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DEVELOPMENT OF A BIOTEST METHOD TO ASSESS INTEGRITY OF ASEPTIC PACKAGES

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Shaun Chenghsiung Chen

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Bruce Harte

Major professor

Bruce Harte

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Development of A Biotest Method to Assess Integrity of Aseptic Packages

by

SHAUN CHENGHSIUNG CHEN

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ABSTRACT

Development of A Biotest Method to Assess Integrity of Aseptic Packages

by

SHAUN C. CHEN

A spray cabinet technique was developed to determine microbial integrity of aseptic packages. It was constructed to include two pumps and 32 nozzles to achieve complete coverage over all surfaces of the test packages. A cell culture of Lactobacillus cellobiosus was sprayed onto the packages through the nozzles during testing. Standard pinhole orifices were used to determine the efficiency of the spray cabinet technique to detect pinholes. A ten micron hole was detected after 15 minutes of spraying, while a 5 micron defect was detected after 30 minutes of spraying. The percent defects increased as the pinhole size increased. The spray cabinet technique provided better detectability compared to an immersion method. Aseptic juice packages were exposed to dynamic testing, and the package integrity assessed using the biotests. A high level of defects was found using the spray cabinet technique. More loss of integrity was observed for packages located in the corner of the shipping cartons. The most damage was observed in corner packages of shipping containers stacked 5 high.

Dedicated to My Parents,

Mr. and Mrs. Chen

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INTRODUCTION

Aseptic packages are widely used for the packaging of perishable products under ambient storage conditions. This is accomplished by maintaining hermetic integrity of the package during transportation and distribution. However, abusive handling of the finished packages may cause defects in the seal area and/or rupture of the package materials, which would result in loss of package integrity. Package defects are a source of both economic loss and public health concern for the food industry and society in general (Bryant, 1988). Loss of package integrity can greatly increase the risk of microbial contamination and potential poisoning hazards. Therefore, packages must maintain their hermetic integrity to protect the product from microbial contamination, because any microbial contamination can be a potential public health threat and could adversely affect a company's reputation. Nowaday, more efficient test techniques are required in the food industry for the trend to microwavable, multi-use, easy-to-open and family size shelf stable products (Harte, 1984). A method, which could provide reliable data that would minimize the problems caused by package defects, would be very beneficial.

Biotest methods are tools used to measure package integrity, and assure sterility of a product/package until

used by consumers (Placencia, 1986a). Tests to determine the hermetic integrity of closed packages after filling and sealing, have been available for the past 25 years (Sizer, 1983). Most biotests are concerned with measuring the biobarrier properties of packaging materials against the penetration of microorganisms (Hartman, 1963; Ronsivalli, 1966; Tallentire, 1984; Reich, 1985; Placencia, 1986), instead of detecting loss of total package integrity (Maunder, 1968).

The selection of microorganisms used in a biotest is based on the characteristics of the packaged food product, especially pH, i.e., the acidity of the food. The two most popular microorganisms used are Enterobacter aerogenes for low-acid products, and Lactobacillus cellobiosus for acid foods (ASTM F 2.4, 1983). The test organisms selected should exhibit the characteristics of ability to utilize the packed food product; ease of handling; non-hazardous nature and ability to produce large amount of gas which contributes to a swelling appearance, or change of pH in the food, making detection of failed packages easier.

Currently, the most common biotest used is the "Immersion Test", which is used to evaluate the construction features of the container and the effect of abusive handling on filled metal cans (Maunder, 1968). In the immersion test, metal cans are dipped into a cell culture for a certain period of time to assess the

microbial integrity of the double seam. Microbial growth inside the can is detected by visual observation of swelling after a period of incubation. However, for paper-based flexible and semi-rigid packages, the paper layer may absorb liquid and cause rupture of the contacting surface due to wicking of water. Therefore, the test itself could result in loss of package integrity, even though there may not have been any inherent defects in the package materials.

To assess the microbial integrity of paper-based packages, a technique referred to as the "Spray Cabinet Method" was developed in this study. Packages were evaluated by directly spraying microorganisms onto the surface of the test units. Defects in the packages were detected by visual and/or compositional change in the packaged products, which occurred due to the penetration of microorganisms through the package. To develop the method, pinhole apertures were used to determine the necessary duration times for spraying and the minimum detectable defect level. Aseptic juice packages were then biotested using the spray cabinet method and an immersion technique to assess the effect of dynamic testing (transportation hazards) on package integrity.

The objectives of this study were:

1. To develop a method to assess the microbial integrity of paper based containers.

2. To measure the efficiency of the spray cabinet technique by applying it to juice packages which had been exposed to transportation hazards.
3. To compare the results to an immersion technique.

LITERATURE REVIEW

I. Aseptic Packaging Systems.

Development of Aseptic Processing and Packaging.

The first aseptic process was developed for milk in cans, in 1921 by Orla-Jensen, Denmark (Hersom, 1985). In 1933, The American Can Company developed a filling machine which used saturated steam under pressure to sterilize the metal cans, it was named the Heat-Cool-Fill (HCF) system. Though technically successful, the HCF system did not survive commercialization (Hersom, 1985). In the 1940's, Martin developed the Dole-Martin process, in which the empty cans were sterilized in an atmosphere of super heated steam (Hersom, 1985). It was the first time aseptic processing was used in the juice industry in the United States (Tillotson, 1984). Currently, aseptic processing has established itself as a feasible and dependable method for commercially sterilizing product, while retaining the high quality attributes of the product (Nelson, 1984).

Development of aseptic flexible packages began in the early 1950's, when Loliger and Reges utilized hydrogen peroxide (H_2O_2) as a sterilant for paper and polymer laminated cartons (Arndt, 1989). This method significantly differed from conventional thermal sterilization, where metal cans were sterilized in an atmosphere of super-

heated steam. In the late 1950's, Verband Molkeric, in Switzerland, was the first company in the world to use hydrogen peroxide to commercially pack aseptic milk in paperboard cartons (Johnson, 1966). Due to its inexpensive construction (polymer /paperboard /polymer /foil /polymer) and ability to extend shelf-life without refrigeration, the advantages of this aseptic system were quickly realized (Arndt, 1989).

