DETECTION OF THE FLUORESCING GROUP OF PSEUDOMONAS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE PREDICTION OF SHELF-LIFE OF DAIRY PRODUCTS

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

in

Food Science and Technology

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November, 1991

Blacksburg, Virginia

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(ABSTRACT)

An enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies has been developed for the detection of the fluorescing group of Pseudomonas. The assay was used as a rapid test (6h) for predicting the shelf-life of pasteurized fluid milk. Milk samples were held at 7°C and tested weekly until determined to be unacceptable by daily sensory evaluation. Sterile milk samples were inoculated with target concentrations of 0 (control), 100, and 1000 cells/ml of Pseudomonas fluorescens on day 0. Samples were tested before and after preliminary incubations. Preliminary incubations conducted include milk alone and milk with broth (1:1) for 18h at 21°C. ELISA and plate counts were performed before and after preliminary incubation to determine the number of pseudomonads present and the relationship between ELISA and plate counts. These numbers were correlated to the shelf-life of each sample, as determined by sensory evaluation. Samples

undergoing a preliminary incubation with only milk gave the best correlation to shelf-life (R=0.86).

ACKNOWLEDGEMENTS

I would like to thank Dr. J. Russell Bishop for his help and guidance over the past two years. I would also like to thank my committee members, Dr. Eigel and Dr. Yousten, for their help. Acknowledgements are due the graduate students in my lab and in the Food Science and Technology Department for keeping my morale up when times got tough. Special thanks to Laura Clark and Rob Byrne for their help and insight. I would also like to thank my family for their support.

Finally, acknowledgements are due DuPont/EBI for their support of this project.

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LIST OF ABBREVIATIONS

ABTS	2,2-amino-di(3-ethyl-benzthiazoline sulfonic acid-6) diammonium salt
BSA	Bovine serum albumin
CVT	Crystal violet triphenyl tetrazolium chloride
DDB	Dairy dilution buffer
ELISA	Enzyme-linked immunosorbent assay
HTST	High-temperature short-time
LPS	Lipopolysaccharide
mPBC	Modified psychrotrophic bacterial count
PBS	Phosphate buffered saline
Pf	Pseudomonas fluorescens
PSM	Petrifilm standard methods
PVRB	Petrifilm violet red bile
SMA	Standard methods agar
SPC	Standard plate count
TSB	Trypticase soy broth
UHT	Ultra-high temperature
VRBA	Violet red bile agar

INTRODUCTION

Many consumers base their purchasing on the sell-by-date which is required on all dairy products. The consumer expects the product to be consumable up to and often beyond this date. Therefore, the processor has the responsibility of estimating the shelf-life date as accurately as possible. The keeping quality of milk is dependent on a number of factors, with post-pasteurization contamination being the primary cause of poor quality milk. Psychrotrophic microorganisms, predominantly the fluorescing group of pseudomonads, which gain access to the milk after pasteurization are generally targeted in a shelf-life study. These organisms, although present in small numbers, can grow rapidly at refrigeration temperatures. Therefore, a method for determining post-pasteurization contamination and potential shelf-life must be sensitive and rapid enough such that effective corrective measures can be taken in the event of a problem. Many of the tests currently available for shelf-life testing tend to be too expensive, too slow, or not sensitive enough to determine the extent of post-pasteurization contamination. The enzyme-linked immunosorbent assay (ELISA) may prove to be a successful technique for determining potential shelf-life. The technique is rapid, reasonably inexpensive, and sensitive.

The objectives of this study were to develop an ELISA for the detection of the fluorescing group of pseudomonads and to test the developed ELISA as a tool for assessing potential shelf-life of inoculated sterile milk samples.

LITERATURE REVIEW

MILK

Milk is a sweet, slightly salty, bland substance produced in the udder of the cow from blood constituents. Cows milk contains approximately 87.1% water, 3.9% fat, 3.3% protein, 5.0% lactose, and 0.7% minerals (ash). This composition makes milk an excellent microbial growth medium (12, 34), second only to blood.

SHELF-LIFE

Shelf-life has been defined as the time from processing and packaging until the product is considered unacceptable for consumption. Baker (6) has stated that although shelf-life reflects the keeping quality of milk, there is no way of accurately predicting shelf-life. In other words, there is no adequate working definition.

Factors which can affect shelf-life of milk include the number of days held raw (48), the pasteurization temperature, the storage temperature of the raw and pasteurized milk, the season, and the extent of post-pasteurization contamination (33). Raw milk held at 5°C for 1, 2, and 3d has been found to have psychrotropic counts

of 4.0×10^5, 2.1×10^6, and 1.1×10^7 cfus/ml, respectively (56). The longer the milk is held raw, the lower the quality due to microbial growth. With a higher number of organisms, more will survive pasteurization (96), along with the production of a higher number of heat-stable enzymes. The pasteurization temperature and time can have an effect on some proteases (7) and spore-forming bacteria as well (25, 26). Pasteurized milk, when stored at 7.2°C, allows for the growth of 1 cell/ml of psychrotrophs to 10^8 cells/ml in 8d (90). Also, given higher temperatures, heat-shocked organisms experience shorter lag phases (6, 47). Yano (101) found that at 5°C the psychrotrophic organisms present had generation times ranging from 6.6 - 12h, giving a shelf-life range of 2 - 5d. At 0°C the bacteria had generation times of 12.2 to 26.1h, with shelf-life ranging from 4 - 13d. Studies show a 5°C increase in temperature can decrease shelf-life by half (17, 47). Hankin, et al. (43) found commercially filled milk cartons held at 1.7, 5.6, and 10°C to have shelf-lives of 17.5, 12.1, and 6.9d. Harmon, et al. (44) found that despite the storage temperature, the maximum number of organisms were present when the product reached its shelf-life. Raw milk, when cooled to 4°C within 15 min did not have a significant increase in microbial contamination after 72h, but when the cooling process was increased to 2h, bacterial numbers did increase (6, 86).

Therefore, milk should be cooled quickly to and held at appropriate temperatures for best quality. Barnard (8) states that temperatures below 7°C are the key to extending shelf-life. Finally, seasonal changes affect milk quality. Higher summer temperatures allow for more optimal growth temperatures (26, 90). In addition Andrey and Frazier (3) noted that differences between barn feeding and pasture feeding affect the types of bacteria present.

Due to changes in processing and consumer trends in the past 40 years, processors need to be more aware of the number of days their product will be consumable. Shelf-life was not such a concern when milk was delivered daily to the consumer. Today the consumer is aware of a product's age and expects the product to be of acceptable quality when purchased (64). A processor's product which does not consistently meet these consumer expectations is not likely to be purchased in the future. Therefore, shelf-life needs to be evaluated rapidly such that effective corrective measures can be taken in the event of a problem (12).

PSYCHROTROPHIC BACTERIA

The types and the numbers of organisms contributing to shortened shelf-life are affected by todays methods of cooling, handling, and sanitation, as well as storage

temperature. The microflora which predominate are those organisms capable of growth at refrigeration temperatures (6, 54). These microorganisms are ultimately responsible for microbial spoilage of milk. These organisms, once referred to as psychrophiles or "cold-loving", are now known as psychrotrophic or "cold-enduring" (53). This term is more appropriate as psychrotroph is defined as those bacteria which are capable of growth at refrigeration temperatures (26, 100), but whose optimal growth temperature is actually higher. Elliot and Michelson, as cited by Cousins (26), found optimal growth temperatures for these organisms to range from 20°C to 30°C, while some preferred 30°C to 45°C. These organisms can multiply relatively rapidly, spoiling milk in as few as 7d at 7°C (10). For this reason, these organisms should be the focus of shelf-life studies. The psychrotrophic microorganisms which cause problems in milk are generally gram-negative, non-spore-forming rods. They are usually aerobic, produce heat-stable metabolites, and are resistant to penicillin and other anti-microbial compounds (12, 65, 100). When milk is produced under sanitary conditions, only about 10% of the total microflora are psychrotrophs, however, milk produced under unsanitary conditions can contain greater than 75% psychrotrophs (90). Psychrotrophic organisms which lead to poor quality milk include species of Pseudomonas,

Flavobacterium, Chromobacter, Acinetobacter, Alcaligenes, Proteus, Escherichia and Enterobacter (12, 30, 90).

