AEROSOL EXPOSURE BIOTESTING FOR PACKAGE INTEGRITY TESTING

by

Scott W. Keller

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

of

MASTER OF SCIENCE

in

Food Science and Technology

APPROVED:

Joseph E. Marcy

Dr. Joseph E. Marcy, Chair

Cameron R. Hackney

Dr. Cameron R. Hackney

Barbara Blakistone

Dr. Barbara Blakistone

Susan E. Duncan

Dr. Susan E. Duncan

January 5, 1995

Blacksburg, Virginia

AEROSOL EXPOSURE BIOTESTING PROCEDURES FOR PACKAGE INTEGRITY TESTING

by

Scott W. Keller

Joseph E. Marcy, Chair

Food Science & Technology

(ABSTRACT)

The objective of this study was to determine how hole diameter, channel length, test organism motility, concentration and aerosol exposure time affected microbiological contamination of sealed flexible pouches. Nickel microtubes with 10 μm and 20 μm hole diameters and lengths of 5 mm and 10 mm were used in various combinations to create seal defects in 128 retortable pouches. A 119,911 cm^3, exposure chamber was used to distribute an aerosol with a particle size of 2.68 μm, infected with motile and isogenically mutated nonmotile *Pseudomonas fragi* TM 849 in concentrations of 10^2 or 10^6 cells/mL. Fifteen and 30 minute aerosol exposure times were used. Six pouches tested positive for test organism growth after a 72 hour incubation period. Pouch contamination via microbial ingress was significant ($P < .05$) for test organism motility (motile) and concentration (10^6 cells/mL).

DEDICATION

This thesis is dedicated to my father Gerald W. Keller.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my research advisor, Dr. Joseph E. Marcy, for his suggestions, guidance, patience, understanding and assistance which, without, my research work would have not been possible.

I would like to extend further appreciation to my committee members, Dr. Cameron Hackney, Dr. Barbara Blakistone and Dr. Susan Duncan, for their interest, advisement and input throughout my research work.

Very special thanks to George H. Lacy for his priceless advices and guidance of bacterial isogeneic mutation, to John Chandler for the construction of the aerosol chamber, and Harriet Williams for her constant support.

Finally, I would like to express my deep appreciation to Mary Jane Thompson whose thoughtfulness touched each page of this text.

TABLE OF CONTENTS

LISTS OF FIGURES

LISTS OF TABLES

I. INTRODUCTION

The rapid expansion of the food packaging industry can be mostly attributed to new applications of flexible and semi-rigid packaging materials (Gnanasekharan and Floros, 1994). Morris (1989) correctly predicted sales of semi-rigid and flexible packaging products for convenience foods to exceed $696 million by 1993. Increased usage of these materials combined with advances in automated processing operations call for the resolution of factors that contribute to post-process contamination.

One hundred percent on-line defect inspection and evaluation of packaged products has been a long time goal of food manufacturers. A current method of package integrity evaluation is biotesting by immersion. Biotesting is not a realistic indicator of package integrity. Most food or medical packages are not immersed in concentrated bacterial suspensions during storage and distribution (Gnanasekharan and Floros, 1994). Also, biotesting is a destructive test method that requires days rather than seconds to receive results.

Market availability of various nondestructive package defect detectors verifies industry's interest in reliable, efficient, and cost effective on-line inspection components. The majority of today's inspection components are based on some variation of the pressure difference principle (Floros and Gnanasekharan, 1992). Equipment capable of detecting package micro-defects were developed without prior knowledge of the threshold defect (pin hole or channel leaker) size critical to sterility maintenance and integrity of the package. The methods of microbial ingress into packaged foods under storage and distribution conditions, threshold leaker size and its effect on post-process contamination must be understood.

II. REVIEW OF LITERATURE

1. Post-Process Contamination of Flexible Pouches

Packaging researchers consider leakers to be the primary source of post process contamination (Anema and Schram, 1980; Davidson and Pflug, 1981; Placencia et al., 1988; Kamei et al., 1991). Methods of leaker detection and standards by which semi-rigid and flexible containers are evaluated were originally developed for metal cans. Gnanasekharan and Floros (1994) state that package types and materials should be considered before detection or test limits are set. They argue that small leaks in cans may permit microbial ingress, whereas similar size holes in aseptic or other packaging may not.

Put and Warner (1972) identified seven factors which historically have contributed to post-process contamination of canned, semi-rigid and flexible packaged food products. They are: the mechanical construction of the container, defect size, double seam and seal integrity, contamination during processing, physical damage of the double seam or seal, sub-atmospheric to atmospheric pressure change, and pressure fluctuations outside the container.

1.1. The Mechanical Construction of the Container

Metal cans are evaluated on the basis of mechanical construction. Cans are characterized by measurable parameters such as seaming, type of lining compound, thickness and position in the double seam or laminate, and the double seam construction (Arndt, 1992).

Semi-rigid and flexible container construction is different from metal cans. Metal alloys are used for the construction of cans while plastics, paperboard, aluminum foil or composites of these materials are used for the construction of semi-rigid and flexible containers. Heat sealability and peelability are the areas of concern in the mechanical construction of semi-rigid and flexible containers (Arndt, 1992).

Parameters such as sealing temperature, sealing pressure and dwell time must operate in the confines of a specific range (material dependent) to ensure adequate sealing. These parameters may be manipulated to accommodate different types or combinations of materials used in the packaging process. For example, a higher sealing temperature used in the packaging process generally requires lower pressure and a shorter dwell time (Arndt, 1992).

Barrier and migration properties of the material(s) used in the construction of semi-rigid and flexible containers must also be considered. Poor barrier material(s) fail to provide gas or chemical permeation into the package, and could result in a reduction of quality and shelf-life of the product (Placencia et al., 1988). Materials which allow migration create opportunity for gas leakage or permeation through seams via channel leakers or through the body wall of the containers via pin hole leaks. Permeation may also occur through the bound polymer layers in laminated barrier plastic sources (Reich, 1983).

1.2. Defect Size

The diameter, profile, shape and length of the leak pathway can have a measurable effect on capillary action within the passage way of the double seam of metal cans. Semi-rigid and flexible container considerations are more complex. Factors such as length to diameter ratio of the leak, the internal geometry of the leak (straight/tortuous or smooth/rough) and the pressure differential across the leak interface, must be considered (Gnanasekharan and Floros, 1994).

1.3. Double Seam and Seal Integrity

The atmosphere outside of the container may contain a variety of air borne contaminants, including toxin producing bacteria (Kamei et al., 1991). Bacterial characteristics such as motility, ability to multiply and multiplication rate are then of great concern (Put and Warner, 1972). These factors inpart or in combination could contribute to post process contamination.

Although most semi-rigid and flexible containers are not retorted, seal integrity is still the main area of concern, especially for aseptically filled or hermetically sealed products (Nichols, 1989). Seal integrity of semi-rigid and flexible containers and double seam integrity of cans, greatly influence shelf-life and, inevitably, consumer confidence in the product. Nichols (1989) reported that seal contamination, poor control of sealing parameters, mechanical alignment problems, and changes in packaging materials contribute to seal failure in semi-rigid and flexible containers.

1.4. Contamination During Processing

Long exposure periods to contaminated liquids may increase the window of opportunity for post process contamination of the canned items. Contaminated cooling water used in production facilities may eventually make contact thru pinholes or channel leakers with the contents of the container. This may result in a bacteria conveyed along with the cooling water, to reach a fertile environment conducive to survival and growth (Put et al., 1972; Shapton and Hindes, 1962).

1.5. Physical Damage of the Double Seam or Seal

Deformations result in special risk when the double seam adjacent to the side seam (the cross-over zone of a can) receives several impacts. Damaged areas of the seam(s) exposed to contaminated liquids during processing, distribution, or other handling may result in product contamination (Arndt, 1992).

Semi-rigid and flexible materials may fail to adequately protect the food product under normal storage and distribution conditions due to physical, mechanical and structural deficiencies of the package. Semi-rigid and

flexible containers are susceptible to burst defects and seal creep due to abuse (shock and vibration or temperature) received during product storage and distribution. Burst resistance must be determined for each material used in the container to reduce the potential for package failure (Arndt, 1992).

Seal creep also involves stressing the seal over a period of time under a specific set of conditions and measuring the time to failure (Matty et al., 1991). Burst resistance and seal creep are important measures of the resistance of the container to potential distribution, temperature or consumer abuses.

1.6. Sub-atmospheric to Atmospheric Pressure Change

During retorting, a vacuum is created due to the pressure difference between the inside and the outside of the can. Opportunity for microbial ingress is great, if a leaker is present, as the pressure differential slowly equilibrate during cooling (Arndt, 1992).

1.7. Pressure Fluctuations Outside the Container

Spoilage of packaged foods may result in visibly distorted containers, especially in canned foods, due to the production of gas by microorganisms. However, some container abnormalities are directly related to overfilling, buckling, or denting while cooling (Kautter et al., 1984). Environmental conditions such as high temperatures and high altitudes also play key roles in the swelling of cans and semi-rigid and flexible containers (Kautter et al., 1984).

2. Mechanical Leak Detection

Gross abnormalities detectable by sight or touch such as, mechanical defects, perforations, delamination, swelling, flex cracks, and malformations, can provide insight as to the cause of the defect. These defects should be measured and noted as the container is inspected (Arndt, 1992). If evidence of microbial intrusion is present, further investigation is required.

Fine examination such as microscopy, SLAM (scanning laser acoustic microscopy), SAM (scanning acoustic microscopy), ultrasound, etc., can be used in combination with direct visual inspection. Fine examination can be

helpful when difficulties exist in detecting problem areas within seals of the containers (Arndt, 1992).

Most leak testing techniques focus on the escape or entry of liquids or gases (pressurized or evacuated) from systems designed to contain them. Gas or liquid escape is a result of pressure differentials formed between two regions (Anderson, 1989; Axelson and Calvin, 1991). Gas, liquid or mass transfer models are currently based on the dynamics of relatively large ($\geq$ 1000 μm) diameter tubes, not 1 μm pin holes (Alves and Boucher, 1963; Fuchs, 1964). Because flexible and semi-rigid food containers may be more easily manipulated by variables within the environment such as abuse during storage and distribution, more opportunities for disfigurement or breach of seal integrity of a complex nature exist than do with cans (Kamei et al., 1991).

The following techniques are accepted methods for leaker detection, identification and characterization within semirigid and flexible containers.

2.1. Air Leak Testing

The objective of this test is to identify leakers by observing a measurable pressure loss within the container. Two methods of air leak testing are the wet method and the

dry method. The dry method requires injection of air into the container until a standard pressure is reached. The standard pressure used for testing should be less than the burst pressure for the package. The process is monitored for a period of 60 seconds (Arndt, 1992).

The wet method uses the same approach as the dry method except that the container is completely submerged. Leaks are detected visually as a result of the presence of bubble streams rather than by pressure changes identified by a pressure gauge (Anderson, 1989; Placencia and Peeler, 1990; Arndt, 1992).