In January, 1981, the Food and Drug Administration approved the use of hydrogen peroxide for sterilization of polyethylene food contact surfaces for food processing. Since then, semi-rigid multilayer cartons have been replacing rigid metal cans and glass containers for aseptic juice products (Tillotson, 1984).

In 1983, Ocean Spray Co. aseptically packed juice drink for the first time in the U.S. market. Currently, the most common aseptic juice package in the U.S. is the 250 ml Brik Pak. About one billion units of fruit drink were sold in this package during 1986 (Stacy, 1987). Of the aseptic systems in the world, about 80% are used to pack milk-based products, and 15% to produce juice packages, and the rest are used for oil, water or sauces (Sacharow, 1986). In the U.S., 1842.8 million gallons of juice packages, valued at 9.8 billion dollars, were sold in 1986 (Stacy, 1987).

Flexible and semi-rigid packages are not as effective

barriers as metal and glass containers. Many factors affect the maintence of package integrity, for instance, damage to aseptic packages can occur during distribution (Table 1) (Harte, 1987). The heat and pressure associated with heat sealing tends to soften the side seal, which can lessen the hermetic integrity of a package (Hultberg, 1981). Improper handling can cause pinholes on the impacted surfaces of packages. This changeover from rigid containers to more flexible forms for shelf stable food requires better quality control, and development of new test methods to assure quality and safety (Harte, 1984). Continuing technological advances in aseptic processing and sterile packaging results in cost savings for manufacturers, and will be a more and more attractive alternative to conventional rigid containers for drinks which require refrigeration (Wolpert, 1987). In the middle 1980's, the food industry became very involved in performing theoretical microbiological studies to determine the exact time and temperature required to destroy spoilage microorganisms, while minimizing damage to food quality during thermal processing. Many "Cool sterilization" methods were developed during this period, such as: U.V. light, Gamma irradiation and membrane filtration to purify juice for aseptic filling (Tillotson, 1988).

Now, a much wider range of materials can be used in

Table 1. Types of damage occurred during transportation and distribution.

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1. Flex cracks caused by vibration of the packages in transit.
2. Pinholes resulting from flexing or handling operation or poor manufacturing and processing.
3. Abrasion damage due to abrasive handling, vibration or shock.
4. Seal failure.
5. Impact damage resulting from dropping or any forms of abuse handling.
6. Compression damage caused by stacking and incorrect warehousing.

(Harte, 1987)

contact with products, allowing development of adequate packages resulting in better performance (Carlson, 1984). Several packaging materials: EVA (Ethylene Vinyl Acetate), polyester, some ionomers and olefin food contact surface materials, have received approval from Food and Drug Administration and can now be sterilized using hydrogen peroxide (FDA Regulation, 21CFR178, March 31, 1984). These materials function to improve the package system by improving the barrier, mechanical properties, and/or heat seal integrity of packages (Arndt, 1989).

Aseptic Processing.

The advantages of aseptic packaging/processing are: reduction in cost of containers and distribution, ease of stacking, and long shelf-life without refrigeration (Tillotson, 1984; Griffin, 1985). Unfortunately, aseptic processing costs are still high, because of more quality control concerns (Griffin, 1985). On the other hand, many concerns are also associated with the process, such as the mechanism of heat penetration from the heating area to the center location, especially for low acid foods (Sacharow, 1987). The main difference of thermal process between low acid and acid products is due to the efficiency of the thermal penetration (Bernard, 1983). Low acid foods require longer time to reach a commercial sterilizing condition because of low heat penetration, which can cause

loss of quality (Nelson, 1984). Many low acid particulate foods require a scraped surface heat exchanger for raw product sterilization (Tarr, 1986). Since low acid foods demand longer sterilizing time, low acid foods may lose some of the advantages associated with aseptic packaging (Sacharow, 1987). For some products, the heating time maybe about seventy minutes (Sacharow, 1986). Long cooking times require more energy and result in quality loss during processing.

The FDA is the primary regulatory body having jurisdiction over aseptic products. The primary responsibility of FDA Regulations is to protect the public health, and help food manufacturers and processors by developing guidelines and procedures to reduce the occurrence of defective containers, which can result in better methods to detect defects in packages before distribution (Quinn, 1984). Food products that have a pH greater than 4.6 and water activity (A_w) greater than 0.85 must comply with Code of Federal Regulations title 21 part 113 (processing of low acid foods in hermetically sealed containers) (Sacharow, 1987). These critical points (pH=4.6, A_w=0.85) control the growth of microorganisms, especially Clostridium botulinum, these microorganisms can cause lethal Botulism poisoning which has been attributed to defective containers in commercially canned low-acid foods (Quinn, 1984).

II. Microbial Hazards on Aseptic Packages.

Microbial considerations.

System considerations include: 1.) Exposure times, temperatures, sterilant concentrations and other critical factors must be adequate to provide microbial safety. 2.) Adequate safeguards must be incorporated within the processing and packaging equipment to protect the systems microbial integrity (Bernard, 1983). Therefore, packages should not contain any holes which are large enough to permit entry of microorganisms, and should also maintain the package barrier properties of oxygen, vapor and flavor, which are critical to the shelf life of product (Harte, 1987).

Packaging Performance and Microbial Contamination.

A hazardous situation may result if contamination of the original material occurs and/or through post processing recontamination. Microorganisms are prevalent in the air, though air does not contain the necessary nutrients and moisture to support microbial growth. However, floating particles in the air may carry microorganisms which can contaminate the raw materials (Lyman, 1984). Many air borne microorganisms accumulate on raw products and the surface of packaging materials, which increases the microbial flora. Factors contributing to food borne illnesses are shown in Table 2. Many bacteria

Table 2. Microbial factors contributing to foodborne diseases.