PSEUDOMONAS SPP.

Studies show that psychrotrophic organisms, comprised primarily of gram-negative bacteria, are the major causes of poor keeping quality, with the pseudomonads dominating (6, 16, 38, 53, 98, 100). White, et al. (99) found that the Pseudomonas comprised 90% of the psychrotrophs in 315 milk samples tested. Studies show the dominant lipolytic and proteolytic populations of milk samples are pseudomonads (58, 71, 83). Of 36 raw milk samples, Shelley, et al. (83) found the lipolytic population of 23 samples dominated by fluorescent pseudomanads, nine with non-fluorescent pseudomonads, and 4 samples with both types.

The Pseudomonas, which belong to the family Pseudomonadacea, are short, gram-negative, aerobic rods. The fluorescing strain produce the pigments, pyocyanin and/or fluorescin (49). In addition, the pseudomonads causing spoilage in milk are psychrotrophic and produce heat-stable lipases and proteases (49, 100).

Since it is difficult and time consuming to biochemically identify the psychrotrophic bacteria, many investigators are examining quicker, easier methods for

identification (41, 69). It is important to be able to identify this group of organisms quickly and to detect them at a low enough level in order to determine the keeping quality of milk. The use of monoclonal and polyclonal antibodies has been investigated. Hancock, et al. (41) produced a monoclonal antibody against an outer membrane protein of Pseudomonas aeruginosa, which could also react with an outer membrane protein of some strains of Pseudomonas fluorescens and Pseudomonas putida.

POST-PASTEURIZATION CONTAMINATION

Psychrotrophic organisms are ubiquitous in nature, with the major source of contamination of milk from poorly cleaned and sanitized plant equipment (26, 76, 89, 90, 100). Many researchers indicate that psychrotrophs do not survive pasteurization, therefore, the main concern of the milk processor is the psychrotrophic microorganisms which gain access to the milk after pasteurization (26, 89, 96, 100). This is known as post-pasteurization contamination. On the contrary, other investigators report that small numbers of psychrotrophs can survive pasteurization (77). Post-pasteurization contamination has been documented as the leading cause of poor quality milk and shortened shelf-life of microbial origin (77). The organisms gain access to the

milk after pasteurization due to ineffective cleaning and sanitizing, cracks, scratches, and pinholes in storage tanks and pipelines, malfunctioning valves, cracks in gaskets, condensation from fillers, and environmental sources (53). Researchers have found a relationship between post-pasteurization contamination and potential shelf-life (15). Therefore, shelf-life testing should be geared toward detecting post-pasteurization contamination, specifically Pseudomonas (98). However, since the initial numbers of organisms present due to post-pasteurization contamination are very low, methods must be sensitive and rapid enough to pick up the contamination. Blankenagel (15) makes five suggestions for successful shelf-life testing: 1) use of preliminary incubation, 2) detection of metabolites, 3) use of selective media, 4) surface plating, and 5) a combination of the above. Some methods employed to determine shelf-life include the detection of post-pasteurization contamination with a 5d, 7°C incubation (30) and the Moselely Keeping Quality Test (32). White and Little (99) conducted a mPBC plus a preliminary incubation in gram-negative broth to predict shelf-life. Some researchers report that flavor scores are better for determining the potential shelf-life than microbiological and chemical tests (6, 42).

SHELF-LIFE TESTING

An ideal test for shelf-life would be accurate, such that the test would relate exact numbers of microorganisms to potential shelf-life, rapid, allowing for effective corrective measures to be taken, simple and economical (15). Some tests currently available for shelf-life determination include the standard plate count, the coliform count, pyruvate concentration, bioluminescence, the direct epifluorescent filter technique (DEFT), the Limulus amoebocyte lysate assay (LAL), and impedance microbiology. The Moseley Keeping Quality Test is the test generally accepted by the dairy industry for determining shelf-life. This test takes 7 - 9 days to complete, by which time the milk will have already been purchased and possibly consumed. This technique utilizes the standard plate count which is time consuming, slow, expensive (51), and not indicative of raw milk quality (97, 98). Also, The standard plate count does not differentiate between organisms surviving pasteurization and those introduced due to post-pasteurization contamination (15). Researchers have found the Moseley Test to have correlations to shelf-life of -0.77 and -0.84 (10, 19). The coliform count only indicates post-pasteurization contamination when coliforms are present, however, these organisms only constitute a small amount of

amount of the contaminants (12, 15). The pyruvate concentration test is rapid, and can be automated to test 80 - 120 samples/h. This test is inaccurate as it assumes the production of pyruvate by all bacteria in milk is uniform (12, 58). The LAL assay is very sensitive and rapid, but the reagents are expensive. The LAL test is desirable as it detects lipopolysaccharides (LPS) both before and after pasteurization (13). Byrne (21) reports a correlation to shelf-life of -0.85 for this method. DEFT is rapid, it can be performed in 25 minutes per sample and costs approximately $.75 - 1.00 per samples (13). Byrne (21) found a -0.78 correlation to shelf-life with this method. The impedance method allows results to be available within 25 - 38 hours after milk has been processed (10), however, this method requires high initial costs for equipment. Bishop and White (11) found a correlation to shelf-life with the impedance method in milk of 0.93.

PRELIMINARY INCUBATIONS

Since the number of psychrotrophic microorganisms present in freshly pasteurized milk is too low to be detected by many laboratory techniques, steps need to be taken to increase these numbers (19). Preliminary incubations are often conducted to achieve a more accurately

detectable level of organisms. The preliminary incubation is based on the fact that as the incubation temperature of a milk sample is lowered, a point is reached where psychrotrophic bacteria continue to multiply rapidly and udder microflora cease multiplying (12, 52). Many studies show the preliminary incubation to be a good indication of potential shelf-life (6, 12), whereas, initial bacterial counts are of limited value for predicting keeping quality (11, 42, 55, 62, 77, 80). Byrne, et al. (19, 20) used a preliminary incubation of 21°C for 18h to increase the gram-negative psychrotrophic bacteria in milk with good correlation to plating techniques and rapid instrumentation. Bishop and White (11) found the modified psychrotroph count (21°C, 25h) and a preliminary incubation of 21°C for 14h correlated to shelf-life. Johns (52) determined raw milk quality with a 55°C, 18h preliminary incubation followed by a standard plate count.

Selective preliminary incubations have been studied using compounds which can inhibit gram-positive growth. Byrne, et al. (20) found benzalkonium chloride (0.1%) to be the preferred inhibitor for selective incubation. This selective preliminary incubation allowed a 3 log increase of pseudomonads on average. Only slight inhibition was found with crystal violet. Phillips, et al. (78) conducted a preliminary incubation of 21°c for 25h in

nisin:penicillin:crystal violet to detect post-pasteurization contamination. Waes and Bossuyt (94, 95) selected for gram-negative organisms using benzalkon-A and crystal violet in a 30°C, 24h preliminary incubation.

