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Detection of Abnormal Milk with Impedance Microbiology Instrumentation

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DETECTION OF ABNORMAL MILK WITH IMPEDANCE MICROBIOLOGY INSTRUMENTATION

by

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Fahad Ali Khayat

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ABSTRACT

Detection of Abnormal Milk with Impedance Microbiology Instrumentation

by

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Utah State University, 1986

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Research Professor: Dr. Gary H. Richardson
Department: Biology (Microbiology)

Mastitic milk was detected by obtaining conductance measurements using an impedance microbiology Bactometer® 120 SC instruments. Conductance readings taken after 30 min at 25°C separated normal and abnormal milks when readings differed by more than 3% from the variance among instrument module wells. Samples blended from four quarters of a cow indicated milk from one quarter was abnormal if the salt level in the abnormal quarter raised the blend conductivity above that of normal samples and variance among the wells. Either solid or liquid substrates that contained bacterial stimulants could be used to accelerate bacterial acid production or to reduce impedance detection times, each without adversely affecting the ability to detect abnormal milk.

However, measurements with liquid substrates varied with the volume of sample in the well. Results suggested that a fixed volume of one ml be used. Such a volume would allow simultaneous detection of abnormal milk and bacterial load on the same sample.

(62 pages)

INTRODUCTION

Impedance microbiology can be used to estimate the total microflora (13) and inhibitory properties, including bacteriophage (31) or anitbiotics (25), in raw milk. Differences in electrical conductivity have been reported when milk was mastitic, as determined with direct current instrumentation (8,10,24,32). The objective of this study was to evaluate the ability of this methodology to simultaneously differentiate between milks with different conductivities while measuring the total microflora of raw milk. This could increase the value of the instrument and reduce the cost of running the sample.

REVIEW OF LITERATURE

ABNORMAL MILK

Conditions of Abnormality

Abnormal milk is a term applied to three conditions which will change the standard composition of milk. Although the term is arbitrary and vague, "abnormality" occurs when:

Colostrum is Found. Colostrum in the milk represents the first secretion of milk after parturition and it is distinguished by a very high serum protein content often with some blood present. This condition is normal for the cow but not accepted as normal milk for the dairy industry.

Contaminants Enter the Milk. This occurs during or after milking and includes almost anything, e.g. water, pesticides, metal ions, plasticizers, disinfectants, and dust.

Milk is Taken from a Diseased Udder. This is mastitic milk. Its composition in severe conditions may be more like that of blood serum. It contains many leucocytes, more somatic cells, and has several other aberrations (32).

Somatic Cells

The term somatic cells describes those body cells present in milk. The somatic cells include lymphocytes, neutrophils, and epithelial cells. The first two are blood cells and the third is produced by the mammary gland. If the somatic cell count exceeds 500,000/ml, it is considered indicative of mastitis (2,32). It is recognized that other factors can affect somatic cell counts, such as the individual cow, the stage of their lactation, the age of the cow, and the mastitis history of the individual cow (32), also

increased somatic cell counts in response to udder trauma such as stepped-on teat in the absence of infectious agents, can give misleading results about microbial infections (22). In normal milk, somatic cells consist of 80% epithelial cells and nine percent neutrophils. In mastitic milk they respectively consist of 47% and 47% with subclinical mastitis, and 26% and 72% with clinical mastitis. The number of lymphocytes change only slightly in different milk conditions. The shift in the number of neutrophils is needed physiologically to destroy invading bacterial cells, foreign proteins, and tissue debris in the areas of tissue inflammation. If milk from a normal, healthy cow was tested for enzymatic activity, it would show the presence of a number of enzymes which are believed to be derived from the secretory epithelial cells of the mammary gland. Their presence may be regarded as a "spilling over" from cells and serum during the milk secretion process (22). This results in an increased number of neutrophils in milk, since they have lipase, phospholipase, and proteinase enzymes which are active in destroying bacteria. These same enzymes also degrade casein which means an economic loss to cheesemakers (2).

Bacteria Involved

The most important source of abnormal milk is the invasion of the udder by microorganisms. Although "the milk inside a healthy udder is sterile" (32, p. 9), some microorganisms inevitably gain entrance (1), even with proper milking and handling.

The predominant types of microorganisms responsible for udder infection are Gram negative bacteria including coliforms, Pseudomonas aeruginosa, Salmonella species, and Klebsiella species. (1, 32). Streptococcus species, also may be involved. Corynebacterium species also have been implicated (1, 24).

Mastitis

Mastitis results from the entrance of pathogenic bacteria into the udder, and causes a change in milk composition with attendant decrease in product yield in cheese making. Mastitic milk contains a wider range and higher concentration of several enzymes than normal milk due to leakage from the basal membrane of secretory cells. Plasmin and other enzymes are spilled over from blood to milk. Plasmins degrade casein and will lead to a decrease in cheese yield. Plasmin in not destroyed by pasteurization (2, 22).

> The result of high somatic cell counts is to decrease the casein content of milk which will result in a change in the casein to fat ratio. Decreased casein is the result of proteolytic damage to milk casein which results in a loss of the enzymatically damaged casein to the whey. A lower amount of casein for curd formation may also lead to higher fat loss in whey (2, p.18).

The relation between somatic cell count and cheese yield is inversely proportional (2). If the somatic cell count is increased, the milk protein composition becomes more like the composition of blood serum. Mastitic milk contains as much as ten times more serum albumin and immunoglobulin as normal milk, but only about half the amount of alpha-lactalbumin and beta-lactoglobulin. Spontaneous lipolysis seems to increase at first with cell count increase but decreases again at very high counts. Hydrogen ion activitiy decreases and the pH of mastitic milk increases about 0.3 units above normal milk pH. The proportion of short-chain fatty acid residues is somewhat higher in mastitic milk, which changes fat compostion (32).

The enzyme catalase can be highly indicative of mastitic milk. Normal milk contains a small amount of catalase present in the leucocytes. However, if the udder is infected, the activity of milk catalase will increase twenty-fold due to the large increase in numbers of

leucocytes, body cells, blood and bacteria, especially staphylococci and aerobic spore formers (20). Alkaline phosphates may show an increase of five-fold in abnormal milk (2). Proper pasteurization is determined by the absence of this enzyme (1).

DETECTION OF MASTITIC MILK

Nature of Mastitis

Since mastitis is an infectious process, the best criterion for detection mastitis is the presence or abundance of udder pathogens in the milk, which requires determining the number of bacteria and types of bacteria involved in the infection. The previous approach is difficult and time consuming, and for this reason there is a great demand for quick and easy diagnostic tests. Mostly, somatic cell counts are used to determine the abnormality of milk, but (as discussed in the previous section) this method is somewhat ambiguous (24, 28). Other indirect tests, including tests for milk components, increased as a result of the infection or damage (e.g. DNA, catalase), turn out to be less accurate than somatic cell counts in determining abnormality of milk. It has been observed that milk from infected or inflamed mammary glands contains a higher salt concentration than does normal milk. In abnormal milk, lactose production is impaired (24, 32). Osmotic pressure is maintained by the transfer of salts from blood serum. This produces and increase in electrical conductivity which makes a better indicator of abnormal milk than high somatic cell counts (32), because it would detect the product of mechanical trauma such as milking machine malfunction.

Screening Tests

Screening tests for determining the presence of somatic cell counts in milk include:

California Mastitis Test. (CMT) This cowside method requires a neutral detergent and pH indicator. The detergent destroys the somatic cell walls and releases the

deoxyribonucleic acid (DNA) filaments that usuallly increase the sample viscosity within fifteen seconds of mixing. In addition, a color pH indicator, bromocresol purple, is included to intensify the appearance of the viscous mass for more accurate estimation of the results and better interpretation of the test. The test also gives a blue color for mastitic milk and a yellow-green color for normal milk. The reaction is scored according to the thickness of the gel formation. The range of somatic cell counts by this method is between 150,000 and 5,000,000/ml. the advantages of this test are rapidity, simplicity, low cost, and convenience (27).

Wisconsin Mastitis Test. (WMT) This is a laboratory procedure that employs the same reagents as the CMT and measures viscosity by the volume of flow through a standard orifice in a fixed time. It requires specific test equipment and conditions for measuring the viscosity (27).