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1. Inadequate holding temperature.
2. Long period of time between preparation and consumption.
3. Infected personnel coming in contact with food.
4. Inadequate thermal processing.
5. Inadequate cleaning equipment.
6. Leftovers.
7. Cross contamination.
8. Obtaining foods from unsafe sources.
9. Loss of hermetic packaging integrity.

(Bryan, 1978)

can survive at refrigeration temperatures (Table 3), if they are still present in the food product after processing. Fortunately, the bacterial load on commercially produced packaging materials is quite small (Hersom, 1985), because there is no carbon source present for microorganisms to use except plasticizers (Roberts, 1986). However, paperboard which can trap bacteria should be adequately isolated from the food contact surface.

Usually, sterilization of packaging materials is accomplished by immersing or spraying hydrogen peroxide (H_2O_2) onto the materials. Hydrogen peroxide may be used in combination with steam, U.V., or irradiation to achieve commercial sterility (Arndt, 1989). Commercial sterility is defined as a condition where the viable spores of microorganisms which are concerned to cause any risk of public health, are incapable of growth in the product under normal handling and storage (Sacharow, 1987). Control of microorganisms in food products to ensure product safety through distribution is essential to any food product (Table 4).

The primary function of a package is to protect the product by maintaining hermetic integrity during transportation and distribution (Drennan, 1987). Historically, post-process failures in metal cans were identified as being due to failure of the double seam, contaminated cooling water, or improper can fabrication

Table 3. Food poisoning bacteria capable of growth at 5°C

Organism/Strain	Characteristics	Food	Disease
C. botulinum type E	Gram postitive anaerobic spore-forming rod	Fish	production of toxin causing nuromuscular paralysis
Yersinia enterocolitica	Gram negative facultative rod	Animal Origin Water	diarrhea
Enterotoxigenic E. coli	Gram negative facultative rod	Animal Origin	production of toxin causing diarrhea
Listeria monocytogenes	Gram positive plemorphic rod	Animal Origin Cabbage	infection
Aeromonas hydrophilia	Gram negative facultative rod	Animal Water	production of enterotoxin causing diarrhea or infection

(Dairy & Food Sanitation. 1977. vol.7 No.2)

Table 4. Control of microorganisms in food products

1. Temperature.
2. Water activity.
3. pH value.
4. Preservatives.
5. Redox potential (Eh).
6. Gases in headspace.
7. Sterilization.
8. Packaging.

(McCormick, 1987)

(Put, 1980). For aseptic flexible packages, most of plastic films more than a half mil thick are not permeable to bacteria (Ronsivalli, 1966). The polymer surface tension prevents passage of bacteria through pinholes (Maunder, 1968).

III. Assessment of Package Integrity.

Historical Studies.

The first method developed to evaluate packaging integrity was in the early of 1960's (Sizer, 1983). The

former studies were concerned with characterization of packaging variables such as:

The effect of pressure (or vacuum) on package integrity. A Seitz filter apparature was modified to permit pressure on one side of a film, and a bacterial medium on the other. A pouch was filled with cell culture and then inserted into a flask containing sugar and a crystal violet mixture. This was used as an indicator of microbial penetration because it would cause color change. Enhanced microbial penetration was demonstrated due to pressure and/or vacuum (Hartman, 1963).

Thickness of plastic films. Several plastic pouches were filled with a suitable bacterial growth medium, and exposed to a cell suspension by immersion of pouches in a slurry containing spoiled fish in water. The turbidity of the medium was then examined as an indicator of the microbial permeability of the films. Films more than half mil thick were not permeable to bacteria (Ronsivalli, 1966).

The surface tension of packaging films. Flexible packages were immersed in water containing a large population of a bacterial species which was capable of growing inside the packed foods during incubation. Films with high surface tension were more likely to prevent bacterial penetration (Maunder, 1968).

Recently, researchers have been more interested in factors influencing microbial contamination, such as:

Cell concentration and effect on recontamination. Microbial contamination increased as the concentration of the test cell culture increased. (Tallentire, 1984); Placentia developed a wet-test technique (Placentia, 1986a), which was referred to as the Membrane Agar Plate Strike-Through Method. In this study, a test packaging film was placed on a solid growth medium in a petri-dish. The film was then inoculated by introducing a pad containing a bacterial suspension on the film surface. The pad was removed after 30 minutes from a petri-dish, the dishes placed in an incubator at 37^{o}C. Colonies on the surface of the agar plate were indicative of microbial penetration. In the wet test method, bacterial penetration of material was assessed using a liquid carrier (Placentia, 1986a); A dry-test method was developed which used an exposure chamber. The test materials were placed in an aerosol chamber and exposed to spores which were released using a nebulizer. A filtering membrane was placed outside the test film and was used to determine the number of cells which penetrated through the test films (Placentia, 1986b).

Evaluation of Packaging Integrity through Biotesting.

A biotest is a method designed to assess the

resistance of sterile packages to penetration by microorganisms, i.e., packages are exposed to microorganisms under controlled conditions, the packages/products are then evaluated for signs of penetration (Anthony, 1986). The results should demonstrate the efficiency of the sterilization method and performance of the hermetic seals (Stevenson, 1987). For a biotest, the selected microorganisms should be able to utilize a wide range of packaged food products, have a non-hazardous nature, be easy to handle, and be capable of producing large amounts of gas that makes detection of failed packages easier. Commonly used microorganisms are Enterobacter aerogenes (ATCC 13048) for low acid foods, and Lactobacillus cellobiosus (ATCC 11739) for acid products.

Lactobacillus cellobiosus are rod shaped, about 0.5-1.0 microns by 3-5 microns, non-motile, and non-flagellate. Colonies vary from smooth to rough, cauliflower or doughnut form. The organisms are heterofermentative, may produce lactate, acetate, ethanol and carbon dioxide. They can survive and reproduce at acid environments (pH values below 4.5), and have inducible growth on gluconate with carbon dioxide gas production. Growth is variable at 15^{o}C, negative above 45^{o}C, and optimum at 30-35^{o}C (Kreig. 1984).