2.2. Burst Testing

Burst resistance, or burst pressure, is defined as the internal container pressure required to cause failure of the container seal (Matty et al., 1991). Burst testing is used to determine the ability of a sealed container to withstand various internal pressure ($kPa/cm^2/sec$) ranges (Arndt, 1992). It is considered a general indicator of abuse resistance. Restrained and unrestrained burst testing are used to identify a separation between packages with strong seals and those with weak seals by determining the pressure at which seal failure occurs (Arndt, 1992). Restrained, or

static burst testing, allows a steady increase of internal pressure (usually 6 kPa/cm^2/sec) to a pressure slightly less than that required to cause seal failure, and is held for 30 seconds (Anderson, 1989). Unrestrained, or dynamic burst testing, involves a steady increase of internal pressure (usually 6 kPa/cm^2/sec) until seal failure occurs. Two factors influence parameters for burst resistance: container pressurization rate and restraint height (Matty et al., 1991). The pressurization rate is defined as the rate at which the internal pressure of the container under use or abuse (test) is increased. The restrained height is defined as the distance above the container flange when the container lid is allowed to flex freely via expansion or contraction (Matty et al., 1991).

2.3. Chemical Etching

Chemical etching allows visual examination of the area(s) suspected of leakage. The laminated seal area is separated into the original constituent components by a process called etching. The etching process removes overlying layers from multi-laminated materials to reveal the seal of the container (Arndt, 1992). This process allows containers with known defects in the seal (found

using the etching technique) to be compared to the exterior of containers suspect of defects before etching.

To facilitate etching, the materials are soaked in tetrahydrofuran (THF) to remove the outer polyester layer by softening the adhesives and or inks (Arndt, 1992). The container sample is then soaked in 6 N HCL to remove any traces of aluminum foil, if applicable. The materials are then rinsed. Ink pattern and dispersion are then observed and checked for leaks and channels within the fused area. The samples may then be placed on an overhead projector so that a more accurate visual inspection maybe rendered (Arndt, 1992).

2.4. Compression Test

The compression test offers three techniques to determine the effects of weight/exterior pressure on the seal integrity of the container: static method, dynamic method, and squeeze test. The sealed sample container is placed on a flat surface while a flat surfaced weight is placed on top. The weight is allowed to remain on top of the container for a predetermined period of time or until deformation resulting in seal breach occurs (Arndt, 1992).

The dynamic method consist of a sealed sample container placed on a flat surface while a continuously increasing force is exerted on the top of the container at a constant rate. The maximum force the container will resist without traces of leakage due to loss of seal integrity is observed (Arndt, 1992).

The squeeze test consists of applying a manual kneading action that forces the contents of the container to come into contact with the interior seal surface area of the container. During the test, the observer checks the container for leaks and delamination around the seal areas and on the exterior walls of the container (Arndt, 1992). Positive results occur when holes are found somewhere on the container, such as in the seal or in the seam and measurable movement of the top plate of the container is observed (Arndt, 1992). Negative results occur when no loss of seal integrity is identified by measurable movement of the top plate of the container (Arndt, 1992). False positive results occur in containers that are underfilled or that simulates failure without loss of seal integrity (Arndt, 1992).

2.5. Distribution Test

Distribution, or abuse, test simulates various stresses such as vibration, compression and impact at levels similar to those expected during normal distribution. All sample containers should be incubated at 37°C under ASTM laboratory conditions of 23 ± 2°C and 50 ± 5% relative humidity for a period of 2 weeks. After testing, all containers are examined for defects or damage as a result of treatment. All containers that do not appear affected by the treatment should be incubated for an additional 14 days at 37°C and visually inspected for indications of defects or damage (Arndt, 1992).

2.6. Electester

The electester allows the detection of changes in the viscosity of liquids within a container as a result of microbial fermentation. Shock waves are used to determine viscosity variations within the package. Packages containing microbial activity due to post process or other contamination will result in the creation of a more viscous fluid that adsorbs more shock waves when compared to noncontaminated containers (Anderson, 1989; Arndt, 1992).

The sample containers to be tested should be incubated at 37°C for 4 days. The containers are rotated 90° and returned to their original position very quickly to create a shock wave for viscosity measurement. The wave formed by the motion is then observed on an oscilloscope and wave lengths compared to noncontaminated containers. Positive results occur when the wave formed by the motion dampens more quickly or slowly than normal. Negative results occur when the wave falls within normal limits, indicating that no microbial contamination is present.

2.7. Electroconductivity

This test is used to identify defects by using electric current. Flexible containers constructed of plastic are generally poor conductors of electricity, therefore any breach in the surface of the plastic container acts as an effective conduit to the current (Axelson et al., 1990; Arndt, 1992). A conductivity meter or probe is placed on outside of the container, while another probe is placed inside of the container. The container is then submersed in 1% NaCl in water (a brine solution). Positive results occur when a current flow is completed, indicating a breach in the

seal. Negative results occur when a current flow does not exist, indicating an intact seal.

2.8. Helium Leak Test

The helium leakage technique is used primarily to study the influence of can structural defects on barrier properties (Put and Warner, 1972; Gilchrist et al., 1985; Gilchrist et al., 1989). Test container preparation consist of evacuation by a 2-stage rotary vacuum pump. When the pressure falls to $< 10^{-1}$ torr, helium is sprayed around the outside of the container. If any quantity of helium penetrates the seams of the container, it is detected by the mass spectrometer according to the amount found (Put and Warner, 1972).

Cautions must be observed when using the helium leak test to detect defects in flexible packages (Arndt, 1992). Sealed pouches are generally placed on their side in a helium pressurization tank. Flexible packages require carefully maintaining the helium pressurization tank at approximately 207 kPa/cm^2. Greater than 345 kPa/cm^2 generally results in false positive results for leakage, whereas less than 207 kPa/cm^2 (between 69 and 138 kPa/cm^2) may reduce the sensitivity of the test. However, problems

have been noted during testing of flexible packages while pressure was carefully maintained at 207 kPa/cm^2. For example, greater than 69 kPa/cm^2 may cause laminated material to distend or stretch near the seal, resulting in temporary closure of channel leakers, especially leakers with a diameter size less than 20 μm (Gilchrist et al., 1985; Gilchrist et al., 1989).

2.9. Manganese Ion Test

The manganese ion test is performed simultaneously with a bio-test. This test is able to detect small traces of Mn^{+2} passing through the compromised areas in, on, or near the container seams (Put and Warner, 1972; Anderson, 1989). Processed containers are mechanically abused and allowed to cool in a 7% solution of $MnSO_4$ containing 10^7 cells/mL (the bacteria used should be insensitive to Mn). After the containers have cooled, the contents of each container are membrane filtered, and the number of bacteria which entered the containers enumerated. The filtrate may be used to estimate calorimetrically the amount of $MnSO_4$ which entered each container.

3. Biotest Method of Leak Detection

The objective of a biotest is to verify post-process contamination of a food container (Folinazzo et al., 1968). Test organism(s) in concentrations typically ranging between 10^7 and 10^9 colony forming units (CFU)/mL are used. Cans used for biotesting are prescreened by immersion in water. Cans that leak 0.01 mL of air at standard temperature and pressure (STP) in 15 seconds are selected for a biotest experiment (Put et al., 1980).

Cans are filled with approximately 1/3 citrate broth containing (by percentages): sodium citrate, 0.25; $Na(NH_4)HPO_3$, 0.1; NaCl, 0.5; in distilled water at a pH 6.8. Filled cans are sterilized and cooled in sterile water. Part of the cooling water surrounding the seam is removed by vaporization in a vacuum system and replaced by water or a citrate broth inoculated with various concentrations of test organisms (Put et al., 1980). Arndt (1992) details a method consisting of a water bath with temperature control and agitation in a solution of *Enterobacter aerogenes* for foods with a pH > 5.0, and a solution of *Lactobacillus cellobiosis* for foods with a pH $\leq$ 5.0, and a concentration of 10^7 CFU/mL. The exterior seam of the container should remain in contact with the infected medium at a constant temperature

for a predetermined period before being washed with tap water. A portion of the containers are to remain wet, while the rest are allowed to dry. After a predetermined time, all containers are allowed to stand at a constant temperature, for various times. Leaks generally become evident during this incubation period (organism dependent) as indicated by container swell (as a result of microbial production of gas with in the container), and broth turbidity. Test organism(s) selected should cause fermentation of the food product if the container is penetrated, but should not be pathogenic. Each container, according to its schedule for standing time period is then removed from the area so that its contents may be membrane filtered to provide a count (CFU/mL) of the infiltrating bacteria (Put et al., 1980). Common modifications to this bio-test include the addition of 0.1% glucose to the citrate broth and variations of standing time. Test microorganisms may also be used at different concentration levels to achieve desired liquid contamination measurements.

3.1. Aerosol Exposure Biotest

Reich (1985) used an airflow-nebulizer approach for controlled microbial exposure to package. The purpose of

the investigation was to determine the effectiveness of an intact package as a microbial barrier under conditions of biological stress (i.e., microbial challenge). The microbial challenge chamber dimensions were 45.7 cm (H) x 45.7 cm (W) x 45.7 cm (L) x 0.635 cm (thick). Entry ports featured a model 645 DeVilbiss™ nebulizer which produced particle sizes ranging from 0.3 to 2.0 mm. Circulating fans of variable volume displacement, from 762 to 6096 cu cm/min were placed within the chamber. Exit ports featured 0.22 μm bacterial filter, and an impinger sampling port. Currents within the exposure chamber were investigated using a smoke test. The chamber was filled with smoke as the orientation of the fan within the chamber was changed to select the fan orientation which yielded the optimum movement and distribution of particles within the chamber. Spores of *B. subtilis* ATCC 9372 were used as the test/challenge organism. All materials were UV sterilized with 12, 47 cm x 25.4 cm x 15.2 cm, 25-W germicidal bulbs, within a 45.7 cm x 12.7 cm x 10 cm exposure chamber. Between exposures the chamber was cleaned and sterilized by a complete interior washing with Cidex®, an alkaline glutaraldehyde solution (Reich, 1985).

Holes were made in Tyvek™ lidding material with a Tesla coil. Thirty five devices (packages) were tested in total. All material was examined with the aid of a scanning

electron microscope (SEM) to verify hole size uniformity. Hole sizes consistently yielded diameters of 40 ± 5 μm (Reich, 1985). Each of the packages were subjected to a microbial challenge concentration of 4 x 10^4 *B. subtilis* spores per cubic meter, a microbial challenge far in excess of what might be found during actual product use conditions (Reich, 1985).

Microbial distribution studies demonstrated a homogeneous dispersion of the challenge organism throughout the chamber. Control medium evaluation units in all orientations and locations within the chamber demonstrated indicator organism growth following 72 hours of ambient incubation. Use of UV radiation to sterilize the surface of microbially challenged packages without penetration of their interiors was also validated (Reich, 1985). This indicated that the complete system represented a consistent and valid method (microbial challege) of the barrier properties of the product.