BACTERIAL ENUMERATIONS

Petrifilm, a dry culture medium produced by the 3M Corporation, was found to be equivalent to the standard plate count (SPC) and violet red bile agar (VRBA) coliform count methods (82). Correlation coefficients for PSM vs SMA in both whole and skim milk and for PVRB vs VRBA were 0.99 and 0.959, respectively (63, 70). The modified psychrotrophic bacterial count (mPBC) developed by Parmelee and Oliveria to enumerate psychrotrophic bacteria at 21°C for 25h has a correlation to the regular psychrotrophic bacterial count (7°C, 10d) of 0.992 with raw milk and 0.996 with pasteurized milk (72).

Researchers have tried various techniques to selectively enumerate psychrotrophic bacteria present. Crystal violet triphenyl tetrazolium chloride agar (CVT) was shown by Olson (73) to inhibit most gram-positive organisms. Research has shown CVT can be used to provide an estimate of the number of gram-negative bacteria in refrigerated milk. Jay (50) found that of 45 psychrotrophs, only 3 did not form

colonies on CVT. Pseudomonas showed maximal growth after 48h. Mallmann, et al. (57) attained good results by inhibiting gram-positive mesophilic bacteria using an anionic wetting solution at 20°C for 72h. Solberg, et al. (85) developed a medium containing cetrimide and 2-hydroxy-2,4,4-trichlorodiphenyloxide in tryptic soy agar for enumerating pseudomanads in foods not containing Alcaligenes, Enterobacter or Citrobacter. Gyllenberg et al. (39) used ammonium lactate agar alone and with triphenyl tetrazolium chloride or crystal violet to select for Pseudomonas in milk as a tool to determine shelf-life. Other compounds employed for selection of gram-negative microorganisms include: sodium desoxycholate, alkyldimethyl benzyl ammonium chloride, methyl dodecyl trimethyl ammonium chloride, alpha-bromolauric acid and alpha-bromomyristic acid (34), crystal violet and neotetrazolium chloride (84), and nutrient agar with alkylaurl sulfonate (61). Other investigators found partial inhibition of gram-positive organisms with monesin in the growth media. In addition, they found neotetrazolium chloride and sodium desoxycholate to allow some growth of gram-positive organisms (79).

HEAT-STABLE ENZYMES

Psychrotrophic bacteria have a direct and indirect

affect on milk shelf-life. They can directly spoil the milk through growth and proliferation. Indirectly, they produce heat-stable lipases and proteases which break down the constituents of milk (24, 26, 37, 55), causing off-odors and flavors. Patel (77) reports the presence of off-flavors, including bitterness, develop after 2 weeks storage at 7°C in the absence of post-pasteurization contamination. However, samples did have high bacterial counts before pasteurization. The off-flavor bitterness is a characteristic of enzymatic breakdown. Adams, et al. (1) report a protease capable of causing milk spoilage in less than 3d when 1000-10,000 bacteria/ml are present at 4°C for 2d. These enzymes on average retain 60% of their activity after pasteurization and 40% after UHT treatment (37). O'Connor (71) states that 1×10^6 cells/ml are required in raw milk for proteolytic spoilage of UHT milk, and could be less for HTST pasteurized milk. One study shows all bacteria isolated from milk produce proteases that survive 149°C for 10 sec. Seventy to ninety percent of the raw milk samples tested contained psychrotrophic organisms which were able to produce heat-stable enzymes (1). Some characteristics of these enzymes are an optimal temperature for activity of 45°C, and an optimal pH of 7-8. (1) Much research has concentrated on the production of these heat-stable enzymes. Mayerhofer, et al. (62) studied a protease

from Pseudomonas fluorescens which retained 71% of its activity after being heated for 60 min at 71.4°C. Garcia, et al. (35) found after a heat treatment of 130°C for 15 sec, 70% of the extracellular proteases from Pseudomonas remained active, with 50% of the enzymes retaining a residual activity higher than 33%. Adams, et al (1) state that the concentration of casein and the pH of normal milk are close to optimal for protease activity. Many investigators have stated that a heat treatment which would destroy these enzymes would probably render the product quality unacceptable (1, 7, 62). However, Barach (7) found he could get 90% inactivation of protease with a low temperature treatment of 55°C for 60 min.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA is a technique by which antigens (antibodies) can be detected based on specific interactions with antibodies (antigens). ELISA refers to all solid phase immunoassays using enzyme-labelled reagents, but the term is most commonly used to describe non-competitive solid phase sandwich assays (92). Different formats of ELISA include competitive and non-competitive assays, including direct and indirect assays. A draw back of the competitive assay is that this assay requires purified antigen (88). The

indirect assay, although involving an extra step, is desirable since the test sample does not come in contact with the enzyme conjugate and, therefore, there is little or no interference from the test medium (88). Enzymes can be either inhibited or enhanced by constituents of the biological medium (14).

The ELISAs potential as a means of bioassay in food quality control has been established. The ELISA technique has acquired popularity due to a number of advantageous properties: 1) the test is applicable to the assay of any compound, 2) the test is capable of high sensitivity, 3) very little to no pretreatment of the sample is needed, and 4) manufacturers produce equipment for the automation of the immunoassays (14). Also, only specific antibodies, which can be purchased or produced in animals, are needed (14).

Materials and reagents

The solid phase is usually constructed of plastic materials such as tubes, paddles, beads, disks, and in particular, microplates. Microplates are popular since they permit batch processing and facilitate reagent additions and washings. Tween 20, a wetting agent, is often employed to prevent non-specific reactivity caused by sample components that adhere directly to the coated plastic surface (92).

Tween 20 also prevents inactivation of peroxidase by polystyrene surfaces (9). A blocking buffer containing BSA or another protein can also be used when necessary to prevent non-specific binding (92). The antibody used for conjugate preparation should be of high affinity and as purified as possible (14, 92). Substrates are usually chromogenic compounds which are initially colorless and upon degradation, produce a colored, soluble product. Fluorescent substrates are becoming more popular due to low detection levels (92).

ELISA Sensitivity

Many factors influence the sensitivity of the immunoassay. The activity of enzymes is affected by substrate and coenzyme concentration, pH, temperature, and ionic strength, thus these factors must be closely monitored (14). Voller (92) states that the antigen should be of low molecular weight to prevent agglutination, precipitation, or steric hinderance of soluble reagents. He also illustrated that a standard curve of shallow slope can better discriminate between similar concentrations of analyte. The more avid the antisera, the more sensitive the assay (14). The type of microtiter plate used can also affect sensitivity. Polystyrene has been shown to take up less protein that polyvinyl chloride (92). The coating of the

solid phase, and thus the sensitivity, can be affected by the purity and concentration of immunoreagent, and diluent, as well as the time and temperature of incubation. The solid phase should be pure or reasonably pure. Cantarero, et al. (22) suggest adding enough protein to give a monolayer, approximately 500ng protein per 6.5 cm^2 of plastic. More protein interactions would be wasteful and not add to increased sensitivity. Diluent successfully used for most proteins are carbonate and phosphate buffers at neutral or alkaline pH. Voller (92) states the typical temperatures for incubation are room temperature and 32°C. Adsorbed antibodies can undergo denaturation or loss of adsorption either upon washing or when other proteins plus non-ionic detergents are added to prevent non-specific adsorption of immunoreagents. Covalent linking of proteins to plastic could overcome these problems and increase the sensitivity and the reproducibility of the assay (45).