Rolling Ball Viscometer. (RBV) This instrument was fabricated in New Zealand. It measures the viscosity of the gel (formed due to DNA released from somatic cells with destroyed walls) in milk samples and provides an direct measure of the level of somatic cells present. The RBV consists of a tube mounted horizontally so that a milk sample with detergent added can be passed through. The tube is then inclined, allowing a stainless steel ball to roll through the mixture. The distance the ball rolls in a fixed time is inversely related to the viscosity developed by the release of DNA from the somatic cells (27).

Modified Whiteside Test. (MWT) This test measures the viscous mass of DNA released from somatic cells when milk is mixed with four percent NaOH. The viscosity and amount of precipitate relates to the number of somatic cells in the milk. This method can be applied to individual quarter and/or blend milk samples. The milk sample is graded by the amount of precipitate formed. This procedure is simple, fast, and inexpensive (27).

Conductivity Measurements. The conductivity of milk, as with any other aqueous fluid, is a function of the electrolytes dissolved in it. The chief constituents responsible for conductivity in milk are the chloride, sodium and potassium ions. In abnoraml milk the Na and Cl ions are the most important and are responsible for an increase from 49 to 78% of total conductivity (8). Milk and blood have the same osmolality. When inflamed mammary glands decrease production of lactose, salt is secreted from the blood to the milk to maintain the same osmolality. Since salt is conductive, but lactose is not, conductivity is applicable to the detection of abnormal milk (10, 24).

Insoluble particles of an emulsion reduce the conductivity of milk by interposing a physical barrier to the ions. Thus, if fat is removed from milk the conductivity will rise around 11%. Jersey milk, being high in fat, has a lower conductivity than Friesian or Holstein milk, other factors being equal (8).

The hand held MAS-D-TEC™ conductivity meter (Fig. 4) is used for cowside measurement of milk abnormality with a 0-9 scale. Any sample above a reading of four indicates abnormal milk (24).

Confirmatory Tests

Confirmatory tests provide more precise counts of somatic cells present. These include:

Direct Microscopic Somatic Cell Count. (DMSCC) A 0.01 ml sample is applied to a slide, dried, heat fixed, stained, dried, and each somatic cell with an identifiable stained nuclus is counted. The advantages of DMSCC include: 1. the ease of preparing permanent milk films, and 2. the ability to recognize bacterial types. The disadvantages of the method include: 1. the high cost of equipment, 2. the potential for a high percntage of error

(introduced if poor techniques are used, since only 0.01 ml is observed), and 3. slowness if many samples are invloved (27).

Electronic Somatic Cell Counter. (ESCC) This instrument technique requires the removal of fat globules by centrifugation or dispersion of fat by chemical methods. Two methods are in use: 1. a semi-automated procedure (ESCC), and 2. an automated procedure (Milk Cell Counter). The advantages of both procedures are: 1. minimal analysis time. 2. ability to store samples after fixing. 3. reliability, and 4. objectivity (since the operator does not have to decide what to count as required in the microscope procedure). The disadvantages of this method are: 1. higher cost of instrumentation. 2. calibration, and 3. standardization (27).

Flourescent Dye (Fossomatic). Preheated fresh or preserved milk samples are mixed with heated buffer and dye (ethidium bromide) solution and stirred. A portion is transferred to a rotating disk that serves as an object plane for a microscope. The dye combines with the DNA of somatic cell nuclei. A xenon arc lamp excites the nuclei-dye complex (at 550 nm) to emit fluorescent light (at 750 nm). Each nucleus that emits energy will be sensed by a photomultiplier and measured as an electrical pulse. The advantages of this method are: 1. the ability to test fresh or perserved samples, 2. fast, and 3. reliable. The disadvantages of this method include: 1. the high cost of instrumentation, and 2. the need for constant standardization during testing (27, 34).

Instrumental Microbiology

Three basic methods have been described for microbial analysis instrumentation, these are:

Direct Counting of Microbes. The direct counting is based upon measuring electrical

resistance between electrodes placed across a fine orifice through which a fluid containing particles flows. The Coulter Counter, Laser Beam, and Flow Microfluorometer instruments function on this principle. They have been used successfully to recognize and enumerate microorganisms in urine samples within a few minutes (6, 23).

Measurement of End Products. The principle of end product measurement relies upon detecting a specific type of product that results from growth; extracellular metabolites, cell wall, cell wall constituents, or a unique cell content. For example measuring the head space gas, as in a headspace analyzer, which detects dimethyl sulfide under specific conditions, and indicates the presence of Proteus mirabilis (7).

Measurement of Growth. The third method is growth measurement. Four types of instruments that use this principle are described with limitations: 1. Microcalorimetry measures a minute amount of heat liberated by metabolizing microorganism. However, Forrest (16, p. 287) comments "This constitutes a powerful potential advantage needing further development before it could be of use to the food microbiologist". 2. Radiometry measures the production of radioactive metabolites. Usually radioactive carbon dioxide is produced as microorganisms consume labeled substrates. This method is mainly used for detection of a very low level of microorganism in blood cultures in the clinical laboratory (9). 3. Turbidity (light absorbance) and light scattering are the most direct and familiar measurements. Because this requires optical clarity, turbidity is inappropritate for many food applications. 4. Impedance measurements are based upon the determination of resistance changes in the flow of alternating current through a conducting medium when the organic and inorganic composition of the growth medium is changed by microbial metabolism (7).

It is the last application, impedance, which was selected to be used in this research.

Impedance, Conductance, and Capacitance

Principle. Impedance is defined as the opposition to the flow of alternating current through a conducting material such as a bactriological growth medium. Microorganisms increase in number and produce more small, highly charged end products, as they metabolize medium constituents. Microorganisms break down complex, less charged molecules (carbohydrates or lipids) to more ionically charged units (lactic acid, acetic acid, or bicarbonate). Charged molecules, polypeptides and proteins are converted to amino acids. Further microbial metabolism will convert amino acids to ammonia and bicarbonate (14). Microoorganisms increase the conductivity of the supporting medium as they grow, by producing pairs from neutral molecules. Reducing the size also increases the mobility of charged molecules. In other words, the dielectric constant increases as existing dipoles become smaller. New dipoles are formed and inducible dipoles are created. Both decrease the overall impedance of the medium as conductivity and capacitance increase (7).

To comprehend the principles of impedance, understanding the rule of the resistance and capacitance in an electrical field is essential. "An analogy of impedance measurement is when a kink occurs in a garden hose which impedes the flow of water, thereby, producing a pressure drop and converting mechanical energy to heat" (14, p. 8).

Derivation of Impedance Equation. In an electrical field, the flow of electrical current (I) will always encounter some resistance (R) which results in a voltage drop (V) and the conversion of electrical energy to heat. Resistance is an energy consuming element obeying Ohm's law (14).

$$V = R \cdot I \qquad R = \frac{V}{I}$$

Conductance (G) is determined by the concentration of mobile ions in the fluid, and is the reciprocal of the resistance to a flow of electrical current through a solution. Solutions of a minimal resistance have a high conductance, and vice versa. Conductance is measured in reciprocal ohms (mhos) or Siemen units or in Bactomatic Inc. parlance "G" (18).

$$G = \frac{1}{R} \qquad G = \frac{I}{V}$$

In an electrical field imposed on an electrolyte solution, ions tend to migrate according to charge. The anions move toward the anode (postitive electrode), and the cations move toward the cathode (negative electrode). The current in solution is due to migration of ions and each ion carries a fraction of the current proportional to its mobility and concentration.

The function of capacitance in an electrical field is to store energy and not dissipate it. A capacitance consists of two conducting surfaces or electrodes seperated by a dielectric material. This prevents current flow when the applied voltage is constant or direct current (DC), but a time-varying voltage or alternating current (AC) produces current proportional to the rate of voltage change (14).

$$\text{Flow of Electrical Current} \quad (I) = C \frac{dV}{dt}$$

where (t) is time and (C) is considered the proportionality constant (capacitance), it is measured in farad or microfarad unit ($1\ \mu F = 10^{-6} F$).

"When two metal electrodes are immersed in a conductive medium, each electrode-solution interface can be represented by a series combination of a capacitor and a resistor" (33, p. 10). If two electrodes are immersed in a conductive solution and alternating sinusoidal potential is applied to the system, the resulting current will depend upon the impedance (Z) of the system. Impedance is a function of resistance (R), capacitance (C), and the applied frequency (f) (cycles/second or Hertz 'Hz'): Note: R = 1/G

$$Z = \sqrt{R^2 + \left(\frac{1}{2\pi f C}\right)^2} = \sqrt{\left(\frac{1}{G}\right)^2 + \left(\frac{1}{2\pi f C}\right)^2}$$

The equation illutstrates that any increase in conductivity and/or capacitance will lead to a decrease in impedance. In other words, conductance or capacitance is inversely proportional to impedance. Bacterial growthis proportional to conductivity and capacitance, and is inversely proportional to impedance. As microorganisms grow, impedance decreases (14).