Using a similar method to that of Reich (1985), Placenia et al. (1986) found this microbial challenge via the exposure-chamber method to be a reliable test of the barrier properties of a packaging product. Placenia et al. (1986) also found that in using this method, a single, 1 μm-diameter pore could be detected 55.6% of the time when a

bacterial exposure of 10^3 CFU/mL was used. Results demonstrated detectability increases as the bacterial concentrations or pore diameter increase (Placenia et al., 1986).

Chen et al. (1991) developed a spray cabinet technique, and added a new twist to the research of Reich (1985) and Placenia et al. (1986). Chen et al. (1991) observed the influence of shock and vibration in addition to microbial exposure in their study.

The objective of the Chen et al.'s (1991) experiment was to determine the microbial integrity of paper board laminate containers. The method assessed the effect of a simulated distribution test on package integrity. Immersion tests were performed simultaneously. *Lactobacillus cellobiosus* ATCC 11739 at a 2.5 x 10^6 cells/mL concentration were used for immersion and aerosol tests. Two hundred juice packages were used in the study; 40 containers were tested as blank controls, 120 packages were sprayed and/or immersion tested and 40 packages were biotested via innoculated spray (20 for a 60 minute exposure, and 20 for a 15 minute exposure). All test containers were 1.89 L flattop (lamination of polyethylene (PE)/paper stock/Surlyn^R /aluminum foil/Surlyn^R/PE) cartons aseptically filled with apple juice (pH 3.9 - 4.1). Each carton was nitrogen-

flushed so that the headspace contained approximately 97% nitrogen and 3% oxygen. Carton dimensions were 19.7 cm (H) x 13.3 cm (W) x 7.6 cm (D) (Chen et al., 1991).

Spray cabinet dimensions were 91.4 cm (L) x 45.1 cm (W) x 61.0 cm (H), constructed of 0.64 cm thick Plexiglas^{R}. The cabinet enclosed a recirculation system that distributed the test organism suspended in inoculated water. Inoculum (sterile *Lactobacilli* MRS broth) was extracted from a 4 L reservoir, pumped (Model 2000-032 Flowjet Corporation, Irvine, CA) through a 32-nozzle system at a pressure of 2.07 x 10^5 kg/m^2, collected and allowed to drain into the reservoir. Nozzles were positioned (60 degree angles) to cover a specific geometry within the cabinet. The reservoir containing the inoculum was replaced with 4 L of a 2500 ppm chlorine solution (NaOCl) and sprayed onto the packages for 30 minutes to kill any microorganisms remaining on the package surface. Containers were removed from the cabinet and incubated for 2 weeks at 35-37°C. An immersion test with identical parameters was performed to compare with the spray cabinet method (Chen et al., 1991).

Three pinhole diameter sizes were used; 5, 10, and 15 μm, and capped over an unspecified number of 30 mL glass vials. Test vials were sprayed or immersed with *Lactobacilli* 2.5 x 10^6 CFU/mL for 15, 30, 60, and 90 minutes

and incubated for 48 hours at 35-37°C). A 2500 ppm chlorine solution via spray exposure was used for decontamination. Microorganism growth within the containers was determined by carbon dioxide production in the headspace of the package. A 300 μl sample of headspace gas was removed from the package with an air-tight syringe and injected into a gas chromatograph. The column packing for the gas chromatograph was Porapak^{R} sieve 5A 60/80. Helium was used as the carrier gas at a flow pressure of 1.72×10^5 kg/m^2. No microbial penetration was observed after 60 minutes of immersion testing. Ten and 15 μm size holes were detected after 90 minutes of immersion testing. Five-μm pinholes were detected after 30 minutes and 20 μm pinholes were detected after 15 minutes. The difference between the spray cabinet technique and immersion method was significant ($P < 0.05$) for the 10 μm orifices (Chen et al., 1991).

Abuse testing was used to physically test package integrity under simulated distribution conditions. A sequential test, consisting of a hydraulic shock test (flat drop), static compression, resonant vibration, hydraulic shock (end drop) and random vibration, was developed. Eighteen packages (15% of total test packages) showed obvious leakage after exposure to the abuse tests (Chen et al., 1991).

4. Threshold Leak Size

Howard and Duberstein (1980) found that under specific conditions certain types of water borne bacteria penetrated 0.2 μm membrane filters and therefore speculated that the minimum hole size critical to sterility maintenance and integrity of the package (i.e., the threshold leak size) is between 0.2 μm and 0.4 μm. This range was selected based on the size of membrane filters used routinely for aseptic packaging and cleanroom applications with little significant microbial contamination. Lampi (1980) showed that bacterial penetration via holes of less than 10 μm was unlikely. Lake et al. (1985), during an extensive 4 year study, found that leaks must be considerably larger than 1 μm for bacterial penetration to occur. Gilchrist et al. (1985) showed that bacterial contamination of cans from cooling water requires pinholes larger than 5 μm. McEldowney and Fletcher (1990[a,b]) found that holes of 1 μm permitted microbial entry under certain conditions. Chen et al. (1991) reported that 5 μm pin holes allowed microbial aerosol penetration. Jarrosson (1992) found that a 20 μm diamter hole with a 5 mm channel length permitted microbiological contamination in Meal Ready to Eat (MRE) pouches. Part of the discrepancy in the study rest in the researchers inability to manufacture and

maintain the size integrity of leaks with diameters smaller than 10-20 μm during retorting and experimentation (Gnanasekharan and Floros, 1994; Jarrosson, 1992).

Gnanasekharan and Floros (1994) cited three major obstacles which must be resolved prior to the development of a reliable, 100% on-line inspection system. These obstacles inculde the size of leaks that occur in real life, the minimum leak size for microbial contamination to occur, and the minimum leak size that can be reliably and rapidly detected.

III. OBJECTIVES OF THE STUDY

1. Rationale

A biotest method via aerosol distribution of a test organism into an exposure chamber was used in this experiment. This experiment differs from previous experiments by three points:

1. Precise, uniform, and maintainable leaks were created. Nickel microtubes developed by the Phillips Laboratory, Edwards Air Force Base, CA, were used to create defects and to ensure size integrity during handling, retorting and experimentation. The microtubes used in this experiment were capable of maintaining structural integrity under greater forces than those used in this experiment.

2. A small, finite range of aerosol particle size was used. Great discrepancies exist in terms of aerosol particle size within the literature. Reich (1985) used a nebulizer that produced particles ranging from 0.3 mm to 2.0 mm. Placenia et al. (1986) used a particle size range was between 0.3 μm and 10 μm. Chen et al. (1991) used 4 L per minute of sterile broth infected with *Lactobacilli;* the aerosol particle size was not specified. A nebulizer that produced aerosol particles which ranged in size from 2.14 μm

to 3.92 μm with a 2.68 μm mass median aerodynamic diameter was used in this experiment.

Nebulizer selection was based on several criteria. The aerosol particle size was larger than that of the test organism to be transported via aerosol. Simultaneously, the aerosol particles were small so that surfaces in the 100% humidity environment created by the aerosol particles were dry to the touch. The test organism remained viable during the aerosol process, and was evenly distributed throughout the exposure chamber. The aerosol method provided a sterile environment ensuring that only the test organism was transferred to the exposure chamber.

3. Vegetative, motile and nonmotile cells of *Pseudomonas fragi* were the test organism(s) in this experiment. Put et al. (1980) used a suspension of *Clostridium intermedius* in a method to biotest glass jars. Effects of organism attributes, such as motility versus nonmotility, on post-process contamination of the jars were not considered in that experiment. Reich (1985) used a bacterial spore suspension of *Bacillus subtilis* ATCC 9372 for his biotesting experiment. A bacterial spore suspension of *Bacillus subtilis* var. *niger* was again used by Placencia et al. (1986) and Placencia et al. (1989). Davidson and Pflug (1981) recovered mesophilic aerobic microorganisms

nonsporeformers and mixed cultures containing aerobic and anerobic sporeformers from spoiled canned foods retrieved at the retail level. However, ingress performance due to organism characteristics, such as spore versus vegetative cell, motility and nonmotility, were not investigated in the experiment. Chen et al. (1991) did not specify whether vegetative cells were used in the bacterial suspension used for the spray cabinet technique.

The indicator organism used in this study was *Pseudomonas fragi* TM 849. *Pseudomonas* species are motile, Gram negative, aerobic, nonsporeforming, polar flagella, catalase-positive rods ranging from 0.5-1.0 μm in diameter and 1.5-5.0 μm in length. Growth of the organism occurs between 4° - 43°C, with an optimal range of 25° to 30°C. A nonmotile form of *Pseudomonas fragi* TM 849 was isogenically mutated and also used as a test organism. The method of isogeneic mutation used was developed by Panopoulos and Schroth (1974) with medium selections developed by Adelberg et al. (1965).

Motile *Pseudomonas fragi* TM 849 was mutated to a nonmotile form to provide a comparison of the effect of motile versus nonmotile organisms on the post-process contamination of flexible pouches. Since a forward reversion method (i.e., removal of motile related

characteristics) of mutation was used, the environmental requirements of each form of the *Pseudomonas fragi* TM 849 organism remained the same, thereby greatly reducing experimental variability inevitably created when several organisms are used (Armstrong et al., 1967; Iino, 1969; Panopoulos and Schroth, 1974; Burkhardt et al., 1993; Lee et al., 1993).

A plasmid marker for kanamycin antibiotic resistance and a photo-phosphorescent tag were used to aid in test organism identification and verification. The kanamycin antibiotic consisted of 10 μL/mL stringent plasmids, and 50 μL/mL relaxed plasmids. Kanamycin's mode of action is bactericidal through inhibition of protein synthesis and translocation which elicits miscoding. The photo-phosphorescent tag caused the test organism to glow when exposed to long wave UV light.

Lower source concentrations (10^2 and 10^6 cells/mL) were incorporated into this experiment than those previously used. The lower source concentrations more closely simulated those found in storage and distribution conditions.

The objective of this study was to determine the seal threshold defect size which allows biological contamination and how it is affected by:

a. hole diameter and channel length (defect dimension),
b. test organisms characteristics such as size and motility (organism characteristics) and,
c. test organism concentration and exposure time (exposure rate).

IV. MATERIALS

1. Pouches

A total of 128 white, translucent, retortable pouches were used in this experiment. They were constructed of polyester on the outside and cast copolymer polypropylene as the sealant with high barrier Saran^{R} sandwiched between as the barrier. The pouches were 20 cm (H) x 17 cm (W), with one end unsealed. Pouches were provided by American National Can Company (Chicago, IL).

2. Sealer

Pouches were heat sealed using a hot jaws sealer (Model PB-H, Packaging Aids Corporation, San Rafael, CA). A sealing temperature of 187.8°C, a 3.0 sec. dwell time, at 207 kPa of pressure was used to create a seal width of 3 mm.