Enzymes

Enzymes are very efficient catalysts, typically converting 10^3 to 10^4 molecules of substrate to product per minute (14). Properties of an ideal enzyme label are: 1) high activity at low substrate concentration, 2) stable at pH required for good antibody-antigen binding, 3) inexpensive, 4) ability to be linked to antibody, or

antigen, with minimum loss of activity, 5) stable under routine storage, 6) availability of purified, soluble enzyme at low cost, 7) absence of health hazards attributed to enzyme, substrate or cofactors, and 8) absence of enzyme and factors affecting enzyme in test fluid (14, 75).

Enzyme labels commonly used include peroxidase, alkaline phosphatase, and B-galactosidase. Horseradish peroxidase is used for general purposes since this enzyme is inexpensive and readily available in a purified form. The most common substrates to be used with horseradish peroxidase, despite the fact they may be carcinogenic, include orth-phenylenediamine (OPD), 2,2-amino-di(3-ethyl-benzthiazoline sulfonic acid-6) diammonium salt (ABTS), and 5-aminosalicylic acid (14, 92). Al-Kaissi and Mostratos (2) compared these chromagens and found them to be of comparable sensitivity at their optimal wavelengths. Alkaline phosphatase suitable substrates include p-Nitrophenyl phosphate, Methyl umbelliferyl phosphate, and Fast red. B-galactosidase substrates include orth-Nitrophenyl-B-D-galactosidase, and Methyl umbelliferyl galactoside (14, 92).

POLYCLONAL ANTIBODIES

Advantages of polyclonal antibodies

Since polyclonal antibodies bind to antigenic determinants covering most of the external surface of an antigen, small changes in the structure of the antigen have little effect on antibody binding (18). However, monoclonal antibodies are more affected by alteration of the antigen since they bind to one unique site (36). Polyclonal antisera also contain antibodies with a wide range of affinities, with the higher affinity antibodies dominating the specificity (36, 59). This heterogeneity allows the antibody population to be covalently modified without loss of reactivity such that binding is not detectable (36). Polyclonal antibodies are applicable over a wide pH range (pH 4-9) and salt concentration (0 - 1M NaCl) whereas monoclonal antibodies have been shown to be affected by minor changes (36, 46). In one study a monoclonal antibody detects antigen at a pH of 7.0 and a salt concentration of less than 100mM, but not at a pH of 8.0 and a salt concentration of 200mM (46).

Antibody production

According to Morris (67) partly by selecting antisera of high avidity, or high kinetic energy, manufacturers can

reduce their kit incubation times. Antibody production occurs in three stages: 1) preparation of the immunogen, 2) immunization and blood collection and, 3) assessment of the antisera. Hurn, as cited by Morris (67), suggests leaving the primed animals for as long as possible before boosting them. However, mixed reports on this subject exist. Morris (67) suggests the animal should not be boosted until the level of circulation of specific antibody has fallen to a steady level. In the assessment of antisera, the emphasis should be on the avidity, not only the titer. Also, the specificity or cross-reactivity of the antisera needs to be determined.

USES OF ELISA IN THE FOOD INDUSTRY

ELISA has been used in the food and dairy industries for quality control and as a means of determining food safety. Many microbial toxins, aflatoxins, and antibiotics have been detected using this technique. Staphylococcal enterotoxins, *Clostridium perfringens* type A toxin and *E. coli* heat-labile (LT) enterotoxin are a few examples (66, 88). Cuartero et al. (27) developed an ELISA to aflatoxin B1 which partially cross reacts with all B and G aflatoxins. Sulfamethazine and chloramphenicol have also been detected by ELISA in dairy products (28, 93).

Most of the immunoassays involving Pseudomonas are directed against the heat-stable enzymes produced by these psychrotrophic organisms. Researchers have used ELISAs to partially characterize heat-stable proteinases from Pseudomonas spp. of dairy origin (4) and for the detection of P1-like enzymes from Pseudomonas spp. (81). Clements, et al. (23) developed an ELISA which could detect .24 - 7.8ng of protease from Pseudomonas fluorescens in ultra-high-temperature pasteurized milk. The problem with detection of heat-stable enzymes from pseudomonads for determining shelf-life lies in the fact that the enzymes are immunologically heterogeneous. Therefore, the detection of Pseudomonas whole cells for shelf-life determination is more desirable. ELISA has been very successful for detecting microbial cells such as Salmonella and Listeria (29, 60). ELISAs have also been successfully used in a milk system. ELISAs have been developed to detect IgA specific for enteropathogenic E. coli (68), bovine somatotropin and zearalenone, (5, 102), as well as other antibodies produced against foreign matter in a milk system (91).

Again, the objectives of this research were to develop an ELISA for the detection of the fluorescing group of Pseudomonas and to use this ELISA to determine the potential shelf-life of milk.

MATERIALS AND METHODS

DEVELOPMENT OF ELISA

POLYCLONAL ANTISERA

Preparation of immunogen

Pseudomonas fluorescens ATCC 949 was inoculated into 400ml of trypticase soy broth (TSB) and incubated at room temperature for 18-24h. Cells were enumerated by direct microscopic count and spread plates. An equal volume (400ml) of 0.6% sterile formalinized saline was added and allowed to stand for 2d at room temperature. When cells were no longer viable, as determined by streak plating, they were harvested by centrifugation (6800-8000 x g , 15min, 4°C). Cells were resuspended in 0.3% sterile formalinized saline (0.85%) and pooled together (final volume 50-100ml). Cell concentrations were adjusted to 6×10^9 cells/ml. Cells were enumerated by a direct microscopic count, aliquoted (7ml), and frozen at -20°C.

Preparation of polyclonal antisera

Polyclonal antibodies were produced against Pf949 and against 2 vaccine mixes of Pf949, Pf13525, *P. fragi*, *P. putida* and *P. aeruginosa* in ratios of V1 (1:1:1:1:1, respectively) and V2 (1:1:2:2:1, respectively), by injecting

young female New Zealand White Rabbits intraperitoneally with whole cells of the target microorganisms. Before primer injection, 5ml of blood was obtained, by bleeding from the ear, for pre-immune sera. The rabbit was immunized with 1.0ml of an aqueous in oil emulsion consisting of 1.0ml immunogen dispersed in 2.0ml Freund's incomplete adjuvant (Sigma F5506) and 2 x 10^8 cells/ml were injected at each immunization. The rabbits were immunized twice a week for 2 weeks and then allowed to rest for 1 week. Immunization occurred again at the end of the third week with a test bleed at the end of the fourth week. Next, the rabbits were immunized once every 2 weeks until the eighth week, then one time every month. The rabbits were bled and titers were checked 7d after each booster. When titers began to decline, blood was obtained by exsanguination. Blood was allowed to clot for 1h at RT and centrifuged (1000 x g, 10min, 4°C). Serum was drawn off, aliquoted and frozen at -20°C.

Assessment of antisera

Antibody titers were monitored by an ELISA in which 100ul samples of formalin treated Pf949 cells (6 x 10^9 cells/ml), diluted 1:250, 1:500, and 1:1000 in coating buffer (1.59g Na_2SO_3, 2.93g $NaHCO_3$, 0.2g NaN_3, to 1 liter in dH_2O. pH 9.6), were coated to a microtiter plate (Dynatech

Immulon 2) for 4h at RT. Antisera dilutions of 1:1000, 1:10,000, and 1:100,000 and a pre-immune sera dilution of 1:1000 were made in PBS-tween (PBS plus 0.5ml Tween 20) and 100ul added, after washing, so that all combinations of antigen and antisera were allowed to react. Washing constisted of adding 100ul of PBS/tween 3 times and discarding. After a 30 min incubation, followed by washing, 100ul of a 1:2000 dilution of goat anti-rabbit IgG peroxidase conjugate (Kirkegaard) in PBS/Tween was added. The samples were incubated for 30 min, washed, and then ABTS Peroxidase Substrate (Kirkegaard, Lot# NA28) (100ul) was added. The reaction was stopped after 30 min by the addition of 2% oxalic acid (100ul). Negative controls were run for each dilution of antisera to determine nonspecific binding. Rabbit IgG standard was initially coated to the plate as a positive control for the conjugate. Microtiter plates were read in a microtiter plate reader (Titertek Multiscan MCC) at 414nm.

