 1975. Adenosine triphosphate measurement in soil and marine sediments. J. Fish Res. Bd. Can. 32:599.

Kemp, C. W. 1979. Adenylate energy charge; a method for the determination of viable cell mass in dental plaque samples. J. Dent. Res. 68:2192.

Kennedy, J. E. and Oblinger, J. L. 1985. Application of bioluminescence to rapid determination of microbial levels in ground beef. J. Food Prot. 48:334.

Knowles, C. J. and Smith, L. 1970. Measurement of ATP levels of intact Azotobacter vinelandii under different conditions. Biochim. Biophys. Acta (Amst). 197:152.

Lampi, R. A., Mikelson, D. A., Rowley, D. B., Previte, J. J. and Wells, R. E. 1974. Radiometry and microcalorimetry - techniques for the rapid detection of foodborne microorganisms. Food Technol. Champaign 28:52.

LaRocco, K. A., Galligan, P., Littel, K. L. and Spurgash, A. 1985. A rapid bioluminescent ATP method for determining yeast contamination in a carbonated beverage. Food Technol. 39(7):49.

LaRocco, L. A., Littel, K. J. and Pierson, M. D. 1986. The bioluminescent ATP assay for determining the microbial quality of foods. In "Foodborne Microorganisms and Their Toxins: Developing Methodology". (eds.) Pierson, M. D. and Stern, N. J. p. 145. Marcel Dekker, New York.

Lee, C. C., Harris, R. F., Williams, J. D. H. and Armstrong, D. E. 1971. Adenosine triphosphate in lake sediments: I Determination. II Origin and significance. Soil Sci. Soc. Amer. Proc. 35:82.

Lee, Y., Jablonski, I. and DeLuca, M. 1977. Immobilization of firefly luciferase on glass rods: properties of the immobilized enzyme. Anal. Biochem. 80:496.

Lehtokari, M., Nikkola, P. and Paatero, J. 1983. Determination of ATP from compost using the firefly bioluminescence technique. Eur. J. Appl. Biotechnol. 17:187.

Levin, G. V., Clendenning, J. R., Chappelle, E. W. and Heim, A. H. 1964. A rapid method for detection of microorganisms by ATP assay. BioScience 14:37.

Levin, G. V., Schrot, J. R. and Hess, W. C. 1975. Methodology for application of adenosine triphosphate determination in wastewater treatment. Environ. Sci. Technol. 9:961.

Littel, K. J. and LaRocco, K. A. 1985. Bioluminescent standard curves for quantitative determination of yeast contaminants in carbonated beverages. J. Food Prot. 48:1022.

Lowry, P. D. and Gill, C. O. 1985. Microbiology of frozen meat and meat products. In "Microbiology of Frozen Foods", (ed.) Robinson, R. K. p. 109. Elsivier Applied Science, London.

Lundin, A. and Thore, A. 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. 30:713.

Mackett, D., Kessock-Phillip, D., Bascomb, S. and Easmon, C. S. F. 1982. Evaluation of Lumac test for the detection of bacteruiria by bioluminescence. J. Clin. Pathol. 35:107.

Martins, S.B., Hodapp, S., Dufour, S.W. and Kraeger, S.J. 1982. Evaluation of a rapid impedimetric method for determining the keeping quality of milk. J. Food Protec. 45:1221.

McElroy, W.D. 1947. The energy source for bioluminescence in an isolated system. Proc. Natl. Acad. Sci. USA. 33:342.

McElroy, W. D. And DeLuca, M. A. 1983. Firefly and bacterial luminescnce: basic science and applications. J. Appl. Biochem. 5:197.

McElroy, W. D., Seliger, H. H. and White, E. H. 1969. Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin. Photochem. Photobiol. 10:153.

McMeekin, T. A. 1982. Microbial Spoilage of meats. In "Developments in Food Microbiology - 1", (ed.) Davies, R. p. 1. Applied Sci. Pub., New York.

Molin, O. M., Nilsson, L. and Ansehn, S. 1983. Rapid detection of bacterial growth in blood cultures by bioluminescent assay of bacterial ATP. J. Clin. Microbiol. 18:521.

Moss, C. W. and Speck, M. L. 1966a. Identification of nutritional components in trpticase responsible for recovery of Escherichia coli injured by freezing. J. Bacteriol. 91:1098.