How Bacterial Growth Changes Impedance. In a conductive solution with two electrodes, changes in conductance are associated with changes in the solution or in the bulk electrolyte. As microorganisms grow, they produce highly charged, end products. For example, one molecule of uncharged glucose is converted to two molecules of negatively charged lactic acid. This increases the conductivity and capacitance of the growth medium and decreases the impedance (30).

The increased response of impedance measurements at low frequencies (100 - 1000 Hz) was a general finding independent of bacterial species, electrode material or electrode configuration. At 100 Hz the impedance signal was almost purely capacitance (C) while at

10,000 Hz it was almost entirely conductance (G). When weakly conductive media (e.g. Nurient Broth or Plate Count Broth) are employed, bacterial metabolism results in clearly detectable changes in the conductance (G) component at 10,000 Hz. A different situation was observed with yeast growth in carbon base medium with ammonium sulfate. Although yeast produced marked changes in the C signal a t 100 Hz, the changes in G were minimal (14). Therefore, selection of a signal (Z, G, C) for monitoring impedance changes in a given product/medium combination should be checked in order to obtain the appropriate results.

Electrical Double Layer Behavior at Interface. The electrolyte near the electrode does not have the same environment as in the bulk solution because ions are subject to attractive forces from the electrodes and repulsive forces from the solution, and vice versa. For example, negatively charged ions are attracted to positive electrodes and repulsed by negatively charged substances in the solution. Consequently, the electrodes develop a certain electrical field structure and the bulk solution develops another with an interface between the electrodes. Molecules and ions form a structure which is a compromise (balance) between the structures dictated by both phases. This interface region is electrically neutral with a potential difference across the interface. The arrangement of charges and oriented dipoles in the interface region of the boundary electrolyte is called the electrical double layer (14).

Figure: 1 The relationship between bacterial growth and impedance curves (Adapted from 14).

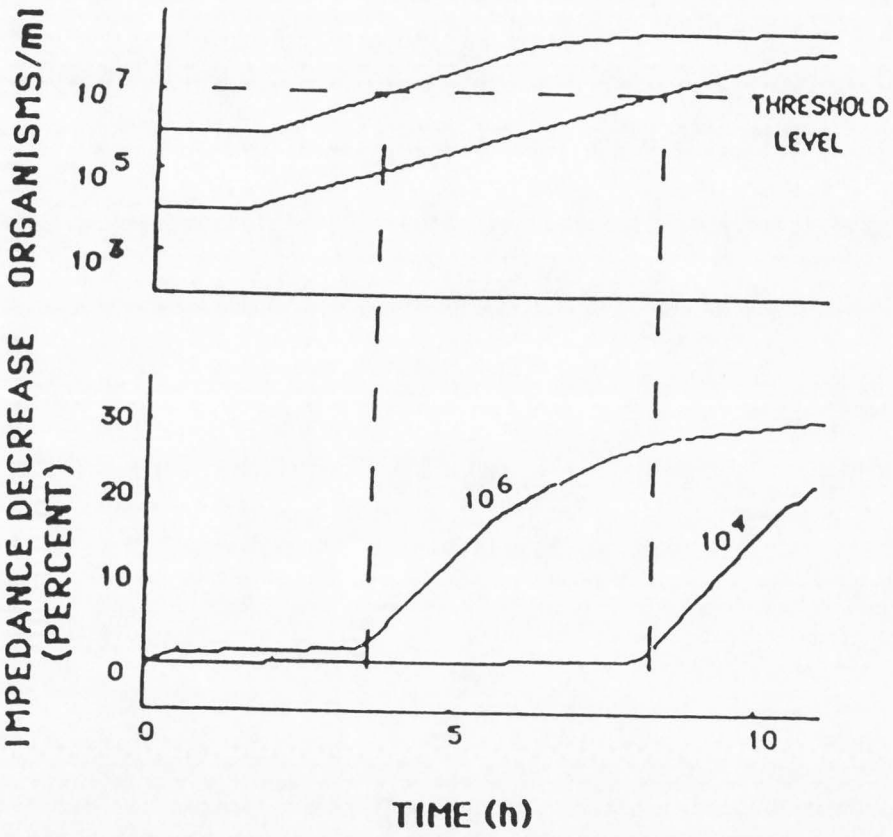
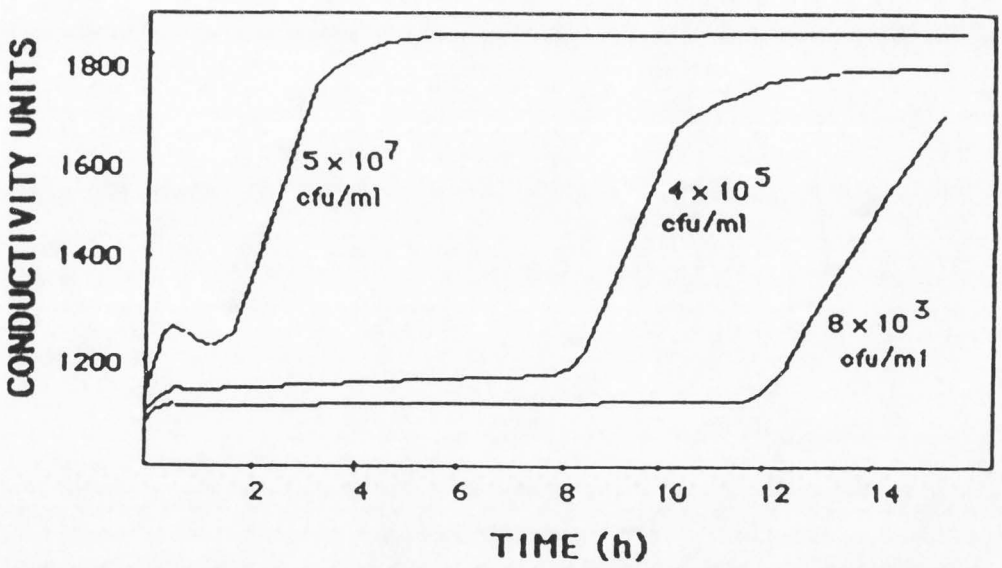

ORGANISMS/ml
10^7
10^5
10^3
THRESHOLD LEVEL
IMPEDANCE DECREASE (PERCENT)
30
20
10
0
10^6
10^4
0
5
10
TIME (h)

Figure: 2 The effect of bacterial contamination level on impedance curves with time (Adapted from (14)).


CONDUCTIVITY UNITS
1800
1600
1400
1200
5 x 10^7 cfu/ml
4 x 10^5 cfu/ml
8 x 10^3 ofu/ml
2
4
6
8
10
12
14
TIME (h)

Impedance Detection Time

Definition. To explain the principle of detection time, one must compare the bacterial growth curve representing two culture inoculum concentrations (10^4/ml, and 10^6/ml) with impedance curve representing both of the inocula (Fig. 1). The threshold level is defined as the point when the bacterial concentration reaches 10^7/ml. In impedance instrumentation the threshold level is equivalent to a point in the log phase of the bacterial growth curve. In Figure 1, the 10^6/ml inoculum growth curve reaches the threshold level in less time than does the 10^4/ml inculum curve. The time required for a culture concentration to reach the threshold level is called Impedance Detection Time (IDT) (7, 14).

Factors Affecting IDT. These are:

1. Concentration of the Microorganisms. In naturally contaminated meat, samples were taken at intervals (Fig. 2), plated for total count, and incubated in the Bactometer® (Impedance Monitoring System). IDT occurred later in samples containing the smaller number of bacteria than in those with higher levels.

2. Generation Time. In order for impedance to give a constant detection time, the microorganisms must have a constant generation time (tg). If the same concentration of microorganisms (10^3/ml) were used, but differed in generation time, the curves of the cultures with shorter generation times will reach the the threshold level in less time than the cultures with longer generation time.

3. Electrode Type. The IDT will differ in the same microorganism if electrodes of different composition are used because the electrodes have different conductance or capacitance sensitivities. Also, location of electrode in the well affects the detection time. Firstenberg-Eden and Tricarico (13) reported electrode location of the bottom of the

test will will give a culture concentration one log cycle lower than with the same electrode located at the top of the test will touching the surface of the solution (13).