3. Exposure Chamber

The exposure chamber was constructed of Plexiglass^{R} of the following dimensions: 63.5 cm (L) x 43.2 cm (W) x 44.5 cm (H) x 0.95 cm (thickness). The volume of the exposure

chamber was 119,911 cm^3. The exposure chamber had a 63.5 cm x 43.2 cm removable Plexiglass^R top, with 8.49 cm (L) x 2.3 cm (H) x 0.47 cm (W), stainless steel handles. Two aerosol entry ports were created by two, 1.91 cm (O.D.) plastic female adapters sealed with 3.49 cm (O.D.) x 2.54 cm (I.D.) rubber gaskets. Nebulizer kits (Baxter model 2D0807, Toronto, Ontario, Canada) with a mass median aerodynamic diameter of 2.68 μm, a geometric standard of 1.85 μm, and a mass of aerosol per minute between 1.1 μm and 4.7 μm were used. The maximum air flow (ml/hour) at 10 liters per minute (Lpm) was 21.9 ml/hour. Four 244 ft^3 size E cylinders, each equipped with CGA 346 air (0 - 15 LPM) flow meters were used for the air supply. Twenty-four 37 mm bacterial air vents (Gelman Sciences, product no. 4210, Ann Arbor, MI) under 103 kPa of pressure were used to filter the contaminated aerosol evacuated from the chamber. Pressure inside the exposure chamber was not measured or monitored. The bacterial vents consisted of a polypropylene housing (3.56 cm [L] x 4.10 cm [W]), a hydrophobic glass filter (effective filtration area of 7.5 cm^2), a maximum operating pressure of 5.3 kg/m^2 bidirectional (a retention minimum of 99.97% for particles $\geq$ 0.3 μm), and featured 6.4 to 9.5 mm graduated hose barbs.

4. Microtubes

Nickel microtubes were used to create seal defects. The microtubes were purchased via a Cooperative Research and Development Agreement (CRDA) from the Phillips Laboratory, Fundamental Technology Division, Carbon Research Lab, OLAC PL/RKFE, Edwards Air Force Base, CA. Four sizes of microtubes were used: 10 μm interior diameter (I.D.) x 5 mm length, 10 μm I.D. x 10 mm length, 20 μm I.D. x 5 mm length and 20 μm I.D. x 10 mm length. All microtubes fell within a mean diameter range of ± 2 microns and a mean length range of ± 1 mm of specified dimensions. Microtubes were formed by extrusion of nickel under unspecified pressure and heat.

5. Pouch Medium

Pouches were each filled with approximately 100 ml of Tryptic Soy Broth (Difco Laboratories, Detroit, MI).

6. Test Organism

Motile and isogenically mutated nonmotile *Pseudomonas fragi* TM 849 were used as the test organism. The test organism was selected from the bacteriological library at

Virginia Polytechnic Institute and State University, Department of Plant Pathology, Physiology, and Weed Science, Blacksburg, VA. A test organism source concentration of 10^2 and 10^6 CFU/mL was used. In this study, an inoculated liquid (source concentration) was converted to an aerosol (final concentration). From a source concentration of 10^2 cell/mL, a final aerosol concentration of 1 cell/10 cm^3 and 2 cells/10 cm^3 was achieved for exposure times of 15 and 30 minutes, respectively. From a source concentration of 10^6 cells/mL a final aerosol concentration of 87 cells/cm^3 and 175 cells/cm^3 were achieved for exposure times of 15 and 30 minutes, respectively. Test organisms were suspended in cartridges containing 500 mL of sterile, nonpyrogenic water and 0.625 ml of Butterfields PO_4 buffer at pH 6.5 - 6.7.

V. METHODS

1. Culture Media

Cultures were prepared on semisolid minimal media (Davis minimal medium with 0.35% Nobel agar), semisolid tryptone media and King's medium B (King et al., 1954). Semisolid minimal media contained (g/L); glucose 1.0 g, K_2HPO_4 7 g, KH_2PO_4 2 g, Na citrate x 5 H_2O 0.5 g, $MgSO_4$ x 7 H_2O 0.1 g, $(NH_4)_2$ $(SO_4)_2$ 1 g, Nobel agar 3.5 g. Semisolid tryptone media used to evaluate motility, contained 1% tryptone (Difco Laboratories, Detroit, MI) and 0.35% agar. Davis minimal broth consisted of (g/L); glucose 1.0 g, K_2HPO_4 7 g, KH_2PO_4 2 g, Na Citrate x 5 H_2O 0.5 g, $MgSO_4$ x 7 H_2O 0.1 g, $(NH_4)_2$ $(SO_4)_2$ 1 g (Adelberg et al. 1965). King's medium B (King, et al., 1954) consisted of (g/L); protease peptone No.3 20 g, glycerol 1 g, K_2HPO_4 2.25 g, $MgSO_4$ 2.25 g, Nobel agar 22.5 g (Arndt, 1992). Davis minimal medium contained 0.03 g/L of 10 μg/L kanamycin antibiotic.

2. Test Organism Identificaton

A plasmid marker for kanamycin antibiotic resistance and a photo-phosphorescent tag were used to aid in test

organism identification. Test organisms were verified by Biologic^{R} Gram negative MicroPlates^{TM} (Biolog, Hayward, CA). The Gram negative MicroPlate^{TM} test consist of 95 biochemical tests that identify a broad range of enteric, non-fermenting, and fastidious Gram negative bacteria (Anonymous, 1993). The ability of a microorganism to utilize or oxidize a preselected panel of different carbon sources is tested (Anonymous, 1993). The test yields a pattern of purple wells which constitutes a "metabolic fingerprint" of the capacities of the inoculated organism. All necessary nutrients and biochemicals are prefilled and dried into the 96 wells of the plate. Tetrazolium violet was used as a redox dye to colorimetrically indicate the utilization of the carbon sources (Anonymous, 1993). The organism to be identified was suspended in prewarmed sterile saline, which meets the prespecified density/turbidity range of the test. One hundred fifty μL of isolate from inoculum blanks with a concentration of 10^{6} cells/mL were introduced into each well. All wells were colorless when inoculated. Color changes resulted when the chemical within a well was oxidized as a result of respiration which allows the test organism to reduce the tetrazolium dye, forming the purple (positive) indicator color.

3. Induction of Mutants

Motile *Pseudomonas fragi* TM 849 cultured in 1983, with a plasmid marker for kanamycin antibiotic resistance, was selected for mutagenesis. Test organisms were treated with 0.5 ml of 250 $\mu g/\mu l$ N-methyl-N'-nitro-N-nitrosoguanidine dissolved in a tris-maleate buffer solution, washed with 0.05 M phosphate buffer (pH 6.9) and grown for two generations to allow for phenotypic expression of the induced mutations. The motile cells were centrifuged and resuspended in 1 ml of 99% glycerol and refrigerated at -75°C until further use.

4. Enrichment and Selection of Nonmotile Mutants

Enrichment for nonmotile mutants was performed according to the method of Armstrong et al. (1967) and Panopoulos and Schroth (1974). Semi-solid tryptone media, which contained 1% tryptone (Difco Laboratories, Detroit, MI) and 0.35% Nobel agar and 1.0 g glucose/L, was used to evaluate motility.

A loop of frozen test organism cells were collected from a 99% glycerol suspension and introduced into a 3 mL

blank of tryptone broth. The blank was placed in a 28°C orbital incubator for 24 hours.

Tryptone plates containing kanamycin were spin inoculated with 100 μL of the tryptone broth solution solution. A motile, isolated colony was selected from the edge of each tryptone plate. Each isolate was introduced into 200 μl of sterile H_2O containing 1.25 mL/L of Butterfield's PO_4 buffer, pH ajusted to 6.5-6.7 and vortexed for 5 seconds. Six 5 μL drops from the 200 μL isolate solution were evenly distributed in a hexagonal arrangement near the outer edge of each semi-solid minimal media petri plate containing kanamycin and allowed to incubate in a noninverted position for 24 hours. Organism growth halos resulted and their dimensions (motile spread diameters) were measured. Five μL of isolate from the center of the smallest halo on each plate was selected. Each isolate was introduced into a test tube containing 200 μL of sterile H_2O containing 1.25 mL/L of Butterfield's PO_4 buffer, pH adjusted to 6.5-6.7. Each tube was vortexed for 5 seconds. The plating procedure was repeated 5 times, after which the medium was changed to semi-solid tryptone agar with kanamycin and a reduced glucose concentration (1%). This procedure was repeated 6 times (10 transfers in total). Two μL of each isolate solution following each plating was used

to create a hanging drop slide to evaluate motility of the test organism. A sample from each isolate solution was used to create a Gram stain slide for identification. After 10 transfers, mostly paralyzed mutants and mutants with morphologically abnormal flagella exhibiting Brownian motion were present. Less than 5% of the field (as specified by count) was occupied by motile organisms. Colonies which demonstrated no motility under the light microscope and reduced spreading ability via stab inoculation in semi-solid medium were purified twice on King's B medium.

Panopoulos and Schroth (1974) found similar mutants (*Pseudomonas phaseolicola*) to remain stable when grown on solid media or in liquid shake cultures for several months. However, the colonies reverted when maintained on semi-solid agar plates for 2 days or longer. Revertants were identified by the appearance of "flares" of the test organism spreading away from the outer edge of the growth site (Panopoulos and Schroth, 1974). Cells from the "flares" were removed after the first transfer and used as the motile test organism.

5. Preparation of Inoculum

Motile and isogenically mutated nonmotile were prepared from dilution blanks inoculated with 100 μL of test organism and containing 99 mL of tryptic soy broth. Cells were suspended in 500 mL cartridges containing sterile, nonpyrogenic water with 0.625 mL of Butterfields PO_4 buffer at pH 6.5-6.7, to create source concentrations of 10^2 or 10^6 cells per mL, as needed. The contents of each inoculated cartridge was distributed via aerosol using nebulizer kits. Inoculums were prepared to limit growth to one log during each repetition trial as shown in Figures 1 and 2.