Moss, C. W. and Speck, M. L. 1966b. Release of biological active peptides from Escherichia coli at sub-zero temperatures. J. Bacteriol. 91:1105.

Newton, K. G. and Rigg, W. J. 1979. The effect of film permeability of the storage life and microbiology of vacuum - packed meat. J. Appl. Bacteriol. 47:433.

Ng, L.-K., Taylor, D. E. and Stiles, M. E. 1985. Estimation of Campylobacter spp. in broth culture by bioluminescence assay of ATP. Appl. Environ. Microbiol. 49:730.

Patel, P. D. and Williams, A. P. 1983. Estimation of food spoilage yeasts by measurement of adenosine triphosphate (ATP) after growth at various temperatures. British Food Manufacturing Ind. Res. Assoc., Res. Rep. 419 Leatherhead, Surrey, England.

Patel, P. D. and Wood, J. M. 1983. Separation of microorganisms from raw meats and their rapid estimation by measurement of microbial ATP. British Food Manufacturing Ind. Res. Assoc., Res. Rep. 419, Leatherhead, Surrey, England.

Paul, E. A. and Johnson, R. L. 1977. Microscopic counting and adenosine 5'-triphosphate measurement in determining microbial growth in soils. Appl. Environ. Microbiol. 34:263.

Peeler, J. T., Messer, J. W., Leslie, J. E. and Houghtby, G. A. 1980. Variation in food microbiological tests used to evaluate analyst performance. J. Food Prot. 43:729.

Pettifer, G.L., Fulford, R.J. and Mabbitt, L.A. 1983. Collaborative trial of the direct epifluorescent filter technique. J. Appl. Bacteriol. 54177.

Pettifer, G.L. and Rodriguez, U.M. 1981. Rapid enumeration of bacteria in heat-treated milk and milk products using a membrane filtration-epifluorescent microscopy technique. J. Appl. Bacteriol. 50:157.

Phillips, J. D. and Griffiths, M. W. 1985. Bioluminescence and impedimetric methods for assessing shelf-life of pasteurized milk and cream. Food Microbiology 2:39.

Picciolo, G. L., Thomas, R. R., Chappelle, E. W., Taylor, R. E., Jeffers, E. J. and McGarry, M. A. 1976. Automated determination of microbial levels for wastewater effluent using luminescent assay. In "2nd International Symposium of Rapid Methods and Automation in Microbiology", (eds.) Johnston, H. H. and Newsom, S. W. B. p. 294. Learned Information, New York, NY.

Pierson, M.D., Collins-Thompson, D.L. and Ordal, Z.J. 1970. Microbiological, sensory and pigment changes of aeobically and anaerobically packaged beef. Food Technol. 24:129.

Rodriguez, U.M. and Pettipher,G.L. 1984. Use of the direct epifluorescent filter technique for predicting the keeping quality of pasteurized milk within 24 hours. J. Appl. Bacteriol. 57:125.

Rowley, D.B., Vandemark, P., Johnson, D. and Shattuck, E. 1979. Resuscitation of stressed fecal coliforms and their subsequent detection by radiometric and impedimetric techniques. J. Food Protec. 42:335.

Schrock, C. G., Barza, M. J., Deming, J. W., Picciolo, G. L., Chappelle, E. W. and Weinstein, L. 1976. Rapid detection of bacterial growth in blood cultures by means of adenosine triphosphate determination. Abstr. Ann. Meet. Am. Soc. Microbiol. p. 37.

Seitz, W.R. and Neary, M.P. 1976. Recent advances in bioluminescence and chemiluminescence assay. In, "Methods of Biochemical Analysis, Vol.23"., (ed.) Glick, D. p.161., Interscience, New York, NY.

Sharpe, A. N., Woodrow, M. N. and Jackson,, A. K. 1970. Adenosine triphosphate (ATP) levels in foods contaminated by bacteria. J. Appl. Bacteriol. 33:758.

Shimomura, O. 1982. Mechanism of bioluminescence. In, "Chemical and Biological Generation of Excited States" p.249., Academic Press, New York, NY.

Sharpe, A.N. 1979. An alternative approach to food microbiology for the future. Food Technol. 33:71.