4. Concentration of Growth Medium. In a dilute medium (low initial ionic strength) a change in conductance due to microbial metabolism will be relatively large as compared to a concentrated medium. Lower media concentrations may result in slower growth and result in a large variation among replicates.

5. Temperature. The two impedance components (capacitance and conductance) are extremely temperature sensitive. A temperature increase of $1^{0}C$ is reported to result in an average increase of 0.9% in capacitance and 1.8% in conductance (15). Generation time is also temperature dependent, therefore, the temperature must be constant to obtain repeatable results with the same microoorganism.

Application of Impedance Measurements. Impedance measurement has been applied in fundamental microbiology for detection of microbial metabolism and growth (18), and in medical microbiology for detection of bacterial growth in blood samples and detection of bacteriuria (29, 30). In food microbiology, impedance measurement has been used: 1. to detect microbial contamination in frozen vegetables (19); 2. to predict shelf life stability in the fruit juice industry (personal communication with Bactomatic Inc.'s vice-president); 3. to rapidly estimate the number of microorganisms in raw meat (11). In dairy science, it has been evaluated in several areas: 1. detection of post pasteurization contamination of milk samples by Gram-negative bacteria in samples less 24 h (5, 7); 2. estimation of microbial levels in fluid milk (which correlates to Standard Plate Counts) (12); 3. detection of the failure of starter cultures due to bacteriophage problems in manufacturing of Cheddar Cheese (31); 4. rapid determination of the potential shelf-life of pasteurized

dairy products (3); 5. estimation of potential shelf-life of cottage cheese utilizing bacterial numbers and metabolites (4); 6. detection fo antibiotics in milk (25); 7. estimation of lactic culture activity (26); and 8. indication of abnormal milk through the conductivity changes associated with increased salt levels (21). It appears capable of quantitating lipolytic and proteolytic activity of enzyme preparations (Poore, D.E. and G.H. Richardson, personal communications).

METHODS AND PROCEDURES

MILK SAMPLES

Raw milk samples were collected from the Utah State University Holstein herd. Approximately 60 ml of unpreserved foremilk samples from individual quarters were aseptically obtained, stored in Whirlpak bags (NASCO, Ft. Atkinson, WI), chilled in ice, and tested within 24 h.

INSTRUMENTATION

A Bactometer® model 120 SC (Fig. 4) and disposable 16 well-modules were provided by Bactomatic Inc. (PO Box 3103, Princeton, NJ 08540). A module is a rectanguler plastic sample holder designed for use with the Bactometer®. Each module contains 16 individual sample wells arranged in two separate rows. Each sample well contains a pair of small stainless steel electrodes attached to a lead frame molded into the module's plastic base. Sample suspensions are pipetted into appropriate test wells which are then covered with sterile plastic caps. Filled modules are loaded into the the Bactometer® incubator by inserting the special metal connecting edge into the appropriate slot to complete the electrical connection (14). The instrument module wells were filled with liquid or solid samples using previously described procedures (13, 17, 25, 27) and in varied volumes as

explained in Results and Discussion section. Incubators were set at 25°C. The instrument readout was plotted simultaneously as Impedance Detection Time (IDT) and conductance (G) or the reciprocal of impedance (I).

A Mas-D-Tec™ conductivity meter (Fig. 5) (Wescor Inc. 459 S. Main, Logan, UT 84321) and a Fossomatic M1215 somatic cell counter (Dickey-John Corp. PO Box 10, Auburn, IL 62615) also were used (34). Standard NaCl solutions used to calibrate the Mas-D-Tec™ conductivity meter and vapor pressure osmometers (27) (provided by Wescor Inc.) Standardized solutions of somatic cells (SCCS, W.L. Henning Bldg. Penn. State Univ., University Park, PA 16802) were used to calibrate the Fossomatic.

MEDIA

Antibiotic-free, low heat nonfat dry milk was reconstituted (RNDM) at 10% (w/v) in deionized water and sterilized at 121°C for 15 min.

Yeast extract-fortified RNDM (YNDM) was prepared by adding 1 ml of sterilized 2% yeast extract (AYE-Light™ Busch Industrial Products Inc., 10877 Watson rd. St. Louis, MO 63127) solution into a module well immediately after adding 1 ml of milk sample (17).

Standard Methods Agar (SMA) and modified SMA (MSMA) were prepared as described in Standard Methods (27). A broth medium (MSMB) was made according to the formula for MSMA but without agar.

Figure 3: The Bactometer® model 120SC

A = Printer
B = Computer processing unit (CPU)
C = Plastic disposable module
D = Bactometer processing unit (BPU)
E = Video screen/key board

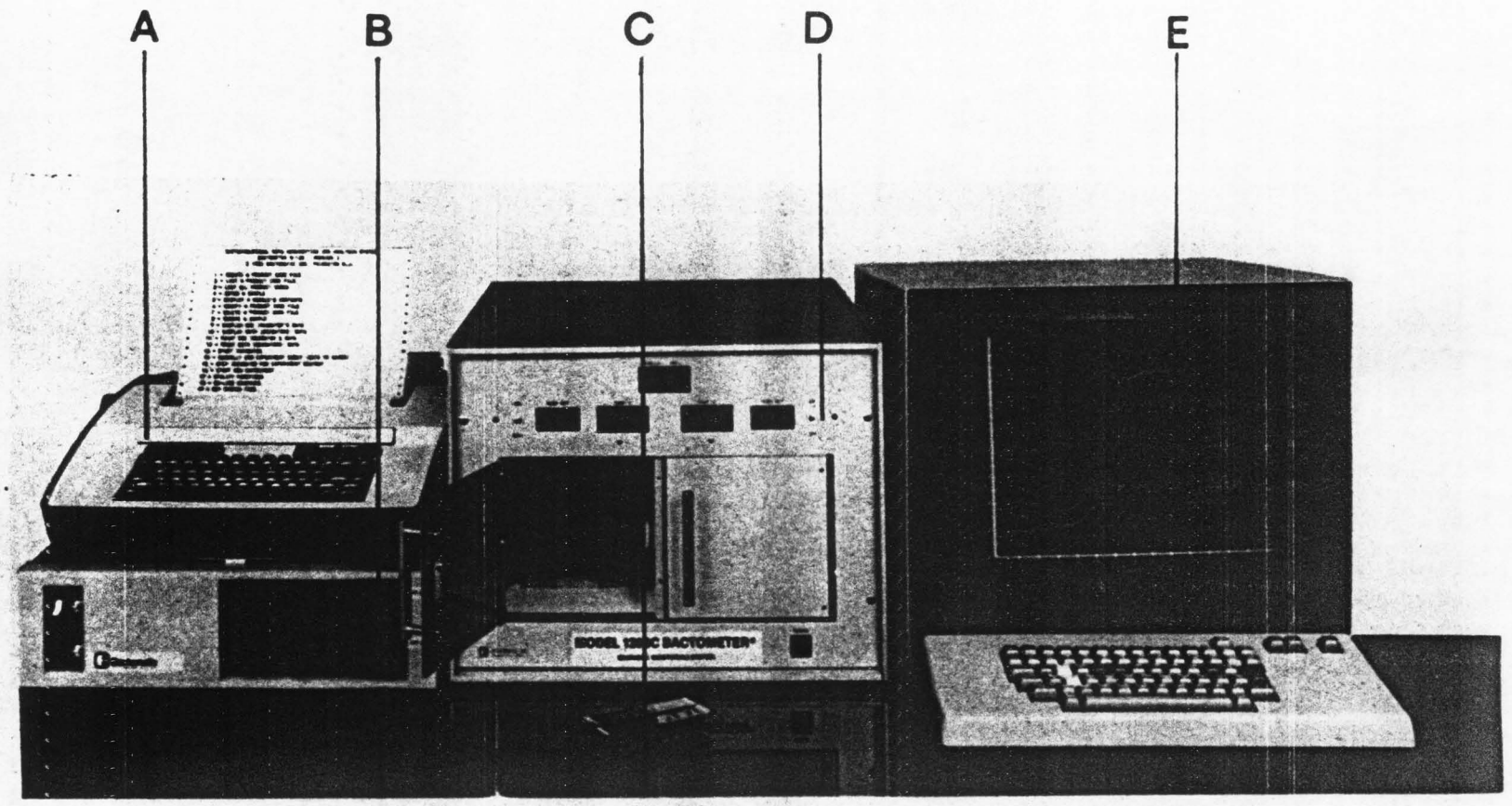

A
B
C
D
E

Figure 4: The MAS-D-TEC™ conductivity meter

MAS·D·TEC™

Early Mastitis Detection

WESCOR

CULTURE

Streptococcus cremoris strain UC310 was obtained from the culture bank of the Department of Nutrition and Food Sciences, Utah State University. It was propagated in RNDM using 1% inoculation and 21°C incubation for 18 h. Dilutions were inoculated into raw milk samples to provide the desired $\log_{10}$ cfu / ml. The viable cells were enumerated using the Standard Plate Count procedure (27).