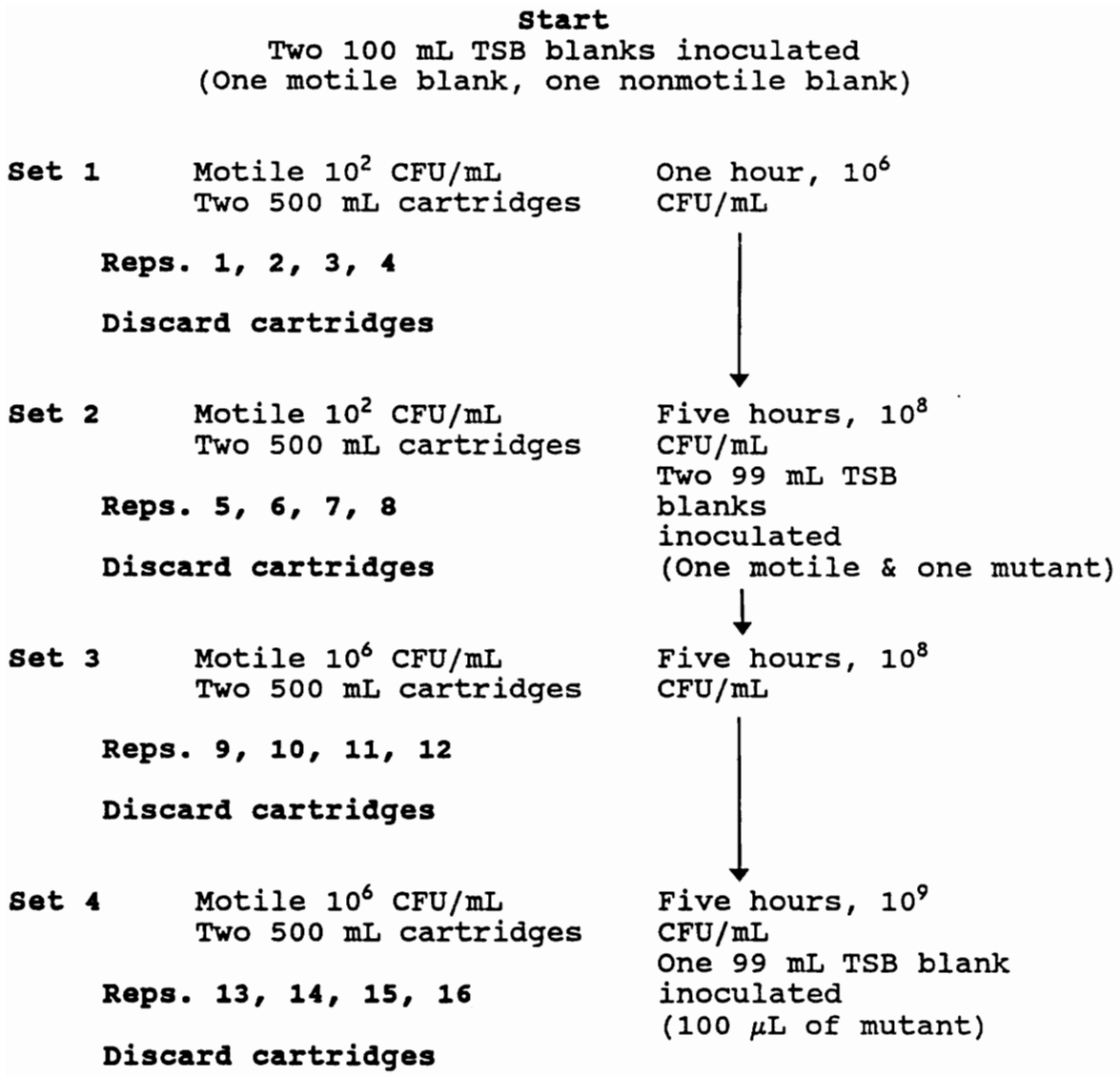

FIGURE 1: SCHEDULE TO MAINTAIN ≤ 1 LOG GROWTH OF MOTILE *PSEUDOMONAS FRAGI* TEST ORGANISM INOCULUM DURING EXPERIMENTAL SETS 1 THROUGH 4.

Start
10^8 TSB blanks inoculated
Nonmotile (mutant)

Set 5 Nonmotile 10^2 CFU/mL
Two 500 mL cartridges

10^8 CFU/mL
inoculated one 99 mL TSB
blank (mutant)

Reps. 17, 18, 19, 20

Discard cartridges

Set 6 Nonmotile 10^2 CFU/mL
Two 500 mL cartridges

Five hours, 10^8 CFU/mL
Two 99 mL TSB blanks
inoculated

Reps. 21, 22, 23, 24

Discard cartridges

Set 7 Nonmotile 10^6 CFU/mL
Two 500 mL cartridges

Five hours, 10^8 CFU/mL

Reps. 25, 26, 27, 28

Discard cartridges

Set 8 Nonmotile 10^6 CFU/mL
Two 500 mL cartridges

Five hours, 10^9 CFU/mL

Reps. 29, 30, 31, 32

Discard cartridges

FIGURE 2: SCHEDULE TO MAINTAIN ≤ 1 LOG GROWTH OF NONMOTILE (MUTANT) *PSEUDOMONAS FRAGI* TEST ORGANISM INOCULUM DURING EXPERIMENTAL SETS 5 THROUGH 8.

6. Manufacture of Package Defects

Placement of the microtubes within the seal area of the 128 pouches prior to sealing created defects of known dimensions. Microtubes were placed in premarked pouches with a magnet. Pouches were placed on a light table while microtubes were adjusted into seal position. Microtube placement and defect dimensions were visually verified with the aid of a light microscope (Model BH-2, Olympus Systems, Lake Success, NY). A second seal was created 2.54 cm toward the package edge, away from the defect seam to ensure sterility (post retort) of the pouch defective seal site and contents, tryptic soy broth (TSB), prior to controlled aerosol exposure.

VI. Experimental Design

1. Split Plot Design

A split plot design using a one way analysis of variance was employed. A split plot design was used so that the influence of variables such as hole diameter could be determined with or without the influence of other variables such as channel length, test organism concentration, motility or aerosol exposure time. The split plot design also allowed relationships between 2 or more variables to be statistically examined. The experimental design incorporated 32 variable combinations, 21 degrees of freedom, for a total error of $32(r - 1)$, where r = sample size per repetition. Each variable and interaction between variables were statistically examined using SAS Proc. ANOVA at a 0.05 level of significance (Schlotzhauer and Littell, 1991).

TABLE 1: TEST ORGANISM CONCENTRATION AND MOTILITY, AEROSOL EXPOSURE TIME, DEFECT HOLE DIAMETER AND CHANNEL LENGTH, INTERACTIONS AND DEGREES OF FREEDOM.

Variables & Interactions	df
A_1 Microorganism Concentration: 10^2, 10^6	1
A_2 Microorganism Morpholgy: Motile, Nonmotile	1
$A_1 * A_2$ Concentration * Morphology	1
A_3 Aerosol Exposure Time: 15, 30 minutes	1
$A_1 * A_3$ Concentration * Aerosol Exposure Time	1
$A_2 * A_3$ Morphology * Aerosol Exposure Time	1
$A_1 * A_2 * A_3$ Concentration * Morphology * Aerosol Exposure Time	1
B_1 Hole Diameter: 10 μm, 20 μm	1
B_2 Channel Length: 5 mm, 10 mm	1
$B_1 * B_2$ Hole Diameter * Channel Length	1
A(7) x B(3)	21

Each of the 128 pouches were sequentially labeled according to their defect dimension(s). Eight experimental sets were created. Each of the 8 sets contained 4 combinations of the experimental variables as shown in Table 2. Thirty-two different combinations (8 sets x 4 different variable combinations per set) of the following experimental variables resulted; defect dimension(s), organism characteristic(s) and exposure time(s). Each experimental set was repeated four times.

TABLE 2: EIGHT EXPERIMENTAL SETS REPRESENTING ALL VARIABLE COMBINATIONS FOR AEROSOL EXPOSURE.

SET 1.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^2	Motile	15
1	10	10	10^2	Motile	15
1	20	5	10^2	Motile	15
1	20	10	10^2	Motile	15

SETS 2.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^2	Motile	30
1	10	10	10^2	Motile	30
1	20	5	10^2	Motile	30
1	20	10	10^2	Motile	30

SET 3.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^6	Motile	15
1	10	10	10^6	Motile	15
1	20	5	10^6	Motile	15
1	20	10	10^6	Motile	15

SET 4.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^6	Motile	30
1	10	10	10^6	Motile	30
1	20	5	10^6	Motile	30
1	20	10	10^6	Motile	30

TABLE 2: EIGHT EXPERIMENTAL SETS REPRESENTING ALL VARIABLE COMBINATIONS FOR AEROSOL EXPOSURE.

SET 5.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^2	Nonmotile	15
1	10	10	10^2	Nonmotile	15
1	20	5	10^2	Nonmotile	15
1	20	10	10^2	Nonmotile	15

SET 6.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^2	Nonmotile	30
1	10	10	10^2	Nonmotile	30
1	20	5	10^2	Nonmotile	30
1	20	10	10^2	Nonmotile	30

SET 7.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^6	Nonmotile	15
1	10	10	10^6	Nonmotile	15
1	20	5	10^6	Nonmotile	15
1	20	10	10^6	Nonmotile	15

SET 8.

Pouch Qty.	Hole Dia. (μm)	Channel Length (mm)	CFU/mL	Organism Motility	Exposure Time (mins)
1	10	5	10^6	Nonmotile	30
1	10	10	10^6	Nonmotile	30
1	20	5	10^6	Nonmotile	30
1	20	10	10^6	Nonmotile	30

2. Experimental Controls for Pouches

Twenty six pouches were used as presence (growth) or absence (no growth) controls. Control pouches received two parallel 3 mm wide seals, 2.54 cm apart, at 187.7°C with a dwell time of 3.0 sec. and a pressure of 207 kPa. One corner from each pouch was cut away to allow filling with 99 ml of tryptic soy broth and a third seal was used to seal the corner in a similar manner. Pouches were retorted for 5 minutes at 121°C, 117 kPa pressure, and cooled under a maintained pressure of 117 kPa in a vertical still retort (Model RDTI-3, Dixie Canner Equipment Co., Athens, GA). Pouches were stored at 4.4°C degrees for 12-36 hours and removed as needed. Thirteen pouches were inoculated with 100 μl of 10^6 cells/mL of the nonmotile (mutant) test organism throught the side wall of the pouches via sterile syringe. A concentration of 10^3 cells/mL was achieved. Pouches were incubated at 28°C for 72 hours. A 2.54 cm^2 area of each pouch was swabbed with a 70% ethyl alcohol and deionized water solution and allowed to air dry. The same area was then swabbed with Cidex^R and allowed to air dry. Tryptic soy broth (100 μl) was removed from the sanitized area via a sterile 27 gauge x 1.58 cm needle with a 3 cc syringe and spin plated on tryptic soy agar petri plates.

Plates were incubated at 28°C for 72 hours. Positive control plate results (13 inoculated pouches) were evident by the presence of the test organism on the TSA plates. A positive plate indicated that the test pouches provided an environment supportive of test organism growth. Negative control plate results (13 noninoculated pouches) were evident by the absence of the test organism on the TSA plate. A negative plate indicated that noninoculated pouches maintained sterility.

3. Exposure Chamber Controls

Relative humidity in the exposure chamber was measured by 8 thermocouples; 4 wet bulb, each with moist, 100% cotton covers, and 4 dry bulb at 4 sites as shown in Table 3. Thermocouple(s) wet and dry bulb readings were verified using a portable relative humidity indicator (Psychron^{R}, Belfort Instrument Co., Baltimore, MD). Wet and dry bulb thermocouples were paired as follows; 1 and 5, 2 and 6, 3 and 7, 4 and 8 as shown in Table 3.

TABLE 3: THERMOCOUPLE TYPE AND PLACEMENT WITHIN THE EXPOSURE CHAMBER.