Sorrells, K.M. 1981. Rapid detection of bacteria content in cereal grain products by automated impedance measurements. J. Food Protec. 44:832.

Stannard, C. J. and Smith, J. 1982. Factory trial of the ATP assay method for estimation of microbial numbers on meat. British Food Manufacturing Ind. Res. Assoc., Tech. Circular 773, Leatherhead, Surrey, England.

Stannard, C. J. and Wood, J. M. 1982. The estimation of viable bacteria in raw meat by measurement of microbial ATP. British Food Manufacturing Res. Assoc., Res. Rep. 379, Leatherhead, Surrey, England.

Stannard, C. J. and Wood, J. M. 1983. Rapid estimation of yeast in fruit juices by ATP measurement. British Food Manufacturing Ind. Res. Assoc., Res. Rep. 379, Leatherhead, Surrey, England.

Stannard, C. J. and Wood, J. M. 1983. The rapid estimation of microbial contamination of raw meat by measurement of adenosine triphosphate (ATP). J. Appl. Bacteriol. 55:429.

Stewart, B.J., Eyles, M.J. and Murrell, W.G. 1980. Rapid radiometric method for detection of Salmonella in foods. Appl. Environ. Microbiol. 40:223.

Strange, R. E., Wade, H. E. and Dark, F. A. 1963. Effect of starvation on adenosine triphosphate concntration in Aerobacter aerogenes. Nature (London) 199:55.

Strehler, B. L. 1968. Bioluminescnce assay: principles and practice. In "Methods of Biochemical Analysis, Vol. 16", (ed.) Glick, D. p. 99. Interscience Publishers, New York, NY.

Theron, D. P., Prior, B. A. and Lategan, P. M. 1983. The effect of temperature and media on adenosine triphosphate cell content in Enterobacter aerogenes. J. Food Prot. 46:196.

Theron, D. P., Prior, B. A. and Lategan, P. M. 1986. Determination of bacterial ATP levels in raw milk: selectivity of non-bacterial ATP hydrolysis. J. Food Prot. 49:4.

Theron, D. P., Prior, B. A. and Lategan, P. M. 1986. Sensitivity and precision of bioluminescent techniques for enumeration of bacteria in skim milk. J. Food Prot. 49:8.

Thore, A., Ansehn, S., Lundin, A. and Bergman, S. 1975. Detection of bacteriuria by luciferase assay of adenosine triphosphate. J. Clin. Microbiol. 1:1.

Thore, A., Lundin, A. and Ansehn, S. 1983. Firefly luciferase ATP assay as a screening method for bacteriuria. J. Clin. Microbiol. 17:218.

Vanstaen, H. 1980. Applicability of bioluminescence for rapid detection of viable microorganisms. Lab. Pract., Dec., p. 1281.

Waes, G. and Bossuyt, R. 1981. A rapid method to detect post-contamination in pasteurized milk. Milchwissenschaft 36:548.

Waes, G., Bossuyt, R. and Mottar, J. 1984. A rapid method for the detection of non-sterile UHT milk by the determination of the bacterial ATP. Milshwissenschaft 39:707.

Waes, G. M. and Bossuyt, R. G. 1982. Usefulness of the benzalkon-crystal violet-ATP method for predicting the keeping quality of pasteurized milk. J. Food Prot. 45:928.

Wakkers, P. J. M., Hellendoorn, H. B. A., OpDeWeegh, G. J. and Heerspink, W. 1975. Applications of statistics in clinical chemistry. A critical evaluation of regression lines. Clinica Chimica Acta 64:173.

Wang, G.I.J. and Fung, D.Y.C. 1986. Feasibility of using catalase activity as an index of microbial loads on chicken surfaces. J. Food Sci. 51:1442.

Warseck, M., Ray, B. and Speck, M. L. 1973. Repair and enumeration of injured coliforms in frozen foods. Appl. Microbiol. 26:919.

Williams, M. L. B. 1971. The limitations of the DuPont Luminescence Biometer in the microbiological analysis of foods. Can. Inst. Food Technol. J. 4:187.

Wood, J. M. and Gibbs, P. A. 1982. New developments in the rapid estimation of microbial populations in foods. In, "Developments in Food Microbiology - 1", (ed.) Davies, R. p. 183. Applied Science Pub., London.

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