STATISTICAL ANALYSIS

Data were anlayzed statistically using the SAS system (SAS Institute, Inc., Cary, NC 27511) on the IBM 4143, at the computer center, Utah State University.

RESULTS AND DISCUSSION

REPEATABILITY

The Bactometer® compares rates of conductance (G) or capacitance (C) within samples. When the rate of change of a conductivity reading exceeds a certain level, an IDT is obtained (Figure 5). The IDT is used to estimate the initial microbial numbers in the sample (13). Initial baseline conductivity values, obtained soon after sample readings are initiated and before IDT values are generated, could thus vary considerably without compromising microfloral estimates (Figure 5). This is because only the conductivity values immediately before and after the IDT are used to estimate microbial numbers. These baseline values vary with the salt levels in the milk and thus can be useful in the detection of abnormal milk (24). The Bactometer® manufacturer has used the term conductance (1/impedance) for this application and uses the symble "G" to express the reciprocal of impedance (13). Baseline readings stabilize in about 30 min as incubator and module temperatures equilibrate and develop gradually with time (Figure 5). Variations in baseline readings within or between modules due to manufacturing differences make it difficult to establish absolute estimates of baseline value for abnormal milk, so the variance associated within modules was first established. Each of 128 wells within eight modules from a single lot had 0.5 ml of pasteurized milk added. The modules were then incubated in the Bactometer® incubator at 25°C for 5 h (Table 1). There were significant differences in the baseline readings among wells ($P<.0001$) and modules ($P<.0001$) but, there was no

consistent pattern bias between modules or wells. The coefficients of variance (CV) were 2.1 and 2.3% for two trials. Thus, differences between normal abnormal milks would need to exceed 2.3% to be detected and relative values between normal and abnormal milk could be used instead of an absolute baseline value of raw milk sample. The sample readings were compared to readings of a standard salt solution in the Bactometer®. Module manufacturing differences may require that different lots of modules be evaluated against standard salt solutions results when appropriate. Absolute values of baseline for normal and abnormal milk from wells containing standard salt solutions could provide reference readings that would minimize differences between module lots.

Figure 5: Typical conductivity curve using the Bactometer®

A = Baseline position In a normal milk

B = Baseline position In an abnormal milk

C = Instrument Detection Time (IDT) for a normal milk

D = Instrument Detection Time (IDT) for an abnormal milk

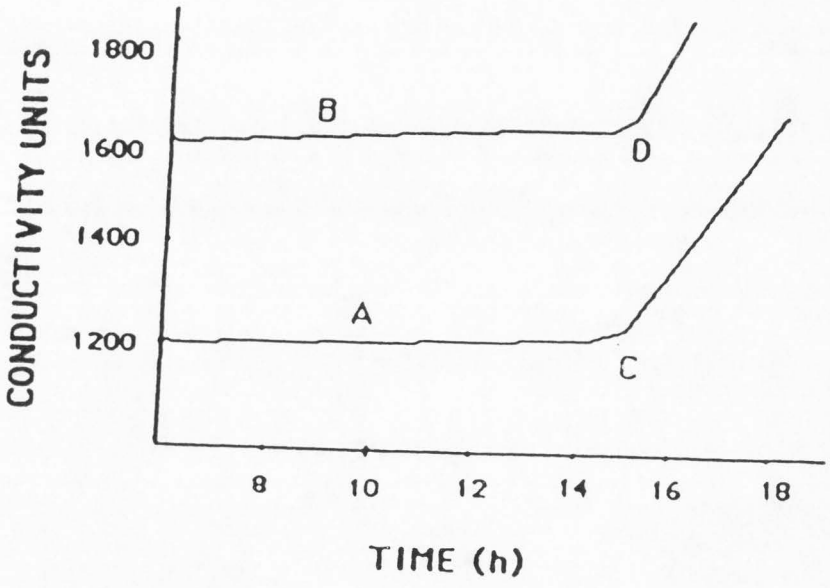

CONDUCTIVITY UNITS
1800
1600
1400
1200
B
D
A
C
8
10
12
14
16
18
TIME (h)

Table 1. Analysis of variance of 128 split samples of pasteurized milk evaluated for baseline development in wells of a Bactometer®120 SC. The incubater was at 25°C for 5 h.

Source	df	SS	F	PR>F
Trial 1, (Mean= 1038.1, CV= 2.1%)				
Module	7	6769.87	1.99	0.0635
Well	15	30507.68	4.18**	0.0001
Error	105	51147.26		
Trial 2, (Mean= 1077.14, CV= 2.3%)				
Module	7	25002.59	5.92*	0.0001
Well	15	35308.97	3.90*	0.0001
Error	105	63299.91		

Figure 6: Conductivity measured by Bactometer® 120 SC when 0.00, 0.05, 0.10, and 0.20 % sodium chloride was added to reconstituted nonfat dry milk (RNDM).

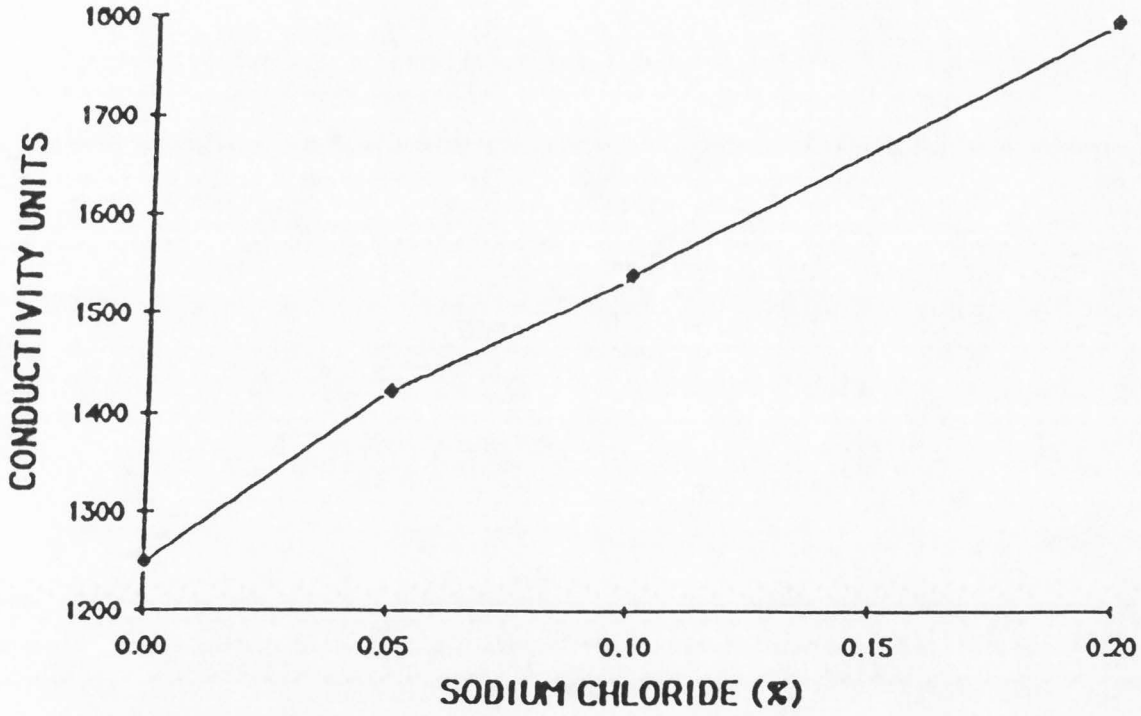

1800
1700
1600
1500
1400
1300
1200
CONDUCTIVITY UNITS
0.00
0.05
0.10
0.15
0.20
SODIUM CHLORIDE (%)

Figure 7: Conductivity baseline values (G) with varying volumes of reconstituted nonfat dry milk (RNDM) and with 2 ml of yeast fortified nonfat dry milk (YNDM).