Biotests are designed to:

1.) Determine the factors which affect microbial penetration through packages (Table 5.). These include: A. The microbial barrier quality of packaging materials: This is influnced by the thickness, surface tension and wettability of the films; B. Characteristics of defects: Microbial penetration is proportional to the size, number of pinholes on the surface, and the shape of the defect. Less penetration is found in a crooked path than a straight path (Bernard, 1984); C. Test microorganisms: The influence of microbial concentration, optimum activity and living conditions (aerobic or anaeribic) must be determined; D. The nature of the food product, such as pH and water activity must be understood; E. Test conditions: Test duration and conditions (e.g., temperature, pH) must be classified.

2.) Charaterize the relationship between packaging materials, microorganisms, and the environment. Packaging materials may attract microorganisms to their surface by electronic or hydrophilic forces (Lyman, 1984), and act as a nutrient source for bacterial growth (Roberts et al., 1986).

3.) Assess not only microbial penetration, but the by-products which are released by living microorganisms

Table 5. Penetrability of microorganisms based on:

A. Properties of film.
   1. weight.
   2. thickness.
   3. permeability.
   4. wettability.

B. Characteristics of defect.
   1. size of pinhole.
   2. number of pinhole.
   3. shape of pinhole.

C. Test microorganisms.
   1. concentration.
   2. mobility.
   3. aerobic or anaerobic.

D. Nature of food product.
   1. pH.
   2. water activity.

E. Conditions of test.
   1. amount of stress.
   2. duration.

(Bernard, 1984; Placentia, 1986a)

in the food product. Microorganisms may produce by-products as a metabolite while growing in the packed foods, the most concern is for toxin producing microorganisms (e.g., Clostridium botulinum). Production of botulinum toxin (and others) may result in lethality.

Most measures of package integrity are destructive, the food industry requires non-destructive tests which can be assembled on the packaging, processing line, and provide sufficient precision. They should also be capable of working on high speed lines. Thus manufacturers would be able to do quality control on the production line, instead of using separate off-line procedures. A perfect test would be a method which would detect one defective package in a pallet (Sizer, 1983).

MATERIALS AND METHODS

Test Microorganisms.

The microorganisms used in the biotest were Lactobacillus cellobiosus (ATCC 11739), obtained from American Type Culture Collection, Rockville, Maryland.

Preparation of Cell Culture.

The cell culture was prepared by inoculating microorganisms into Lactobacilli MRS broth which was purchased from Difco Co., Detroit, Michigan (See Appendix A for ingredients). The broth was prepared by adding 55 g of the dehydrated medium to one liter distilled water, the broth was sterilized in an autoclave using the condition at the pressure of 15 psi (1.02 atm or 1.03×10^5 kg/m/s^2) and 121^{o}C for 15 minutes. The cell culture was prepared by inoculating 1 ml stock cell culture into 9 ml sterilized broth, and incubated at 37^{o}C for 24 hours to regain optimum activity. For each biotest, initial concentration of about 10^7 cells/ml cell culture was used.

Test Orifices and Packages.

Standard orifices were obtained from Buckbee-Mears Co., St. Paul, Minnesota, and were used to determine the efficiency of the biotest procedures. Orifices were used

as a mean to indicate the minimum size of pinhole that could be detected in a specific test condition (see orifice test section for more details). The orifices were made of nickel alloy containing a specific pinhole aperture in the center. Each orifice was 0.00254 cm thick and 0.635 cm in diameter. Three sizes of pinhole, 5; 10 and 15 micron, were used in the study, which were determined by using microscopy (American Optical Co.).

The packages were one-half gallon (1.89 L) flat-top cartons containing apple juice. The initial pH of the juice was in the range of 3.75-3.85. The cartons were nitrogen-flushed before sealing. The headspace contained more than 95% nitrogen and about 2% carbon dioxide, and the rest oxygen. The carton construction was a lamination of polyethylene (PE) / paperboard / Surlyn / aluminum foil / Surlyn / PE. The carton dimensions were 19.7 cm height x 13.3 cm width x 7.62 cm deep, and were obtained from a local manufacturer.

Agar Plates and Standard Cell Count Method.

The Direct Plate Count Technique was used to determine the cell number in a suspension. The assumption was made that each viable cell will develop into a colony, so that the colony counted on the plate is related to the number of viable cells in the bacterial suspension. Cell numbers in the sample suspension were determined by

directly counting the numbers of cells on agar plates.

To determine cell number, an agar solution was prepared by mixing 2 g of bacto agar with 100 ml of Lactobacilli MRS broth. This solution was then sterilized using the same conditions as previously described. Twenty ml of the agar solution were aseptically poured into a petri dish (9 cm in diameter), while the agar was still hot (55-60^{o}C). The agar plates were then held at room temperature for 24 hours until the agar surface solidified and the extra water evaporated.

To measure the number of cells in the test cell culture, one ml of the microbial suspension was diluted in sequence to reach an appropriate concentration of cells for the standard plate count (30 - 300 cells per plate). The dilution procedure was as follows: One ml of the cell culture was mixed with 9 ml of sterilized distilled water, 1 ml of this diluent was then mixed with 9 ml of sterilized distilled water. The dilution procedure was repeated five times to obtain a 1/100,000 dilution, because the initial concentration of the test cell culture was about 10^{7} cells/ml. The final diluent component (0.1 ml) was then transferred onto the surface of an agar plate, and spread out to obtain equal distribution. After incubating the plate for 48 hours at 37^{o}C, the number of colonies was counted visually. The number of cells was calculated using the following equation:

Cell Conc. = Colonies number x 10^6

where,

Cell Conc. is cell concentration expressed cells/ml cell suspension.

Colony number is the number of the colonies counted per plate expressed as number/0.1 ml diluent.