MONOCLONAL ANTIBODIES

Preparation of inner and outer membranes

Pseudomonas outer membranes were prepared for production of monoclonal antibodies in mice. Cells were harvested by centrifugation (8000 x g, 4°C, 10min) after

growth in 4-liters of TSB for 18 - 24h at RT. Pellets were resuspended in a total of 20ml 0.03M Tris buffer (pH 8.0), containing 1mg DNase, 1mg RNase, and 20% sucrose (W/V). The cell suspension was passed 2 times through a French Press at 15,000 lb/in^2. An aliquot (2ml) of lysozyme (1mg/ml) was added; after 10 min phenylmethanosulfphonylfluoride (PSMF) was added to a final concentration of 1mM. This preparation was centrifuged (1000 x g, 4°C, 10min) to remove cell debris. The supernatant was collected and 14ml of 0.03M Tris buffer (pH 8.0) added. The preparation was stored at -20°C until used.

Inner and outer membranes were prepared by sucrose density gradient ultracentrifugation in which 4ml of 70% sucrose made up the bottom layer, 4ml 60% sucrose made up the upper layer and supernatant preparation was carefully layered on top. Tubes were centrifuged at 183,000 x g (SW40 rotor) overnight (12-18h). Bands were collected, washed twice with dH_2O, and were collected by ultracentrifugation at 183,000 x g for 1.5 - 2h. Pellets were resuspended in a small amount of 0.03M tris buffer (pH 8.0) plus .002% NaN_3. Protein determinations (Biorad assay) were performed on solubilized membrane preparations. The 2-keto-3-deoxyoctonic acid (KDO) and the succinate dehydrogenase (SDH) assays were also preformed to detect the outer and inner membrane markers, respectively. KDO was performed by

the following method: 120ul of outer membrane (0.05 - .10 mg protein) was added to 150ul 0.04N periodic acid solution in 0.125N sulfuric acid and incubated 20 min at RT. Three-hundred ul of 0.2M sodium arsenite solution in 0.5N HCl was added and allowed to set for 2 min at RT; 1.2ml of 0.3% thiobarbaturic acid solution (TBA) (pH 2.0) was added followed by heating for 15 min at 100°C. Sample was cooled to RT and filtered (0.45um) to remove precipitate. Absorbance was read at 548nm (74). The SDH assay was performed by adding xml 50mM Tris buffer (pH 8.0), 20ug membrane protein (sample), 50ul 0.5 M succinate (disodium in 50mM Tris buffer, pH 2.0) and 50ul 0.2 M KCN (in 50mM Tris buffer, pH 2.0) in the order listed. The preparation was then incubated for 5 min followed by the addition of 30ul 2,6-dichlorophenol-indophenol (0.667 mg/ml in 50mM Tris buffer, pH 2.0) and 20ul phenazine methosulfate (PMS) (0.5mg/ml in 50mM Tris buffer, pH 2.0). The PMS was prepared fresh daily. The final volume was 1.0ml and timing began immediately following the addition of PMS. Absorbance values at 600nm were obtained at 30 sec intervals for 15 min at RT (31).

Determination of LPS

LPS was extracted from outermembrane preps by trichloroacetic acid (TCA) precipitation and by phenol

extraction (40). LPS was measured before and after extraction by the KDO assay as described above.

Production and assessment of monoclonal antibodies

Four to eight week old BALB/c mice were immunized with outer membrane preparations and formalin treated cells. Thirty ug of outer membrane or 200ul (3.0×10^8 cell/ml) of immunogen were injected intraperitoneally (IP) every 7d until the titers maximized. The first injection was done in 50% Freund's complete adjuvant (Miles Lab, 64-285), all subsequent injections were done in 50% Freund's incomplete adjuvant. Immunogen was injected IP for 3 consecutive days prior to fusion with NS-1 myeloma cells (ATCC). The production of monoclonal antibodies was done at the Anaerobe Laboratory at VPI & SU under the direction of Dr. Tracy D. Wilkins.

ELISA was performed for assessment of ascities fluid and hybridoma supernatant as described above for polyclonal antibodies except: 1) microtiter plate was coated with 1:250 of the immunogen, 2) goat anti-mouse IgG conjugate was used, and 3) mouse IgG standard was used at a 1:1000 dilution as a positive control. One-hundred ul of hybridoma supernatant was loaded directly into the wells. The ascities fluid was diluted 1:10 - $1:10^4$. The hybridomas produced were screened against LPS to determine which of the

monoclonal antibodies were specific for proteins. LPS (50ug) prepared by phenol extraction was coated to a microtiter plate for screening.

SHELF-LIFE STUDY

Milk sample preparation

Sterile two-percent milk samples were inoculated with target concentrations of 0 (control), 100, and 1000 cells/ml of Pseudomonas fluorescens ATCC 949. One-hundred ml samples of milk were steam sterilized for 3 min and held at 7°C until inoculated the following day. Cells were grown up in TSB at 21°C for 24h; an aliquot (1ml) was transferred to a sterile 99ml 2% milk blank for 18 - 24h at 21°C to provide a final concentration of approximately 1 x 10^8 cells/ml. The cells were diluted in 99ml 2.0% milk blanks to 10^5 and 10^4 cells/ml. An aliquot (1ml) of each dilution was added to milk samples to give final target concentrations of 1000 and 100 cells/ml. Plate counts were performed to determine exact numbers of organisms inoculated.

Bacterial enumerations

Bacteria were enumerated by the crystal violet triphenyl tetrazolium chloride count (Standard methods agar

plus 1ppm crystal violet and 50ppm triphenyl tetrazolium chloride (TTC)) (CVT) (49), the modified psychrotrophic bacterial count (mPBC) (21°C for 48h) (72), and by the coliform count (32°C, 24h) (70). mPBC and coliform counts were performed on petrifilm.

Preliminary incubations

Preliminary incubations were performed with milk alone (control), milk with broth (1:1), and milk with broth and benzalkonium chloride (1:1:0.1). Samples (10ml) were incubated at 21°C for 18h.

Standard curve construction

A standard curve was obtained each testing day. cells (1×10^8 cell/ml) were grown up as described previously and serially diluted in dairy dilution buffer (DDB: in SMEDP). Dilutions ranged in concentration from 10^7 to $10^{3.5}$. Dilutions of standards were coated on the microtiter plate simultaneously with the samples. Exact numbers of cells plated were determined by CVT and mPBC counts.