Thermocouple #	Wet/Dry Bulb	Placement
1	wet	geometric center
2	wet	bottom corner
3	wet	top corner
4	wet	bottom center fold
5	dry	geometric center
6	dry	bottom corner
7	dry	top corner
8	dry	bottom center fold

Ambient temperature during each trial ranged from 26°C to 28.8°C. Two cartridges containing 500 mL of sterile, nonpyrogenic water under 345 kPa of pressure facilitated a combined flow of 20 L/minute of H_2O saturated aerosol into the 119,911 cm^3 exposure chamber. Three consecutive trials were performed (two, 15 minute and one, 30 minute trials). During each trial, 100% humidity was achieved within 3 minutes after aerosol initiation, without fluid droplet formation or accumulation.

3.1. Particle Distribution

Two cartridges containing 500 ml of tonic water containing quinine (Golden Crown Co., Bluefield, VA) under 345 kPa of pressure facilitated a combined flow of 20 L/minute of H_2O saturated aerosol into the 119,911 cm^3 exposure chamber. Six trials (2, 5 minute, 2, 10 minute, 2, 15 minute) were performed. Particle distribution was observed and defined as a result of the phosphorescence quality of quinine within the tonic water solution with the aid of a long wave UV light. Particle visually appeared evenly distributed throughout the exposure chamber.

3.2. Sanitation Effectiveness via Aerosol

Ten petri plates with semisolid media were inoculated (1 mL of inoculum via spin plate inoculation) with the test organism. Five petri plates were placed individually within the exposure chamber for 5 minutes. Four petri plates were placed in the corners (1 petri plate in each corner) and 1 petri plate was placed in the center of the bottom panel of the exposure chamber. Two cartridges containing 500 mL of sterile, nonpyrogenic water with 5 mL of a liquid sanitizer XY-12 (Klenzade, St. Paul, MN), active ingredient sodium hypochlorite 8.4%, inert ingredients 91.6%) under 345 kPa of pressure to introduce 20 L/minute of H_2O saturated aerosol into the 119,911 cm^3 exposure chamber. After sanitizer exposure, the chamber was evacuated with 40 kPa through two bacterial air vents (Gelman Sciences, product no. 4210, Ann Arbor, MI) for 5 minutes. Ambient air was allowed to enter the chamber through nebulizer air channels. The 5 plates were removed prior to the sanitizer aerosol, and allowed to incubate at ambient temperature (25°C) for 72 hours. The 5 plates remaining were exposed to a sanitizer aerosol for 5 minutes. After sanitizer aerosol exposure, plates were removed from the exposure chamber and incubated at ambient temperature (25°C) for 72 hours. Petri plates were checked

for presence or absence of test organism growth. Petri plates not exposed to the sanitizer aerosol were identified as positive for growth. Petri plates exposed to the sanitizer aerosol were identified as negative for growth.

Fourteen petri plates with TSA were numbered (1-14) and arranged in 5 rows (4 rows of 3 plates, 1 row of 2 plates) on the bottom panel within the exposure chamber. Two cartridges containing 500 mL of sterile, nonpyrogenic water with a 10^6 CFU/mL concentration of the test organism, under 345 kPa of pressure facilitated a combined flow of 20 L/minute of H_2O saturated aerosol into the 119,911 cm^3 exposure chamber. All petri plates were exposed to the inoculum aerosol for 5 minutes. A 2 kPa vacuum was maintained throughout the exposure period to assure chamber pressure equilibrium. After inoculum exposure, the chamber was evacuated with 40 kPa through two bacterial air vents (Gelman Sciences, product no. 4210, Ann Arbor, MI) for 5 minutes. All odd numbered plates (1, 3, 5, 7, 9, 11, 13) were removed and incubated at ambient temperature (25°C) for 72 hours. Even numbered plates (2, 4, 6, 8, 10, 12, 14) remained in the chamber and were exposed to a chemical sanitizer aerosol. Sanitizers were delivered via two cartridges containing 500 mL of sterile, nonpyrogenic water with 5 mL of liquid sanitizer under 345 kPa of pressure

facilitated a combined flow of 20 L/minute of H_2O saturated aerosol into the 119,911 cm^3 exposure chamber. After chemical sanitizer aerosol exposure, the chamber was evacuated with a 40 kPa vacuum pressure for 5 minutes. Even numbered petri plates were removed and incubated at ambient temperature (25°C) for 72 hours. Petri plates (odd numbered plates 1, 3, 5, 7, 9, 11, 13) which were exposed via aerosol to the test organism, and not exposed to a chemical sanitizer aerosol were identified as positive for growth. Petri plates (even numbered plates 2, 4, 6, 8, 10, 12, 14) which were exposed to the test organism aerosol, and exposed to the chemical sanitizer aerosol were identified as negative for growth.

Swab samples (5, 10 cm x 10 cm) obtained from the side(s) and bottom panel(s) of the exposure chamber after exposure to the test organism aerosol, and chemical sanitizer, were placed in TSB tubes and incubated at 28°C for 72 hours. All swab sample(s) results were negative for growth.

3.3. Microtubes

Four microtubes (1, 100 μm [O.D.] x 10 mm [l] x 10 μm [I.D.]; 1, 100 μm [O.D.] x 10 mm [l] x 20 μm [I.D.]; 1, 100

μm [O.D.] x 5 mm [l] x 10 μm [I.D.]; 1, 100 μm [O.D.] x 5 mm [l] x 10 μm [I.D.]), were tested for gas flow. Each tube was fitted into a 27 gauge x 1.27 cm needle, sealed with silicon and attached to a model #1001, Gastight^{R} (Hamilton Co. Reno, NV) syringe. The syringe was submerged into a 1 L beaker filled with water, and compressed to force ambient air through the microtube. All tubes tested demonstrated flow via bubble formation through the end of the microtube. Eight microtubes, 2 of each size (hole diameter and length) were evaluated via light microscopy (Model BH-2, Olympus Systems, Lake Success, NY) for interior hole diameter variation. A variation of ± 2 μm was observed for both 10 μm and 20 μm hole diameters.

3.4. Experimental Repetition(s)

The exposure chamber was sanitized via two cartridges containing 500 mL of sterile, nonpyrogenic water with 5 mL of XY-12 liquid sanitizer. Under 345 kPa of pressure, the two cartridges produced a combined flow of 20 L/minute of H_2O saturated aerosol. The aerosol entered the 119,911 cm^3 exposure chamber via plastic tubing. After a 5 minute exposure to chemical sanitizer, the exposure chamber was evacuated with a 40 kPa vacuum for 5 minutes.

Pouches were removed from refrigerated conditions (4.4°C degrees) as needed in groups of 4. Pouches were allowed to achieve ambient temperature (25°C) prior to aerosol exposure. A cut was made along the top seam from each side, progressing towards the defect area in the center of the pouch. A 45° angle was cut from each side of the defect to avoid damaging the microtube. Excess pouch material was removed, and each pouch of the experimental set was placed on a plastic hanging rack and secured via autoclaved wood clamp. The rack with the 4 pouches (1 of each defect dimension) was placed inside the exposure chamber, and the top secured. Two size E - cylinders filled with filtered air, each with a 0-15 mL air flow meter and a cartridge containing 500 mL of sterile, nonpyrogenic water with a 10^2 or 10^6 cells/mL source concentration (either motile or nonmotile) of the test organism, under 345 kPa of pressure facilitated a combined flow of 20 L/minute (10 L/minute per flow meter) of H_2O saturated, infected aerosol into the exposure chamber. A 4 kPa vacuum was used on the filtered exit port to aid in achieving equilibrium within the exposure chamber. Pouches remained in contact with the infected aerosol at ambient temperature (25°C) within the exposure chamber for 15 or 30 minutes.

After infected aerosol exposure, the chamber was evacuated with 40 kPa through two bacterial air vents (Gelman Sciences, product no. 4210, Ann Arbor, MI) for 5 minutes. Pouches were removed from the exposure chamber and incubated at 28°C for 72 hours. Two cartridges containing 500 mL of sterile, nonpyrogenic water with 5 mL of XY-12 sanitizer under 345 kPa of pressure replaced the inoculated cartridges. The two cartridges produced a combined flow of 20 L/minute of saturated H_2O into the exposure chamber. This measure was used to reduce the potential for post exposure contamination. The process was repeated for each experimental set (i.e., repeated 32 times).

3.5. Test Organism Growth Confirmation During Experimental Set(s) Repetitions

Every fourth set, two TSA petri plates (plates A and B) were placed on the floor of the aerosol chamber prior to the start of a pre-selected repetition set. Plates were labeled according to the experimental set number. Plate A was removed with the pouches upon completion of aerosol inoculation and evacuation of the chamber. Plates were incubated at 28°C for 72 hours. Plate B was subjected to an aerosol of sanitizer within the exposure chamber for 5

minutes. After exposure and chamber evacuation, plate B was removed and incubated at 28°C for 72 hours. Plates were evaluated based on the presence or absence of test organism growth.

Twelve pouches without seal defects were also used for experimental controls. Six pouches were immediately incubated (at 28°C for 72 hours) upon initiation of the experiment. Six pouches were placed in pre-selected experimental sets with defective pouches (i.e., 5 pouches within the chamber). Following experimental treatment and incubation of all pouches (defective and control), 2 mL of TSB growth medium was extracted from each pouch and spin plated onto TSA petri plates. Each petri plate was labeled with an identification number which matched that of the pouch from which the inoculum was retrieved. All pouches (140 total) were incubated in an environmental room at 28°C for 72 hours.

VII. RESULTS AND DISCUSSION

Fourteen control plates of TSA were used to confirm organism viability during aerosol testing and to evaluate the effectiveness of the sanitizing step, prior to aerosol exposure of the pouches with manufactured defects. Control plates which demonstrated post aerosol test organism growth were designated, "positive". A "positive" designation for plate A indicated test organism presence, viability, as well as an effective inoculum aerosol method. A "positive" designation for plate B indicated test organism viability, as well as an ineffective sanitizing procedure. Plates which yielded no growth of test organism were designated "negative". A "negative" designation for plate A indicated no test organism presence or viability, and an ineffective aerosol method. A "negative" designation for plate B indicated no test organism presence or viability, and an effective sanitizing procedure. All A plates were positive. All B plates were negative.

Petri plates which represented the experimental controls or defective pouches by identification number, were evaluated based on the presence or absence of test organism growth. Plates which demonstrated test organism presence and growth were designated "positive". Pouches designated

as "positive" indicated microbial presence from microbial ingress via manufactured defect. Plates yielding no test organism presence or growth were designated "negative". Pouches designated "negative" indicated an absence of microbial presence from microbial ingress via manufactured defect. Plates with growth received the following treatment: representative CFU selection, Gram stain, long wave UV inspection, and visual verification via light microscope.

Eight experimental sets (each repeated 4 times) yielded 6 positive pouches. Four positive pouches had a 10 μm hole diameter, 10 mm channel length, and were exposed to a 10^6 cells/mL source concentration of the motile test organism, for 15 minutes. One positive pouch had a 20 μm hole diameter, 10 mm channel length, and was exposed to a 10^6 CFU/mL source concentration of the motile test organism for 30 minutes. One positive pouch had a 20 μm hole diameter, 5 mm channel length, exposed to a 10^6 CFU/mL source concentration of the motile test organism for 30 minutes as shown in Table 4.