10^6 is a dilution factor. Each dilution expresses a negative exponent, i.e., 10^{-1}. In the procedure, the sampled cell culture was diluted five times, so the final concentration of diluent was one hundred thousandth of the initial sampled cell suspension. Since the diluent pippetted on each plate was only 0.1 ml, the colony number is multiplied by 10. Therefore, to determinate the number of cells in the cell suspension 10^5 is multiplied by 10 to reach 10^6.

An example of the calculation is shown in Appendix B.

Decontamination of the Experimental Units and the Spray Cabinet.

The tested orifice sets and/or juice packages and the spray cabinet were decontamined after biotesting by using a chlorine solution. A 2500 ppm hypochlorite solution (NaOCl) was used to sanitize the system for 30 minutes (Banwart, 1981). Determination of the effect of the NaOCl concentration on inhibition of microbial growth was

measured (Appendix C).

Spray Cabinet Technique.

The test orifices and packages were sprayed with the microbial suspension in a closed cabinet, with dimensions of 91.44 x 45.08 x 60.96 (L x W x H) cm. The material of the cabinet wall was made of 0.64 cm thick Plexiglass (Fig. 1). The cabinet encloses a recirculation system in which two pumps (Model 2000-032, Flowjet Corporation, Irvine, Ca) are installed. The pumps pull fluid from a reservoir and force it through a 32-nozzle system (Fig. 2) within the confines of the spray cabinet. The nozzles were mounted on the top, bottom and two main sides of the cabinet. The fluid was drained through four bottom drains back into the reservoir. One pressure line containing a "Y" shaped valve was used to switch lines to pump the fluid back through the cabinet or for draining the fluid out.

The spraying pattern was designed to cover a specific package geometry which required that the test units be set on a stand in the cabinet in order to achieve full coverage. The stand was constructed from PVC (polyvinyl chloride), and was used to support samples in the center of the cabinet. The spray suspension was delivered through the orifice discs at a pressure of 30 psi (2.07×10^5 $kg/m/s^2$) and an angle of 60 degrees. All surfaces of the

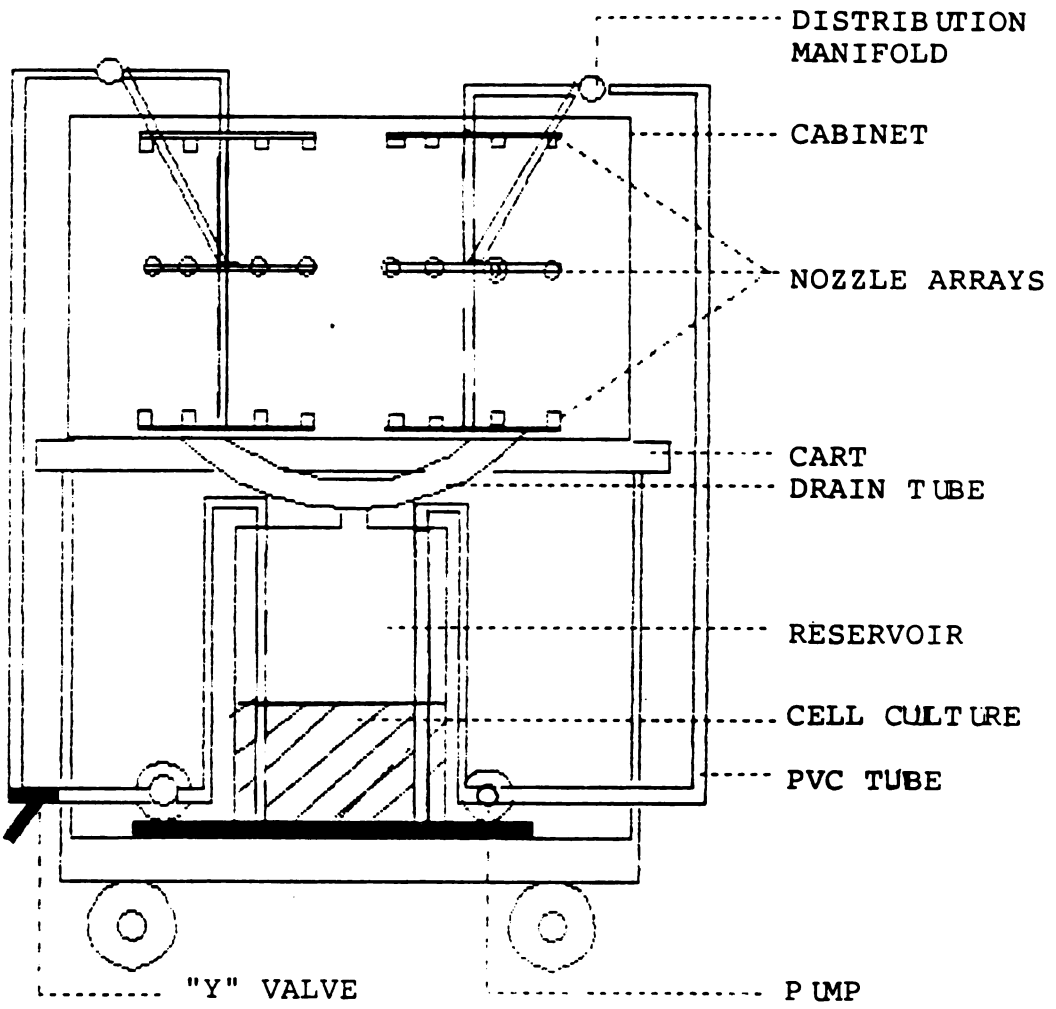


Fig. 1a. Diagram of the Spray Cabinet, front view.