ELISA

Samples (100ul) were coated to the plate for 4h at RT (or overnight at 4°C) in dairy dilution buffer at the appropriate concentrations to fit with in the standard

curve. After incubation, the samples are discarded by shaking out the microtiter plate and the plate was washed 3 times. Washing was conducted by adding 100ul of PBS/Tween (pH 7.3) to the microtiter plate, discarding, and repeating 2 more times. Next, the anti-Pf949 antibody was diluted 1:1000 in PBS/Tween and 100ul were added to the plate. After a 30 min incubation, the antisera was discarded and the plate was washed. The conjugate was diluted 1:2000 in PBS/Tween, and 100ul were added to the plate for a 30 min incubation. After discarding the conjugate and washing, 100 ul of ABTS substrate was added. The reaction was stopped by the addition of 100ul 2% (W/V) oxalic acid after 30 min. Results were read as previously described. Both antibody and conjugate were stored at -20°C.

Experimental design

On day 0, the freshly inoculated samples were assayed by ELISA. Duplicate samples were coated on the plate undiluted and as a 1:10 dilution in DDB. CVT, mPBC, and coliform counts were also conducted.

Aliquots of each sample were also collected on day 0 to conduct preliminary incubations. Following preliminary incubation (day 1), samples were tested by ELISA, CVT, and mPBC. Samples for ELISA were diluted 1:10 and 1:100 in DDB.

Samples were analyzed one week later following the same

procedures as described above. Samples for ELISA were diluted 1:10 and 1:100 in DDB before preliminary incubation and 1:100 and 1:1000 after preliminary incubation.

Sensory evaluation

The original inoculated samples were held at 7°C and analyzed every other day by sensory evaluation to determine shelf-life. As samples approached their shelf-life the testing frequency was increased to daily. The product's shelf-life was considered to be the day before samples were determined unconsumable. Sensory evaluation was performed by three experienced judges. A score of 5 or below on the ADSA scale was considered unacceptable.

RESULTS AND DISCUSSION

DEVELOPMENT OF ELISA

Evaluation of Polyclonal Antisera

Cross reactivity and sensitivity

The best antibody titer was against Pseudomonas fluorescens ATCC949 (1.29×10^{-4}), as expected (Table 1). The titer was approximately the same for Pf13525 (1.27×10^{-4}) but dropped off against the other species of pseudomonads tested, including, P. fragi, P. putida, and P. aeruginosa.

Live cells of the fluorescing group of pseudomonads, including, Pf13525, Pf948, P. putida, and P. aeruginosa were tested for cross-reactivity with the anti-Pf949 sera (Table 2). In addition, P. fragi, Staphylococcus aureus, Bacillus cereus, and Escherichia coli were tested for cross-reactivity by the same ELISA procedure. Results indicated inconsequential cross-reactivity for B. cereus, and S. aureus (Table 3). These organisms were detected at 10^6 and 10^8 cells/ml in buffer and milk, respectively. Neither B. cereus nor S. aureus are likely to be present at such high concentrations and, therefore, should not interfere with the detection of targeted organisms. However, since both organisms are thermoduric, a possibility exists for

TABLE 1

Titer of anti-Pf949 antisera against other Pseudomonas as determined by ELISA.

ORGANISM	TITER OF ANTI-PF 949 ANTISERA
P. fluorescens 949	1.29×10^{-4}
P. fluroescens 13525	1.27×10^{-4}
P. fragi	1.21×10^{-2}
P. putida	2.25×10^{-2}
P. aeruginosa	4.79×10^{-2}

Titers determined with formalin-treated cells.

TABLE 2

Cross-reactivity of anti-Pf949 antisera against microorganisms belonging to the fluorescing group of *Pseudomonas*

	Cells/ml detected	
	Diluent	
Microorganism	Milk	Buffer
Pf 949	10^5	10^4 - 10^5
Pf 13525	10^6	10^5 - 10^6
Pf 948	$>10^6$	10^5 - 10^6
P. putida	$>10^6$	10^5 - 10^6
P. aeruginosa	$>10^6$	10^6

Experiments conducted with live cells diluted from 1×10^7 to 1×10^4 in milk and buffer.

TABLE 3

Cross-reactivity of anti-Pf949 antisera against microorganisms other than the fluorescing group of pseudomonas

Microorganism	Cells/ml detected	
	Diluent	
	Milk	Buffer
P. fragi	$>10^6$	10^5
B. cereus	10^8	10^6 - 10^7
S. aureus	10^8	10^6
E. coli	ND*	ND

*E. coli was not detected at 10^9 cells/ml.

Experiments were conducted with live cells diluted from 10^9 - 10^5 cells/ml, except for P. fragi which was diluted from 1 x 10^7 to 1 x 10^4 cells/ml.

gram-positive contamination. The ELISA developed would indicate that a problem exists, but would not be able to distinguish which organisms were responsible. This cross-reactivity could be due to the rabbit having had previous exposure to these organisms. E. coli was not detected at 10^9 cells/ml.

Coating media

Antibody specificities for live and formalin-treated cells were compared in different coating media (Table 4). Cells (live and formalin-treated) were suspended in PBS or milk and diluted in PBS or milk to give 3 combinations prior to testing by ELISA. Live cells suspended in PBS and diluted in PBS were detected at the same level as formalin-treated cells, but the absorbance value was higher for the live cells (0.358 vs 0.187). Live cells suspended in milk and diluted in PBS were detected at lower levels than the formalin-treated cells (Table 4). These results are encouraging since the test samples were live cells suspended in milk and diluted in buffer. Buffer was used as a diluent rather than milk because the assay has greater sensitivity in buffer. These results were supported by Stein-Erik (87) who found a 10-fold decrease in sensitivity for detection of the heat-stable proteinase from Pseudomonas fluorescens P1 in milk compared to buffer.

TABLE 4

Detection of formalin-treated cells and live cells of *Pseudomonas fluorescens* in PBS and skim milk

Pf949 detected	Stock soln/ diluent	Number of bacteria per ml
Formalin	PBS/PBS	$3 \times 10^3 - 3 \times 10^4$*
treated cells	MILK/MILK	$>3 \times 10^6$
	PBS/MILK	3×10^5
Live cells	PBS/PBS	$3 \times 10^3 - 3 \times 10^4$*
	MILK/MILK	3×10^5
	MILK/PBS	$3 \times 10^2 - 3 \times 10^3$

*Live cells suspended in PBS and diluted in PBS had higher absorbance readings than the formalin treated cells.

The effect of different coating buffers on detection of live cells was determined. Coating buffer, PBS, and DDB were compared. Coating buffer appeared to hinder the assay of live cells (data not shown). Absorbance readings for 10^7 cells/ml were only half the intensity obtained in PBS or DDB. No difference could be detected in linearity or sensitivity when cells were dispersed in PBS or DDB. DDB was chosen as a more convenient buffer for a dairy plant to use.

Detection of dead cells

The ability of the antisera to detect dead cells was also determined. Equal numbers of live cells at a concentration of 10^9 cells/ml were inoculated into duplicate milk samples and steam sterilized to kill the cells. Live cells were re-inoculated into two samples, one containing steam sterilized cells and one with no cells previously inoculated. All samples were tested by ELISA. No difference was observed in the ability of the antibody to detect cells in DDB or milk samples which had been steam sterilized. Live and dead cells were detected to a level of 10^5 cells/ml. Since a raw milk sample is not likely to contain 1×10^5 cfu/ml of pseudomonads, which is the limit for the standard plate count for grade A raw milk, dead cells should not be detected. However, detecting dead cells

would be desirable since raw milk quality affects the shelf-life. The effects of pasteurization on the cells is not known.

Effects of freezing on cells

The effects of freezing on cells was also determined. Live cells were frozen in sterile skim milk and in PBS. The amount of time frozen and the number of freeze thaw cycles the cells endured were tracked. ELISAs were run over a period of 19d for frozen cells (thawed once) and for cells enduring 3 - 4 freeze/thaw cycles. The ELISA results were not affected by the freezing processes.