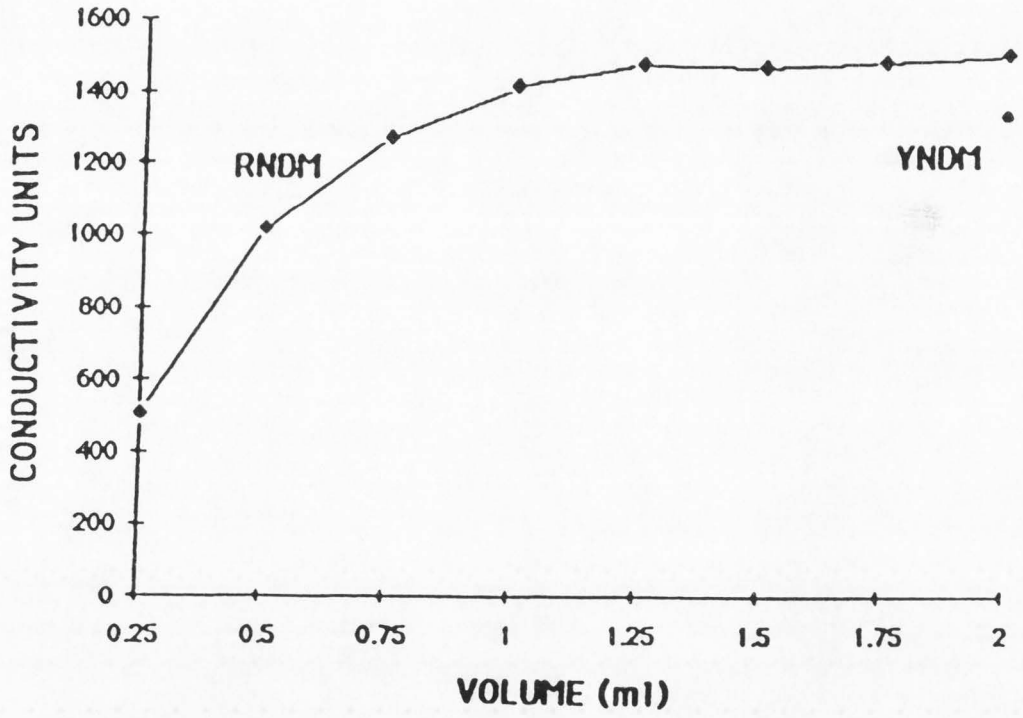

1600
1400
1200
1000
800
600
400
200
0
CONDUCTIVITY UNITS
RNDM
YNDM
0.25
0.5
0.75
1
1.25
1.5
1.75
2
VOLUME (ml)

Table 2. Means of quadruplicate baseline readings from normal and abnormal milk samples tested in individual wells in a Bactometer® 120 SC. Samples were also tested on a Mas-De-Tec™ instrument, and a Fossomatic (Somatic cell counter) instrument.

Cow No.	Quarter	Somatic cell count (thousands)	Mas-De-Tec™ reading	Bactometer® Quarter (G)	Baseline values Blend (G)
5458	FR	10	0	1,130	1,010
	FL	10	1	1,190	
	RR	5	0	1,010	
	RL	14	0	1,140	
5394	FR	152	3	1,360	1,260
	FL	54	2	1,300	
	RR	18	1	1,200	
	RL	10	1	1,160	
5506	FR	9	2	1,220	1,190
	FL	4	2	1,260	
	RR	9	1	1,180	
	RL	11	2	1,190	
5430	FR	13	2	1,280	1,360
	FL	23	2	1,330	
	RR	5	2	1,270	
	RL	18	2	1,300	
4636	FR	15,940	*	*	1,870
	FL	9,320	8	1,990	
	RR	2,760	7	1,770	
	RL	9,380	4	1,480	
5347	FR	6,640	9	1,890	1,410
	FL	500	0	1,230	
	RR	2,130	0	1,160	
	RL	2,420	1	1,230	
4678	FR	1,629	9	1,790	1,910
	FL	5,230	9	1,920	
	RR	8,930	9	1,960	
	RL	6,670	9	1,760	

5046	FR	6,070	9	1,980	1,500
	FL	1,420	3	1,390	
	RR	750	3	1,300	
	RL	1,430	5	1,440	

* Too viscous.

DETECTION OF ADDED SALT

A total of thirty-two wells were inoculated with 0.5 ml solutions of 10% RNDM containing 0.0, 0.05, 0.1, or 0.2% added NaCl and incubated at 25°C for 5 h. Analysis of the resultant baseline measurements (Figure 6) (24) showed the conductivity means were linear (R2 = 0.99) with NaCl additions. The CV values were 2.2, 3.2, 2.2, and 1.9% ,respectively, for the four levels studied. Instrument repeatability was thus linear through the range of normal and abnormal milk (24).

VOLUME OF SAMPLE

Duplicate wells were charged with 0.25 to 2 ml of sterilized RNDM and incubated. Another duplicate set of wells of 2.0 ml volumes was fortified with 2% yeast extract (33) to determine the effect of stimulant addition in the event stimulation was needed to accelerate the growth of microbes due to testing. Conductivity increased as sample volume increased (Figure 7), but the change was insignificant when volume exceeded 1 ml. Stimulant addition would change conductivity depending upon the ionic composition. The reduced salt content of the yeast extract reduced conductivity in this case.

DETECTION OF ABNORMAL MILK

Individual quarter samples were obtained from eight cows known to have mastitis as

determined by the Mas-D-Tec™ conductivity meter (24). Quadruplicate samples were tested using the Bactometer® and the Mas-D-Tec™ conductivity meter. The samples were also tested using the Fossomatic somatic cell counter. The lowest conductivity reading was 1010 G and the highest was1990 G with CV values on the Bactometer® between 1.9 and 2.3% on quadruplicate samples (with a range of 97% of abnormality) (Table 2). Both conductivity instruments correlated well (R2 = 0.95). The linear regression line was

$$Y = 1112.39 + 97.1X$$

where Y = Bactometer® conductivity baseline

and X = Mas-D-Tec™ conductivity readings.

The correlation between conductivity baseline values and somatic cell counts were lower as expected (3)(R2 = 0.67).

$$Y = 1252.55 + 0.08X$$

where Y = Bactometer® conductivity baseline

and X = Fossomatic somatic cell counter (X 1000).

Quarter samples were blended and tested in quadruplicate. The lowest conductivity baseline reading was 1010 G and the CV was 2.1%. The highest was 1910 G with a CV = 1.5% within each sample, and range of 89% between normal and abnormal milk. Results correlated well (R2 = 0.98) with the mean conductivity of the four quarters of individual cows. Since a blended sample of abnormal milk will have a higher mean than milk from normal quarters, abnormal milk in blended quarter samples can be detected. The readings were increased by 18% (cow # 5046) to 29% (cow # 5347) above the mean of milks from a normal udder; there was no increase in the average reading of a blended sample in which all samples had low and

near equal values. The instrument can thus detect the milk from an abnormal quarter in a blend sample if the blend baseline is high enough (cow # 5347, and 5046) or if the computer was progammed to compare readings to previous baseline readings in memory where all four quarters may produce slightly higher readings. Accurate judgements would be possible where readings from all quarters were high (cow # 4636 and 4678).

Figure 8: Conductivity (G) measured by Bactometer® 120 SC when 1.0 ml normal and abnomal (mastitic) milks were added to 1.0 ml modified standard methods broth (MSMB), 0.5 ml standard methods agar (SMA), 1.0 ml yeast fortified nonfat dry milk (YNDM), and 0.5 ml modified standard methods agar (MSMA).