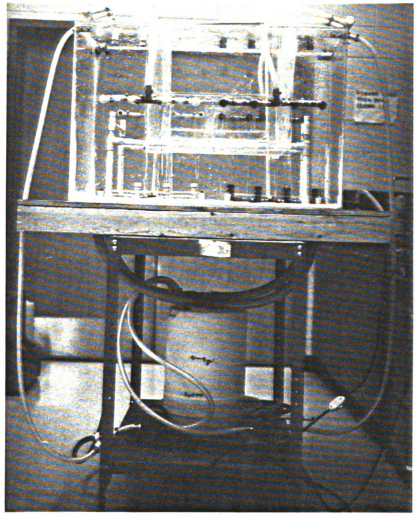
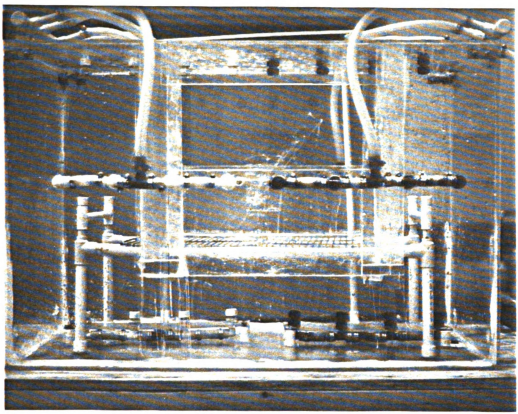
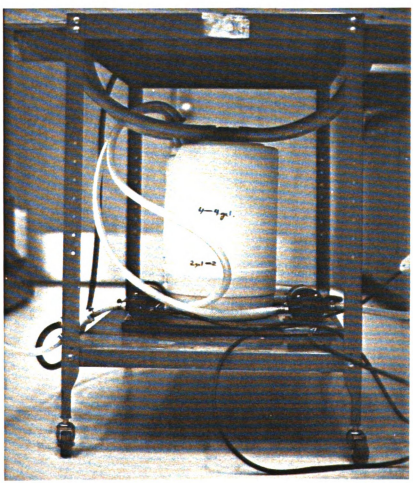


Fig. 1b. Photograph of the Spray Cabinet. The Spray Cabinet is constructed of two parts (shown above), the upper part is the test chamber and spray nozzle sets (right above), the lower part is comprised of two pumps and a reservoir (right bottom).





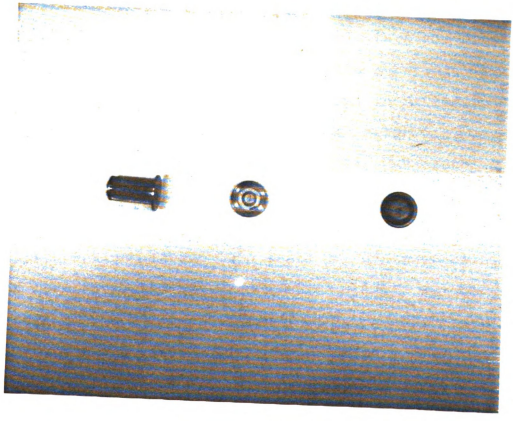


Fig. 2. The spray nozzle set with components.
From left to right: Stainer, Core, Orifice Disc.

sample packages were exposed to the microbial suspension, due to the misting effect inside the cabinet (Fig. 3). The spraying system works well for fluid materials with viscosity approximating water. Each pump was installed with a pressure safety switch, which would turn the pump off automatically if clogging occurred while spraying.

The following is the sequence of steps in performing the spraying cabinet technique: (1) The reservoir was filled with 4 liters of sterile Lactobacilli MRS broth (4 liters were the minimum required for the pumps to reach the necessary pressure level during spraying). Ten ml of the cell suspension (about 10^9 cells /ml of suspension which had been activated at 37^oC for 24 hours) was then inoculated into the 4 liters of broth to achieve 10^7 cells /ml cell culture for testing. (2) Both pumps were then turned on to spray the microbial suspension onto the test packages for 60 minutes. (3) After spraying, the used microbial suspension was pumped out through the "Y" valve into a waste tank, (4) Four liters of a 2500 ppm hypochlorite solution (NaOCl) was filled into the reservoir and then sprayed onto the test units for 30 minutes to kill any microorganisms remaining on the surface of the test units. (5) The chlorine solution was then pumped out into the waste tank, and the cabinet and samples were then rinsed with tap water. (6) The samples were taken from the cabinet and incubated at 37^oC for two

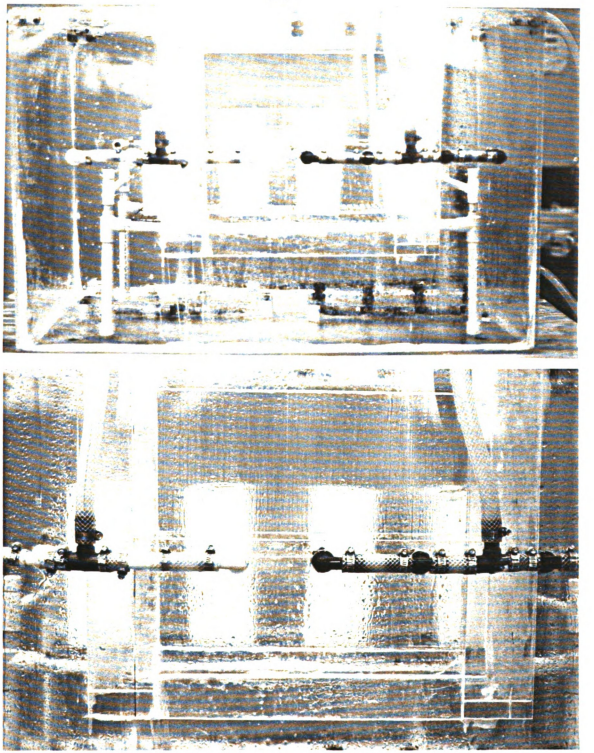


Fig. 3. The test juice packages were placed on a PVC stand in the cabinet (above). Packages were then sprayed with cell culture, complete coverage was achieved due to the misting effect inside the chamber (bottom).

weeks. During incubation, juice package samples were inspected for sign of microbial growth, such as the increase of cell number, production of carbon dioxide, change in pH and package physical appearance, like swelling.