Effects of milk fat

Possible interference by milk fat was examined. Whole raw milk, obtained from the Virginia Tech dairy, was centrifuged at low speed and filtered to give skim milk. The whole and skim samples were steam sterilized and inoculated with live organisms. The amount of milk fat did not appear to interfere with the assay at either high or low detection levels when samples were diluted in PBS.

ANTI-PSEUDOMONAS SPP. ANTIBODIES

The anti-Pseudomonas species antiseras (anti-V1 and anti-V2) were produced in hopes of increasing the

sensitivity of our ELISA. The anti-V1 sera yielded slightly higher titers to all the Pseudomonas species and to the vaccine mix V2 (Table 5). Therefore, the anti-V1 sera was used in comparison to the Pf949 antisera. The antisera developed against Pf949 was found to be more sensitive than the antisera prepared against V1 when different concentrations of Pf949 were used as antigen (Table 6). The anti-V1 sera was more sensitive when 20% to 14% of the total number of pseudomonads were Pf949, however, it is not likely to find Pseudomonas fluorescens at these lower levels.

MONOCLONAL ANTIBODIES

The mice challenged with 30ug of the outer membrane preparation became immediately ill and later died. The mice had received the equivalent of 1.8×10^{12} cells, which would contain enough LPS to kill the mice. Extraction of LPS by both the phenol extraction method and the TCA precipitation method gave approximately 50% recovery of protein with 35 - 65% LPS still remaining. Therefore, the mice were challenged with whole cells of formalin-treated Pf949 (immunogen) at a level of 3×10^{8} cells/ml instead of outer membranes.

Hybridomas were produced which reacted with LPS and proteins on the outer membrane of Pf949, however, problems

TABLE 5

Comparison of anti-*Pseudomonas* species antiseras to various *Pseudomonas* species and to the vaccine mixes

Species tested	Absorbance values at 414nm	
	Anti-V1 antisera	Anti-V2 antisera
Pf949	0.25	0.23
Pf13525	0.37	0.35
P. fragi	0.25	0.23
P. putida	0.26	0.22
P. aeruginosa	0.13	0.03
V1[1]	0.58	0.69
V2[1]	0.71	0.65

[1]V1 and V2 have the same compositions as previously described.

All preparations consisted of 3×10^6 cell/ml with antisera diluted $1:10^5$.

TABLE 6

Comparison of the polyclonal antiseras produced against Pf949 and the V1 mixture to various preparations of *Pseudomonas* spp.

	Absorbance values at 414nm	
Percentages of Pf949	Anti-V1 antisera	Anti-Pf949 antisera
100% Pf949	0.12	0.27
90% Pf949[1]	0.16	0.32
80% Pf949[1]	0.16	0.30
70% Pf949[1]	0.17	0.36
50% Pf949[1]	0.16	0.26
V1(20% Pf949)[2]	0.32	0.28
V2(14% Pf949)[2]	0.45	0.30

[1]Remaining percentage of sample was divided evenly between *P. fragi*, *P. putida*, and *P. aeruginosa*.

[2]V1 and V2 have the same compositions as previously described.

All preparations consisted of 3×10^6 cell/ml with antisera diluted $1:10^6$.

with growing them up and keeping them viable were experienced. Also, the monoclonal antibodies produced reacted only with Pf949, whereas the polyclonal antibodies showed preferred cross-reactivity with the other Pseudomonas spp. Therefore, research efforts were focused on the utilization of the polyclonal antisera for the ELISA and the shelf-life study.

SHELF-LIFE STUDY

A typical standard curve from the ELISA is illustrated by the plot of absorbance at 414nm vs bacterial numbers of Pf949 (Fig 1). This curve is linear between 10^6 and 10^4 cells/ml with a correlation of 0.998. Standard curves conducted with each experiment were linear with correlations ranging from 0.910 to 0.998.

Since absorbance values could not be compared due to different dilution factors, results were based on bacterial numbers obtained from the standard curve. The mPBC gave a good indication of the actual number of organisms detected by the standard curve. The numbers obtained from the standard curve and those from the mPBC were not significantly different based on Duncan's Multiple Range Test (Table 7). However, the CVT count was in a different Duncan grouping for all data sets except samples which were not subjected to a preliminary incubation. The CVT count

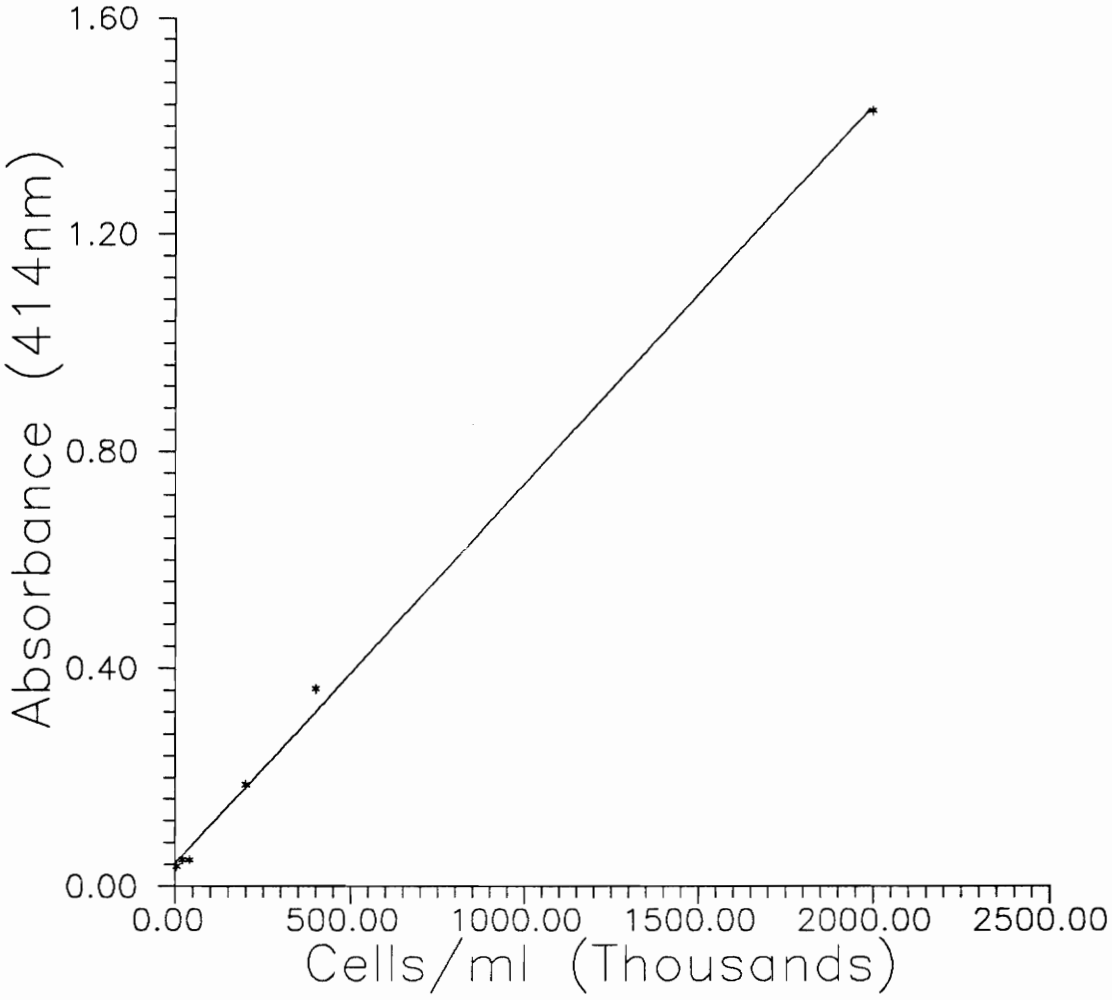

FIGURE 1: Standard curve of absorbance at 414nm vs bacterial number, R=0.998 (n=6)

TABLE 7

Results of Duncan's Multiple Range Test for bacterial numbers based on the CVT count, the mPBC, and the ELISA standard curve

	DUNCAN GROUPINGS		
	Sample (pre PI)	Broth PI	Milk PI
CVT	B	B	B
mPBC	A/B	A	A
ELISA CONC	A	A	A

was not significantly different from the mPBC count, but it was from the ELISA standard curve.