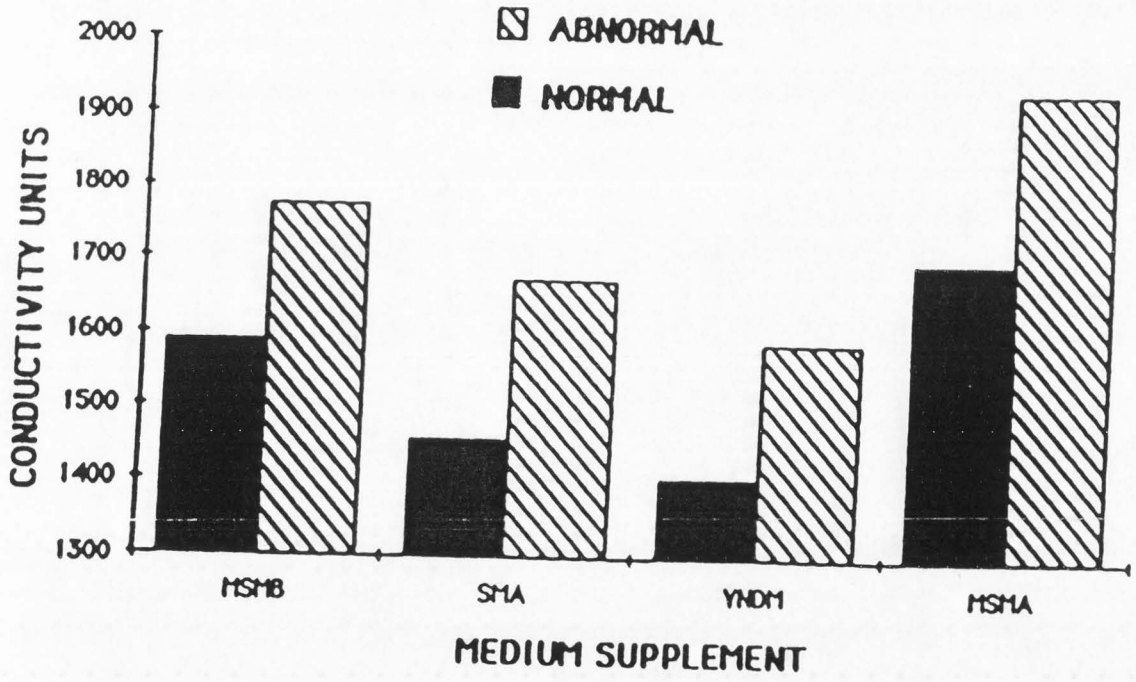

ABNORMAL
NORMAL
CONDUCTIVITY UNITS
2000
1900
1800
1700
1600
1500
1400
1300
MSMB
SMA
YNDM
MSMA
MEDIUM SUPPLEMENT

Figure 9: Effects of added lactic culture to changes in impedance detection times (IDT) using 1.0 ml modified standard methods broth (MSMB), 0.5 ml standard methods agar (SMA), 1.0 ml yeast fortified nonfat dry milk (YNDM), and 0.5 ml modified standard methods agar (MSMA) plus 1.0 ml raw milk sample.

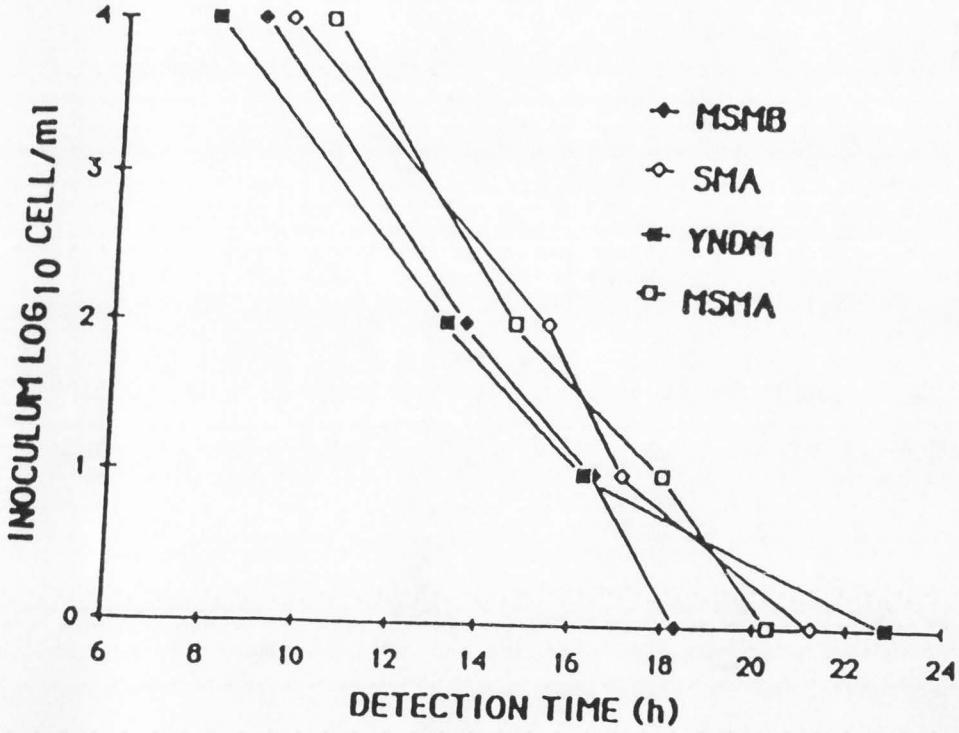
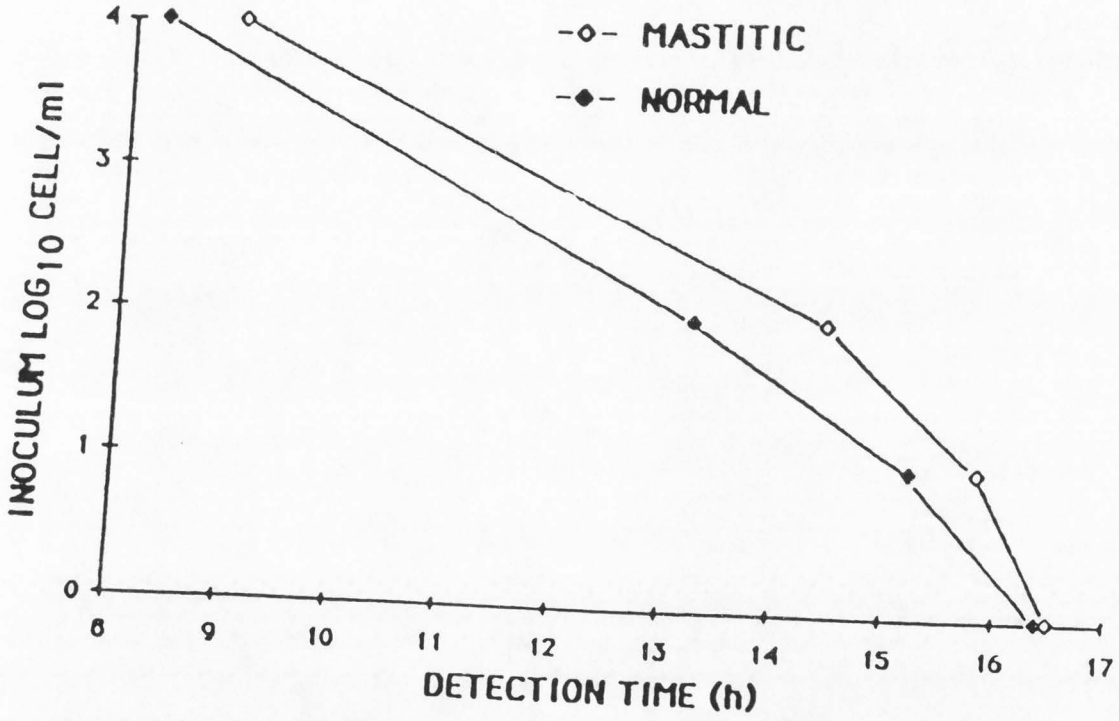

INOCULUM LOG10 CELL/ml
0
1
2
3
4
6
8
10
12
14
16
18
20
22
24
DETECTION TIME (h)
MSMB
SMA
YNDM
MSMA

Figure 10: Effects of added lactic culture upon changes in impedance detection times (IDT) in 1.0 ml normal and mastitic milk samples plus 1.0 ml (2%) yeast extract (YNDM).


MASTITIC
NORMAL
INOCULUM LOG10 CELL/ml
0
1
2
3
4
8
9
10
11
12
13
14
15
16
17
DETECTION TIME (h)

MEDIA MODIFICATIONS

Previous research indicates that IDT values used to estimate the total initial microflora in milk can be shortened by adding stimulants to the milk in the incubation wells (13, 17, 31). Solid media added to a module well was better correlated with standard plate count data (13) than when liquid media was used (17). We used both solid and liquid media in wells to see if the buffering in media would override the ability to detect subtle differences in conductivity. Accurate measurement of differences between normal and abnormal samples was possible with either media (Figure 8).

Readings from normal and abnormal samples differed by 12 to 15%. When a lactic culture (UC310) was added to each medium, the shortest IDTs were obtained with YNDM liquid medium (Figure 9) but estimation of abnormal milk was obtainable with any of the media . The addition of yeast extract (YNDM) reduced the baseline reading in RNDM (Figure 7).