TABLE 4: EXPERIMENTAL VARIABLES WHICH RESULTED IN MICROBIAL INGRESS BY Pseudomonas fragi VIA AEROSOL EXPOSURE CONDITIONS.

Variable Combinations
10 μm hole dia. * 10 mm channel length * 10^6 CFU/mL * motile * 15 min exposure time
10 μm hole dia. * 10 mm channel length * 10^6 CFU/mL * motile * 15 min exposure time
10 μm hole dia. * 10 mm channel length * 10^6 CFU/mL * motile * 15 min exposure time
10 μm hole dia. * 10 mm channel length * 10^6 CFU/mL * motile * 15 min exposure time
20 μm hole dia. * 10 mm channel length * 10^6 CFU/mL * motile * 30 min exposure time
20 μm hole dia. * 5 mm channel length * 10^6 CFU/mL * motile * 30 min exposure time

1. Interactions

Forty eight variable interactions were statistically examined in the split plot design model. Four interactions were significant at the $\alpha = 0.05$ level of significance as shown in Table 5. Individual variables were reported as statistically significant only if they were a component of each positive pouch defect. Variables A_1^b (10^6 CFU/mL test organism source concentration) and A_2^d (motile test organism morphology) were found statistically significant and present in all 6 positive pouch defects. Variables A_3^e (15 minute aerosol exposure time), B_1^g (10 μm hole diameter) and B_2^j (10 mm channel length) were present as components in 4, 4 and 5 positive pouches respectively, but were not individually statistically significant. However, the interaction between variables A_3^e (15 minute aerosol exposure time) and B_1^g (10 μm hole diameter) was statistically significant as shown in Table 5.

TABLE 5: EXPERIMENTAL VARIABLES WHICH SIGNIFICANTLY CONTRIBUTED TO MICROBIAL INGRESS BY *Pseudomonas fragi* VIA AEROSOL EXPOSURE CONDITIONS.

Variables	Number of Positive Pouches With Variable(s)	P > F
Motility	n = 6/64	0.0001
Concentration (10^6 CFU/mL)	n = 6/64	0.0001
Concentration (10^6 CFU/mL) and Motility	n = 6/64	0.0001
Exposure Time (15 minutes) and Microtube Diameter (10 μm)	n = 6/64	0.0097

Several other experimental variables and variable interactions may have strongly influenced the process of microbial ingress. A 10 mm channel length (B_2^j) was a component of the defects in 5 of the 6 positive pouches but not found statistically significant. Three interactions common to 5 of the 6 positive pouches were also not found to be statistically significant. They are as follows: interactions between 10^6 CFU/mL test organism source concentration (A_1^b) and a 10 mm channel length (B_2^j); motile test organism (A_2^d) and a 10 mm channel length (B_2^j); 10^6 CFU/mL test organism source concentration (A_1^b), motile test organism (A_2^d) with a 15 minute aerosol exposure time (A_3^e). Statistically, channel length and aerosol exposure time did not significantly contribute to microbial ingress into the positive pouches.

2. Effect of Aerosol Exposure Time and Concentration on Threshold Size

Four pouches with a 10 μm hole diameter and a 10 mm channel length resulted positive after a 15 minute aerosol exposure time. Two pouches with a 20 μm hole diameter and a 5 mm channel length tested positive for microbial ingress after a 30 minute aerosol exposure time. Positive pouches

with a 10 μm hole diameter required less aerosol exposure time to allow microbial ingress than pouches with a 20 μm hole diameter.

Pouches which tested positive for microbial ingress in this study were exposed to a source concentration of 10^6 CFU/mL inoculed water. With a source concentration of 10^6 CFU/mL inoculated water, final test organism concentration within the exposure chamber increased from 87 CFU/cm^3 of air to 178 CFU/cm^3 of air as aerosol exposure time increased from 15 minutes to 30 minutes, respectively. The number of positive pouches decreased from 4 to 2 with the increase of test organism final concentration.

Chen et al. (1991) results differed from those stated above. Positive containers with 5 μm pinholes exposed to 30 minutes of spray inoculum tested positive for microbial ingress. Also, 20 μm pinholes exposed to 15 minutes of spray inoculum tested positive for microbial ingress (Chen et al., 1991). The ability to detect small pinholes increased with increased spray exposure time.

Anema and Schram (1980) found that pouches with 100 μm holes varied in the rate of contamination from 18% to 100% as the concentration of the challenge suspension increased from 10^2 to 10^8 CFU/mL. And by increasing the exposure time

from 1 to 30 minutes, the contamination rate increased from 15% to 58%.

3. Comparison of Spray Cabinet Technique and Immersion Method to Aerosol Exposure Biotesting

Results varied between the spray cabinet technique, immersion and aerosol exposure biotesting methods primarily due to the method of inoculum delivery and exposure to test containers. For example, the inoculum delivery and exposure system used by Chen et al. (1991) more closely simulated immersion rather than aerosol biotesting due to the volume of fluid sprayed onto the juice cartons used in the experiment. The pressure of the spray (2.07×10^5 kg/m^2) may have the contributed significantly to microbial ingress into the cartons (Chen et al., 1991).

Several factors such as pressure, test organism concentration, dynamic flow, defect size and length are thought to influence microbial ingress into packages. Unfortunately, analytical models to evaluate properties such as dynamic flow within either pinholes or channels are antiquated. Dynamic flow models (Alves and Boucher, 1963; Fuchs, 1964) are based on studies of relatively large ($\geq$ 1000 μm) diameter tubes rather than the 5 μm, 10 μm or 15 μm

pinholes used by Chen et al. (1991) or the 10 μm and 20 μm microtube diameters used in this study. Therefore, predictions based on current dynamic flow models concerning relationships between leaker size and microbial ingress, may result in fallacious conclusions.

Gilchrist et al. (1989) immersed 26 trilaminated polyethylene, aluminum and polypropylene pouches with manufactured defects (laser holes) of 17 μm to 81 μm in a TSB 10^8 *E. coli*/mL for 2 hours and agitated every 15 minutes. Gilchrist et al. (1989) found that bacteria passed through holes down to approximately 22 μm in diameter. Upon completion of the biotesting procedures, the 22 μm laser made hole was remeasured. The final hole diameter measured 5 μm, for a size reduction of 17 μm or 77%.

Throughout this study, defects were uniformly maintained by using microtubes. Variations were not found between microtube diameters before and after the experiment.

A flow rate of 10 Lpm pasted through each of the two 500 mL cartridges, for a total of 21 mL of inoculated aerosol per 15 minutes of exposure time. A total of 42 mL of inoculated aerosol was produced per 30 minutes of exposure time. From a source concentration of 10^2 cells/mL, a final aerosol concentration of 1 cell/10 cm^3 and 2 cells/10 cm^3 was achieved for exposure times of 15 and 30

minutes, respectively. From a source concentration of 10^6 cells/mL a final aerosol concentration of 87 cells/cm^3 and 175 cells/cm^3 were achieved for exposure times of 15 and 30 minutes, respectively.

Six of 128 pouches, or 9.4% of the pouches exposed to an aerosol test organism source concentration of 10^6 cells/mL tested positive for microbial contamination. Placencia et al. (1986) exposed filters with microholes ranging from 47 μm to 67 μm to an aerosol concentration of 8.55 spores/cm^2 for a period of 15 minutes. Placencia et al. (1986) found this method detected a single microhole 55.6% of the time. Jarrosson (1992) immersion biotested 150 pouches using a 4 x 10^8 cells/mL concentration of *E. coli* 9637. Each of the 150 pouches was characterized by a 20 μm hole diameter and a 5 mm seal width. Of the 150 pouches, 144 or 96% of the pouches tested positive for microbial contamination.

Reich (1985) stated that aerosol concentrations of > 10^4 cells/cm^2 are far in excess of what might realistically be found in product-use conditions. Reich (1985) also stated that the probability of detecting one microhole increases as pore or hole diameter increases. Specifically, he found that the probability of detecting one microhole with a pore or hole diameter in the range of 0.8 μm to 12 μm

is less than 10^{-12} to 10^{-16} (Reich, 1985). Microhole diameters of 0.8 μm to 12 μm were identified as the lower and upper limit, respectively, of the low probability range for microhole detection (Reich, 1985).

In this study, 66% of the positive pouches were in the low probability range (i.e., positive pouches with a hole diameter of 10 μm). Therefore, the aerosol exposure biotesting method used in this study can be used to detect microholes via microbial ingress, previously considered statistically improbable to detect.

4. Uncontrolled Factors

Several uncontrolled factors may have contributed to the results obtained in this experiment, such as: test organism stress condition(s), hostile environment (within exposure chamber and on side panels of the polymer pouches during incubation period), and nutrient availability within microtube(s).

Test organisms selected for nonmotility were exposed to stress conditions (pH change, nutrient deprivation and antibiotic marker) to facilitate mutagenesis. During aerosol exposure, stressed test organisms were inoculated into cartridges containing sterile, nonpyrogenic water (with

0.625 mL of Butterfields PO_4 buffer), at pH 6.5-6.7, in the absence of nutrient materials. Potentially, an opportunity for reduction or an increase of vegetative test organism cells/mL existed. Cartridges containing inoculum for aerosol exposure were used a maximum of 2 hours.

The chemical sanitizer aerosol provided an effective sanitizing step following inoculum aerosol exposure. However, residual chemical sanitizers could potentially interact with water particles in a high humidity environment (100% within exposure chamber). This interaction could provide a vehicle for pouch side wall and defect site contact with the chemical sanitizing agent. Aerosol particle sizes of 2.68 μm mean aerodynamic mass were used to reduce the probability of this occurrence.

Test organism motility loss may have resulted during the aerosol exposure process or from charge differentiation. Charge differentiation may be recognized (via chemo-receptors or Van der Waals forces, etc.) by the test organism as an inhibitor (repulsion) or a promotor (affinity) of attachment to the pouch surface or defect site. Potentially, the surface of the polypropylene pouches and the nickel microtubes could provide a source of charge. The charge potential of the pouch material or the microtubes were neither measured or evaluated. Microorganisms within

an aerosol may orient themselves based on charge repulsion and or affinity more freely than the same microorganisms in a liquid or solid medium. In this study, airborne microorganisms which landed in or near the microtube hole may have been influenced by affinity or repulsion charge forces. The affinity or repulsion charge forces may have caused the test organism to attach temporarily or permanently to the interior wall of the microtubes. Organism immobilization at the tube surface (adsorption) is the first of three stages which lead to the formation of a biofilm (Notermans et al. 1991). Adsorption is followed by the formation of polymer bridges (consolidation). Ultimately growth and spreading of the organism over the surface (colonization) results in biofilm formation (Notermans et al. 1991). Partial or total obstruction of the microtubes may have occurred as a result of biofilm formation resulting in the low number (6 out of 128) of positive pouches.