Immersion Test.

Test package samples were submerged in a microbial suspension of Lactobacillus cellobiosus containing about 10^7 cells/ml for 60 minutes. These samples were immersed such that the cell culture covered the entire surface of the test units. The samples were removed from the cell culture, and dipped in a 2500 ppm chlorine solution for 30 minutes. Samples were then rinsed with tap water, and incubated at $37^{o}C$ for two weeks. The packaged juice was then sampled to determine if microbial growth had occurred due to the penetration of microorganisms during biotesting.

Orifice Test.

A challenge test was devised to test the efficiency of both the spray cabinet and immersion techniques, for the determination of the microbial integrity of 1.89 L paperboard packages by using predetermined size orifices.

Three different pinhole size orifices (5, 10 and 15 microns) were used to test the spray cabinet and immersion

techniques, each hole size was replicated six times for each test. A 0.48 cm hole was drilled in the center of a rubber stopper, and an orifice was placed over the hole, and sealed around the edges with silicone gel to prevent entry of microorganisms except through the orifice.

Test procedures were as follows, ten ml of Lactobacilli MRS broth was filled into a 30 ml glass vial, placed in an autoclave and sterilized under a pressure of 15 psi (1.03×10^5 kg/m/sec^2) and 121^{o}C for 15 minutes. Each sterile broth/vial was then aseptically sealed in a laminar flow cabinet (Contamination Control Incorporated, model 1160, Lansdale, PA) with a sterilized rubber stopper containing a specific size orifice, and clamped with an aluminum cap (Fig. 4).

The vials were placed up-side-down during testing in both the spray cabinet and immersion methods (Fig. 5). This was done to ensure that the test cell culture was in contact with the orifices during testing, test vials were sprayed or immersed for durations of 15, 30, 60 and 90 minutes. Vials were then placed right side up during decontaminating after biotesting, to avoid the chlorine solution (2500 ppm) from going through the pinhole into the broth which could prevent the microorganisms from growing in the vials. When immersion or spraying was accomplished, the vials were inspected to determine if microbial growth had occurred in the broth by visual

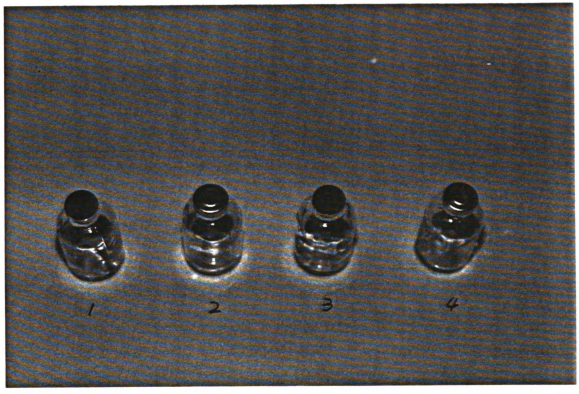


Fig. 4. The orifice unit for determining the efficiency of the biotests.
Vials from left to right are:
1.) The negative control which is the vial sealed by a rubber stopper without drilling a hole in the center and placing an orifice.
2.) A negative control vial clamped by an aluminum cap .
3.) The test unit is a vial sealed by a rubber stopper with an orifice which possesses a specific pinhole size.
4.) A clamped test unit by an aluminum cap.

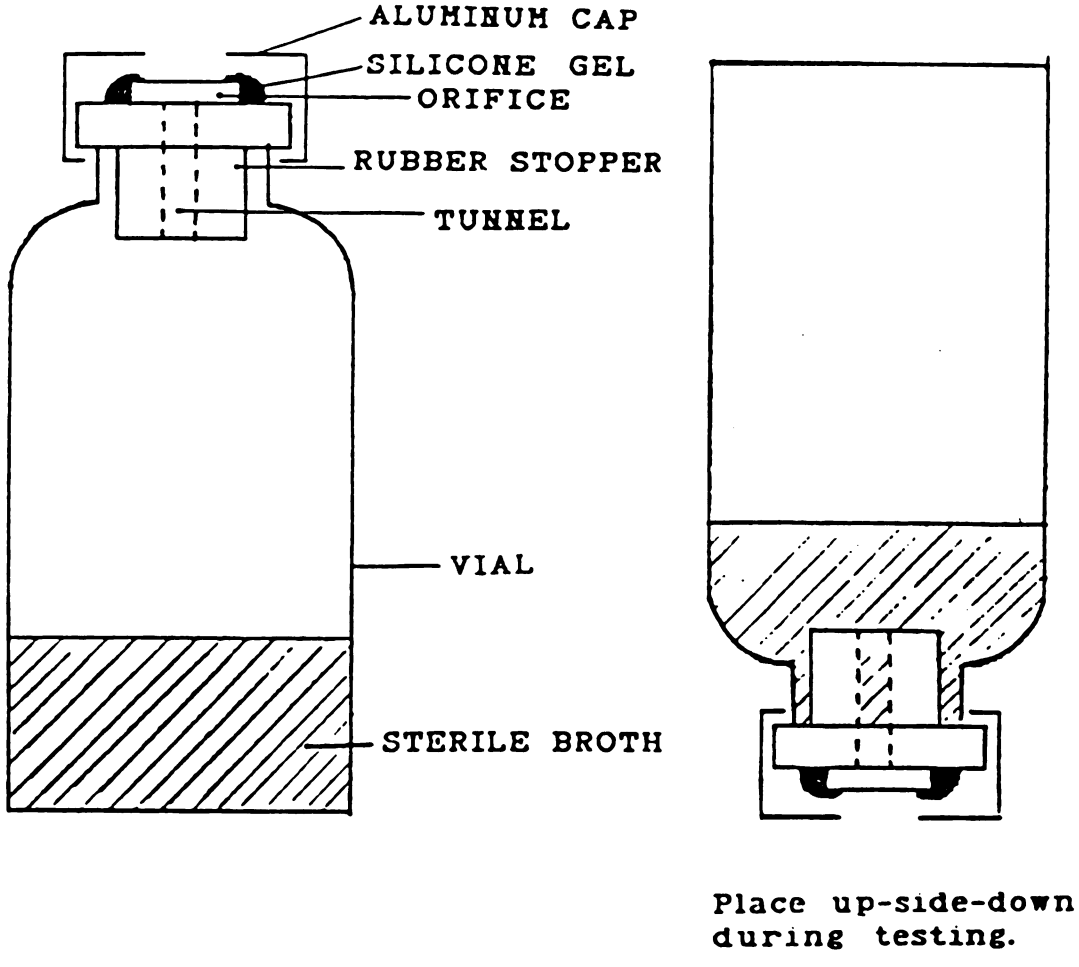


Fig. 5. Diagram of a vial with an orifice for determining the efficiency of biotest methods.