ESTIMATION OF ABNORMAL MILK AND INITIAL MICROFLORA

Eight normal and eight mastitic samples were obtained. Different concentration of lactic culture (UC310) were added using raw milk samples as a dilulent. Duplicate 1.0 ml samples were added to module wells containing 1.0 ml (2%) YNDM, 0.5 ml SMA, wells containing no medium to assess levels of bacteria and and abnormal milk stimultaneously. Conductivity and IDT values were determined (Figure 9, 10). The shortest IDT values were obtained with liquid medium, the longest IDT's were with milk samples incubated alone.

Mastitic milk increased IDT values by about 1 h when culture was added, suggesting residual inhibition. Heat treatment (66°C for 4 min) of raw milk samples before culture addition eliminated the differences in IDT. This suggests a bias against mastitic milk providing the same estimate of microbial load as normal milk. Residual somatic cells or natural milk inhibitory properties probably caused this bias. Such bias could be corrected by adjusting the computer output according to sample baseline value. Conductivity results indicate that milk samples incubated alone gave the highest conductivity range (51%) between normal and abnormal milk; liquid and solid media gave the lowest conductivity range (41, 43%) between normal and abnormal samples. Additional work is needed to elucidate the application of Bactometer® impedance instrument to milk conductivity.

A single milk sample could be used in an impedance instrument to detect both abnormal milk and microbial activity in raw milk (13,24). The sample would be incubated for 30 min or until a stable baseline is established. Longer incubation may be required if solid media were used in the assay. The instrument would then compare baseline values to estimate abnormal milk. Error associated with volume differences would be reduced if 1 ml volumes were used instead of 0.5-ml volumes (27). The instrument baseline varies with salt levels in milk due to mastitis or other abnormal condition. The instrument's computer could be programmed to compare baselines between the current sample with stored data from the previous test(s) to estimate significance of the differences. Sample incubation would continue until IDT data were generated and microbial load estimated.

CONCLUSIONS

1. Since the coefficients of variance of The Bactometer® module wells was found to be between 2.1 & 2.3%, only those abnormal milk samples with significantly greater variance than 2.3% could be detected using baseline measurements.
2. Different added sodium chloride concentrations gave a high correlation of determination (R^2=0.99) with conductivity baseline means, therefore the Bactometer® could be used to differentiate between samples having different salt concentrations.
3. Although the volume of sample inoculated into Bactometer® wells was proportional to the conductivity baseline, the change became insignificant when the volume exceeded 1.0 ml, which allows the use of 1.0 ml as an optimum inoculum. However, volume stimulant addition changed the conductivity depending upon the ionic composition of the stimulant. For example, yeast extract reduced the base line, and increased the growth of the bacteria resulting in fast IDT results.
4. A correlation of determination of R^2= 0.95 was obtained when The MAS-D-TEC™ conductivity meter was the reference method for the Bactometer®; a correlation of determination of R^2= 0.67 was obtained when the Fossomatic somatic cell counter was the reference method; therefore the Bactometer® could be used for determination of abnormal milk.
5. Since either a liquid or a solid medium could be used in the module well for determination of cell counts, any of the media could be used to diferentiate between normal and abnormal milk samples.

6. A single milk sample could be used in an impedance instrument to detect both abnormal milk and initial microbial activity.

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APPENDIX

Table 3. Least Significant Differences (LSD) for repeatability of the eight modules of The Bactomter® 120 SC. A 0.5 ml inoculum of pasteurized milk incubated for 5 h at 25°C. First trial.

Module position	Number of wells in a module	Mean (G) value	Grouping*
3	16	1045.3	A
8	16	1044.2	A
6	16	1043.1	A B
5	16	1041.7	A B
7	16	1040.6	A B
1	16	1036.9	A B C
4	16	1028.3	B C
2	16	1024.3	C

* Means of the same letter are not significantly different.

Table 4. Least Significant Differences (LSD) for repeatability of 128 module wells of The Bactomter® 120 SC. A 0.5 ml inoculum of pasteurized milk incubated for 5 h at 25°C. First trial.

Well position	Number of wells with same position in eight modules	Mean (G) value	Grouping*						
3	8	1066.6	A						
4	8	1060.1	A	B					
1	8	1055.9	A	B	C				
7	8	1053.9	A	B	C	D			
6	8	1047.1	A	B	C	D	E		
16	8	1043.8		B	C	D	E		
13	8	1038.6		B	C	D	E	F	
5	8	1037.9			C	D	E	F	
2	8	1033.9				D	E	F	G
9	8	1033.8				D	E	F	G
8	8	1033.3				D	E	F	G
12	8	1030.9					E	F	G
15	8	1028.4					E	F	G
14	8	1017.5						F	G
11	8	1014.1							G
10	8	1013.0							G

* Means of the same letter are not significantly different.

Table 5. Least Significant Differences (LSD) for repeatability of the eight modules of The Bactomter® 120 SC. A 0.5 ml inoculum of pasteurized milk incubated for 5 h at 25°C. Second trial.

Module position	Number of wells in a module	Mean (G) value	Grouping*
3	16	1095.8	A
2	16	1093.6	A
8	16	1083.4	A B
1	16	1080.7	A B
5	16	1076.3	B C
7	16	1073.4	B C
6	16	1062.8	C D
4	16	1051.3	D

* Means of the same letter are not significantly different.

Table 6. Least Significant Differences (LSD) for repeatability of 128 module wells of The Bactomter® 120 SC. A 0.5 ml inoculum of pasteurized milk incubated for 5 h at 25°C. Second trial.

Well position	Number of wells with same position in eight modules	Mean (G) value	Grouping*				
7	8	1108.8	A				
5	8	1097.9	A	B			
6	8	1096.9	A	B			
4	8	1092.6	A	B			
8	8	1092.1	A	B	C		
1	8	1084.6	A	B	C		
15	8	1081.1		B	C	D	
3	8	1075.6		B	C	D	E
13	8	1075.3		B	C	D	E
2	8	1074.1		B	C	D	E
14	8	1067.9			C	D	E
12	8	1059.6				D	E
9	8	1059.4				D	E
16	8	1058.4				D	E
10	8	1056.3					E
11	8	1053.8					E

* Means of the same letter are not significantly different.

VITA

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ARABIC TRANSLATION OF THE CONCLUSIONS

تلخيص للبحث

١- حيث ان معامل الاختلاف بين اوعية جهاز البالكتوميتر كانت بين ١,٠ و ٢,٠ من المئة ، لذلك فإن عينات الحليب الملون التي يفارق اختلاف اكبر من ٢,٠ من المئة عن الحليب الغير ملون يمكن معرفتها بطريقة قياس قاعدة الخط .

٢- عند وضع تركيزات مختلفة من محلول الملح أعطت علاقة متبادلة (r^2 = ٠,٩٩) مع قاعدة الخط الايصالية ، لذلك يمكن استخدام جهاز البالكتوميتر للتفريق بين عينات تحتوي على تركيزات ملحية مختلفة .

٣- بما ان الجسم السائل الملحي المضاف الى اوعية جهاز البالكتوميتر يتناسب طردياً مع قاعدة الخط الايصالية ، فإن التناسب يصبح ضعيفاً عندما يتعدى حجم السائل الملحي واحد ملم . لذلك فإن واحد ملم هو افضل كمية مناسبة تضاف الى الاوعية . وعند اضافة مواد مثيرة فإن الايصالية تتغير حسب التركيز الايوني للمواد ، مثالاً على ذلك ان اضافة ملامح الخميرة قللت قاعدة الخط وزادت من نمو البكتيريا ، ولذلك حصلنا على (IST) سريع .

٤- لقد حصلت علاقة متبادلة (r^2 = ٠,٩٥) بين جهاز ماس-ري-تك ميتر الايصالية المستخدم كمرجع وجهاز البالكتوميتر . وعلاقة متبادلة (r^2 = ٠,٦٧) بين جهاز الفوماتك سل كاونتر (تعداد الخلايا الدقاقية) المستخدم كمرجع وجهاز البالكتوميتر ، لذلك يمكن استخدام جهاز البالكتوميتر لتحليل الحليب الملوث .

٥- حيث انه يمكن استخدام البيئة الجسم او السائلة في الاوعية لمعرفة عدد البكتيريا ، فإن أيّ من هذه البيئات يمكن استخدامه كذلك لمعرفة الحليب الصحي من الحليب الملوث .

٦- يمكن استخدام عينة حليب واحدة في جهاز الايصالية لمعرفة الحليب الملوث وعدد البكتيريا الموجودة فيه .