Scatterplots of log number of bacteria (Pf949) vs shelf-life (in days) were constructed with all 3 sets of data, and predictive equations were obtained. The correlation coefficients (Table 8) indicate that the samples with a preliminary incubation conducted in milk alone gave the best results when related to shelf-life. The quadratic relationship for this sample set gave correlations of -0.861 and -0.741 for R and R^2 respectively. The samples which were given a preliminary incubation in broth (1:1) were of limited value for predicting shelf-life, with only the linear predictive equation having values of significance. Samples undergoing no preliminary incubation were not linearly or quadratically related to shelf-life. Only the scatterplot (Fig 2) and predictive equation for the best relationship are illustrated. The quadratic predictive equation obtained for samples with a preliminary incubation conducted in milk alone is:

$$SL = -0.4091Y^2 + 3.7277Y - 0.2497$$

SL is shelf-life of sample (in days)

Y is the CFU/ml obtained from the ELISA

TABLE 8

Correlation coefficients obtained for all data sets based on linear and quadratic relationships

	Correlation coefficients			
	Linear		Quadratic	
Sample set	R	R^2	R	R^2
No PI	-0.0332	-0.0011*	-0.6105	-0.3727
PI(MILK)	-0.8186	-0.6701	-0.8607	-0.7408
PI(BROTH)	-0.8341	-0.6957	-0.8383	-0.7027*

*The coefficients for the predictive equation were not significant at the 0.05 level.

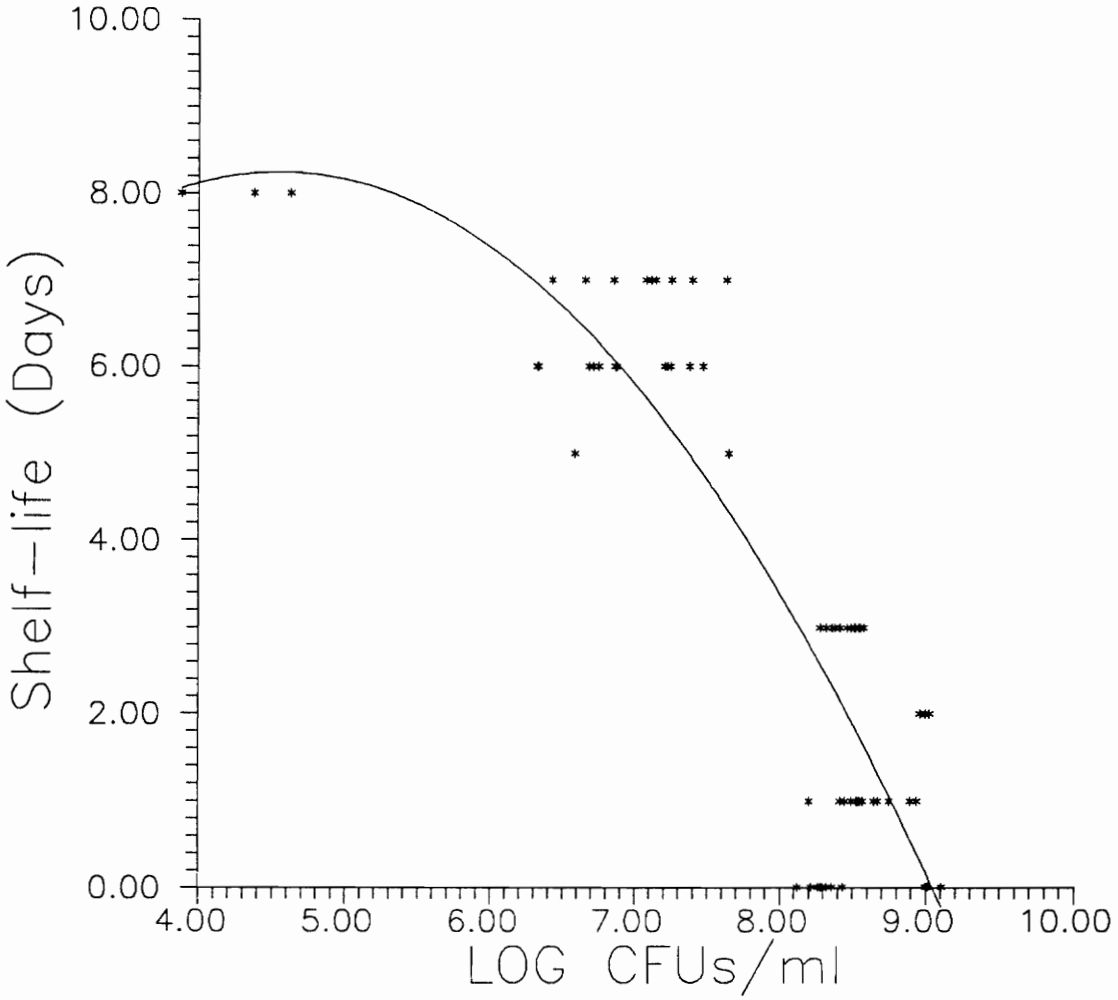

fIGURE 2: Scatterplot of shelf-life (in days) vs LOG CFU/ml from the PI-ELISA method. (n=76)

A problem with the samples which were not given a preliminary incubation was that no data could be obtained from day 0. The shelf-life range was too narrow since there were too few organisms to detect. Benzalkonium chloride (0.1%) was found to interfere with the detection of cells in the ELISA. Therefore, the selective PI was dropped.

An experimental limitation was the sample size of 100ul. By coating only 100ul of sample to the plate, a representative sample was not obtained, especially when the number of cells in the sample was low and drawn from a large population (100ml). With the preliminary incubated samples, aliquots (100uls) were drawn from 10ml rather than 100ml, plus, the number of cells in the samples was greater. Therefore, a more representative sample should be attained with the preliminary incubated samples.

CONCLUSIONS

The ELISA that has been developed is unique from other ELISA methods since the assay is designed to detect a broad spectrum of microorganisms. We are looking to detect a group of psychrotrophic microorganisms, not just one particular organism. This diversity is a key to quality control in the dairy industry as not just one organism contributes to spoilage. For this reason, polyclonal antibodies are more desirable for a shelf-life determination. The monoclonal antibodies produced were too specific, with little cross-reactivity, whereas the polyclonal antisera produced did show some preferred cross-reactivity with the fluorescing group of pseudomonads.

The ELISA performed in conjunction with a preliminary incubation with milk alone for 18h at 21°C shows the most potential as a shelf-life test. The test is both rapid and sensitive. The total test time, including preliminary incubations, is 24 hours, which is much shorter than the 7 - 9 days required for the Moseley Keeping Quality Test. The ELISA allows for effective corrective measures to be taken by the processor. The test is sensitive down to 10^4 cells/ml and is relatively inexpensive.

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