The presence or absence of TSB growth medium within the microtube may have contributed to the low number of positive pouches. The flow of broth from the pouch interior, through the microtube, to the pouch exterior was not measured. The motile *Pseudomonas fragi* used in this experiment requires a liquid or semi-solid medium to demonstrate motile

characteristics. While exercising motility, the microorganism elongates (Lee et al., 1993). During elongation the diameter of the microorganism reduces axially. The diameter reduction and elongation of the motile microorganism may have facilitated movement through the microtubes, which resulted in positive pouches.

VI. SUMMARY AND CONCLUSIONS

Pouch contamination via microbial ingress was affected by test organism motility (motile) and test organism concentration (source concentration of 10^6 cells/mL). It is the opinion of this author that the individual effects of hole diameter and channel length play a less influential role in aerosol contamination when viewed individually, than do factors such as organism motility and concentration in post-process microbial contamination of pouches.

Howard and Duberstein (1980) found that under specific conditions certain types of water borne bacteria penetrated 0.2 μm membrane filters and therefore they speculated the minimum hole size critical to sterility maintenance and integrity of the package (i.e., the threshold leak size) is between 0.2 μm and 0.4 μm. This range was selected based on the size of membrane filters used routinely for aseptic packaging and cleanroom applications with little significant microbial contamination. Factors such as microorganim(s) motility or concentration were not considered. Lampi (1980) showed bacterial penetration via holes of less than 10 μm was unlikely. Lake et al. (1985) suggested holes larger than 1 μm were needed for bacterial penetration to occur. Chen et al. (1991) reported that 5 μm pin holes allowed

microbial aerosol penetration. Jarrosson (1992) found that a 20 μm hole diameter and a 5 mm channel length permitted microbiological contamination in Meal Ready to Eat (MRE) pouches. Placencia et al. (1986) found that the average microhole for the five materials used in their experiment varied from 47 μm to 67 μm in diameter. The variation of microhole size rest in researchers' inability to manufacture and maintain the size integrity of leaks with diameters smaller than 10-20 μm during retorting and experimentation (Gnanasekharan and Floros, 1994). Microtubes were used in this experiment to maintain hole size of 10 μm and 20 μm with a variation of ± 2 μm.

The ability to control and reduce the variation of microtube diameters provided an opportunity to examine the importance of organism motility and concentration to post process contamination of flexible pouches. This researcher considers the threshold microhole diameter to be < 10 μm when using test organisms that are motile, rod shaped and have an average length of approximately 1 μm to 2 μm. This researcher also considers an airborne microbial concentration of ≥ 87 cells/cm^3 to provide a favorable environment for microbial ingress into flexible pouches with ≥ 10 μm interior diameter defects.

VIII. REFERENCES

Adelberg, E.A., Mandel, M. and Chen, G.C.C. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *E. coli* K12. Biochem. Biophys. Res. Communic. 18:788-795.

Alves, G.E. and Boucher, D.F. 1963. Fluid and particle mechanics. In "Chemical Engineering Handbook," R. H. Perry, C. H. Chilton and S. D. Kirpatrick (Eds.), 9-10, 4th Ed. McGraw Hill, New York.

Anderson, G.L. 1989. Leak testing. In Nondestructive Evaluation and Quality Control: Metals Handbook, 9th ed.: 57-70.

Anema, P.J. and Schram, B.L. 1980. Prevention of post-process contamination of semi-rigid and flexible containers. J. Fd. Prot. 43(6):46-464.

Anonymous. 1993. GN MicroPlate instructions for use. Biolog, Hayward, CA 94545.

Armstrong, J.B., Adler, J. and Dahl, M.M. 1967. Nonchemotatic mutants of *Escherichia coli*. J. Bact., 93(1):390-398.

Arndt, G.W. 1992. Examination of flexible and semirigid containers for integrity. In "Bacteriological Analytical Manual," 7th ed., Ch. 21A, pp. 322 - 368. AOAC, Arlington, VA.

Axelson, L. and Calvin, S. 1991. Aseptic integrity and microhole determination of packages by gas leakage detection. Packaging Technol. and Sci., 4:9-20.

Axelson, L., Calvin, S. and Nordstrom, J. 1990. Aseptic integrity and microhole determination of packages by electrolytic conductance measurements. Packaging Technol. and Sci., 3:141-162.

Burkhardt, J.K., McIlvain, J.M., Sheetz, M.P. and Argon, Y. 1993. Lytic granules from cytotoxic T cells exhibit kinesin-dependent motility on microtubules in vitro. J. Cell Sci., 104:151-162.

Chen, C., Harte, B., Lai, C., Pestka, J. and Henyon, D. 1991. Assessment of package integrity using a spray cabinet technique. J. Fd. Prot., 54(8):643-647.

Davidson, P.M. and Pflug, I.J. 1981. Leakage potential of swelled cans of low-acid foods collected from supermarkets. J. Fd. Prot., 44(9):692-695.

Floros, J.D. and Gnanasekharan, V. 1992. Principles, technology, and application of destructive and nondestructive package integrity testing. In "Advances in Aseptic Processing Technology," R.K. Singh and P.E. Nelson (Ed), 157-188. Elsevier Science Publishers, New York.

Folinazzo, J.F., Kiloran, J.J. and Maunder, D.T. 1968. Bio-test method for determining integrity of flexible packages of shelf-stable foods. Fd. Technol. 22:615-618.

Fuchs, N.A. 1964. Classification of aerosols: size and shape of aerosol particles. In "The Mechanics of Aerosols," Ch.4., pp. 107-180, McMillan Co., London.

Gilchrist, J.E., Rhea, U.S., Dickerson, R.W. and Campbell, J.E. 1985. Helium leak test for micron sized holes in canned foods. J. Fd. Prot. 48(10):856-860.

Gilchrist, J.E., Shah, D.B., Radle, D.C. and Dickerson Jr., R.W. 1989. Leak detection in flexible retort pouches. J. Fd. Prot., 52(6):412-415.

Gnanasekharan, V. and Floros, J.D. 1994. Package integrity evaluation: Criteria for selecting a method. Part I. Package Technol. & Eng. 3(6):67-72.

Howard, G. and Duberstein, R. 1980. A case of penetration of 0.2 μm rated membrane filters by bacteria. J. Parenteral Drug Assn., 34(2): 95-102.

Iino, T. 1969. Genetics and chemistry of bacterial flagella. Bacteriol. Rev., 33(4). 454-475.

Jarrosson, B.P. 1992. Closure Integrity Testing of Heat Sealed Aseptic Packaging Using Scanning Acoustic Microscopy. Virginia Polytechnic Institute and State University, Department of Food Science and Technology, M.S. Thesis.

Kamei, T., Sato, J., Natsume, A. and Noda, K. 1991. Microbiological quality of aseptic packaging and the effect of pin-holes on sterility of aseptic products. Pack. Technol. and Sci., 4:185-193.

Kautter, D., Lynt, R., Landry, W., Lanter, J. and Schwab, A. 1984. Examination of canned foods. Ch. 23. In Bacteriological Analytical Manual, 6th ed., 23.01-23.20. AOAC, Arlington, VA.

King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. and Clin. Med., 44:301-307.

Lake, D.E., Graves, R.R., Lesniewski, R.S. and Anderson, J.E. 1985. Post-processing spoilage of low-acid canned foods by mesophilic anaerobic sporeformers. J. Fd. Prot., 48(3):221-226.

Lampi, R.A. 1980. Retort pouch: The development of a basic packaging concept in todays high technology era. J. Fd. Process Eng., 4:1-18.

Lampi, R.A., Schlz, G.L., Ciavarini, T. and Burke, P.T. 1976. Performance and integrity of retort pouch seals. Fd. Tech., 30(2):38-48.

Lee, J., Akira, I., Theriot, J. and Jacobson, K. 1993. Principles of locomotion. Nature 362:167-171.

Matty, J.T., Stevenson, J.A. and Stanton, S.A. 1991. Packaging for the 90's: convenience versus shelf stability or seal peelability versus seal durability. Fd. Pack. Technol., 4(4):74-90.

McEldowney, S. and Fletcher, M. 1990[a]. The effect of physical and microbiological factors on food container leakage. J. Appl. Bacteriol. 69:190-205.

McEldowney, S. and Fletcher, M. 1990[b]. A model system for the study of food container leakage. J. Appl. Bacteriol. 69:206-210.

Morris, C.E. 1989. Convenience foods: the second revolution. Food Eng. 61(9):69-82.

Nichols, Phillipa. 1989. Container integrity of heat sterilized food. PIRA Packaging Division, Slough, England.

Notermans, S., Dormans, J. and Mead, G.C. 1991. Contribution of surface attachment to the establishment of micro-organisms in food processing plants: a review. Biofouling, 5:21-36.

Panopoulos, N.J. and Schroth, M.N. 1974. Role of flagellar motility in the invasion of bean leaves by *Psedomonas phaseolicola*. Phytopathology. 64:1389-1397.

Placencia, A.M., Arin, M.L., Peeler, J.T. and Oxborrow, G.S. 1988. Physical tests are not enough. Med. Dev. & Diag. Ind. 10(9): 72, 74-78.

Placencia, A.M., Oxborow, G.S. and Peeler, J.T. 1986. Package integrity methodology for testing the biobarrier of porous packaging. Part I: membrane agar plate strike - through method", Med. Dev. & Diag. Ind. 8(4):60-65.

Placencia, A.M. and Peeler, J.T. 1990. Relationship of bubble point to microbial penetration in medical packaging, TAPPI J. 73:255-7.

Placencia, A.M., Peeler, J.T., Arin M.L. and Oxborrow, G. S. 1989. Microbial penetration and the porosity of medical-grade packaging materials, TAPPI J. 72:102-5.

Put, H.M.C. and Warner, W.R. 1972. The mechanism of microbiological leaker spoilage of canned foods: a review. J. Appl. Bacteriol. 35:7-27.

Put, H.M.C., Witvoet, H.J. and Warner, W.R. 1980. Mechanisms of microbiological leaker spoilage of canned foods: biological aspects. J. Fd. Prot. 43(6): 488-497.

Reich, R.R. 1983. A comparison of the microbial barrier properties of vented and non-vented luer guards. Partic. Microb. Cont. 2(4):46-49.

Reich, R.R. 1985. A method for evaluating the microbial barrier properties of intact packages. Med. Diag. Ind. 7(3):80, 82, 86-88.

Schlotzhauer, S.D. and Littell, R.C. 1991. SAS system for elementary statistical analysis. 3rd ed. Cary, NC: SAS Institute Inc.

Shapton, D.A. and Hindes, W.R. 1962. Somes aspects of post processing infection. Proc. 1st Int. Congr. food Sci. technol., London, 206.

VITA

The author, Scott Keller, was born August 29, 1960 in Joplin, Missouri, U.S.A. In May 1983, he received his B.S. degree in psychology from Missouri Western State College, in St. Joseph, Missouri. In 1986 he received his M.B.A. from Northwest Missouri State University in Maryville, Missouri.

In January of 1993, he entered Virginia Polytechnic Institute and State University to begin graduate study in pursuit of a Master of Science Degree in Food Science and Technology.