inspection for turbidity after 48 hours of incubation at 37°C. The results provided an indication of the minimum pinhole size that the microorganisms could penetrate in a specific time period.

Determination of Microbial Growth.

Following microbial challenge, several measurements were used to detect microbial growth, and thus violation of package integrity.

1.Direct Cell Count Method:

To monitor the cell number in the packed juice, a pinhole was drilled through the surface of the package using a sterile needle (size 25G 5/8) , and sealed immediately with silicone gel to prevent contamination. Juice was then sampled from the package using a 1 ml 25G 5/8 disposable sterile syringe (Becton Dickinson Co, Rutherford, NJ) by inserting the needle through the silicone layer and hole after fourteen days of incubation at 37°C. The cell number was determined using the standard cell count method as decribed previously.

2.Gas Chromatographic Analysis.

Carbon dioxide is produced as a by-product of growth of Lactobacillus cellobiosus. Production of carbon dioxide was determined by analyzing the gas composition in the

headspace of the packages using a Gas Chromatograph.

Headspace gas (300 ul) in the package was withdrawn using an air-tight syringe (Hamilton Co., Reno, Nev) at the end of the incubation period, and directly injected into a AGC III Gas Chromatograph (Carle Co., Anaheim, CA). The G.C. conditions were as follows:

Column packing	-----	porapak and molecular seive 5A 60/80 mesh (Carle Co., Anaheim, CA)
Carrier gas	-----	Helium
Flow pressure	-----	25 psi (1.72×10^5 kg/m/sec^2)
Flow rate	-----	40 ml/min
Bridge setting	-----	thermistor
Oven Temperature	-----	80^{o}C
Output (range)	-----	256
Chart speed	-----	0.5 in/min
Sample quantity	-----	300 ul

3.Measurement of pH:

Lactobacillus cellobiosus can produce lactic acid while consuming carbon as an energy source. To determine the change in pH of the juice, a pH meter, Digital ionalzer model 501 from Orion Research Co., was used. Three ml of juice were taken from the test packages at the end of incubation for pH measurement.

4.Visual Inspection of Swelling:

The most significant characteristic indicative of microbial growth in the packages is swelling due to gas production. It is a non-destructive measurement which maintains the integrity of the packages. In this determination, the depth of the juice packages were measured every three days during incubation to detect swelling.

Effect of Transportation on Package Integrity.

A distribution simulation test was used on the juice packages. The dynamic test was composed using hydraulic, compression, vibration, end drop and random vibration tests. Tests were based on standard methods: ASTM D-3332-77, Mechanical-Shock Fragility of Products Using Shock Machine; ASTM D-4577-86, Compression Resistance of A Container under Constant Load and ASTM D-999-75, Vibration Testing of Shipping Containers. Juice packages were evaluated with respect to then dynamic response to drop, compression, and vibration hazard. Packages were then tested for their microbial integrity.

Eight juice packages were placed in an 27 x 34 x 20 cm (L x W x H) carton case. Dynamic tests were accomplished using the following sequence:

1. Hydraulic shock test.

   Each individual case was dropped from an 18 inches equivalent on a shock table to simulate abusive loading (Model 846 shock test system, MTS Systems Corporation, Minneapolis, MN). The velocity change was 117.8 in/sec (2.992 m/sec) and the duration 5 milliseconds.

2. Compression test.

   A single case was tested by 2-tier loading equivalent to simulate warehousing condition by using model 76-5K container compressor, Lansmont Corporation, Pacific Grove, CA. The test level was 158.4 lbs (71.914 kg) on top loading.

3. Vibration test.

   Five cases were stacked on a MTS 840 vibration system (MTS Systems Corporation, Minneapolis, MN), and tested at a stack-resonance of 8 HZ and amplitude of 0.5g's for 30 minutes to simulate transportion.

4. End Drop test.

   Cases were tested by dropping from a 12 inches equivalent to simulate abusive handling, using the MTS 846 shock test system (MTS Systems Corporation, Minneapolis, MN). The velocity change was 96 in /sec (2.438 m/sec) and duration 5 milliseconds.

5. Vibration test.

   Five stacked cases were tested under random vibration conditions which were held at stack-resonance between 3 HZ to 100 HZ and amplitude 0.5 g's for 30 minutes on a MTS 840 vibration system (MTS Systems Corporation, Minneapolis, MN).

Experimental Design and Statistical Analysis.

The experimental design required two hundred juice packages (25 cases). A flow chart of the experimental design is shown in Figure 6. Five cases of juice packages (40 packages) were used as blank controls, and were inspected for microbial integrity after being held at room conditions (25^{o}C, 50% RH) for two weeks without being subjected to dynamic input or biotesting. One hundred and twenty packages were sprayed or immersion biotested to determine violation of package integrity after exposure to the dynamics hazard study. Forty packages were selected for testing 60 minutes and twenty for 15 minutes in each biotest. Eight original juice packages were used as a negative control for the different biotests and test durations.

The results were analyzed by using the factorial experiments (Gill, 1987) to measure sample response to different treatments and interactions. A two-factor model was used to determine the confidence of test efficiency of

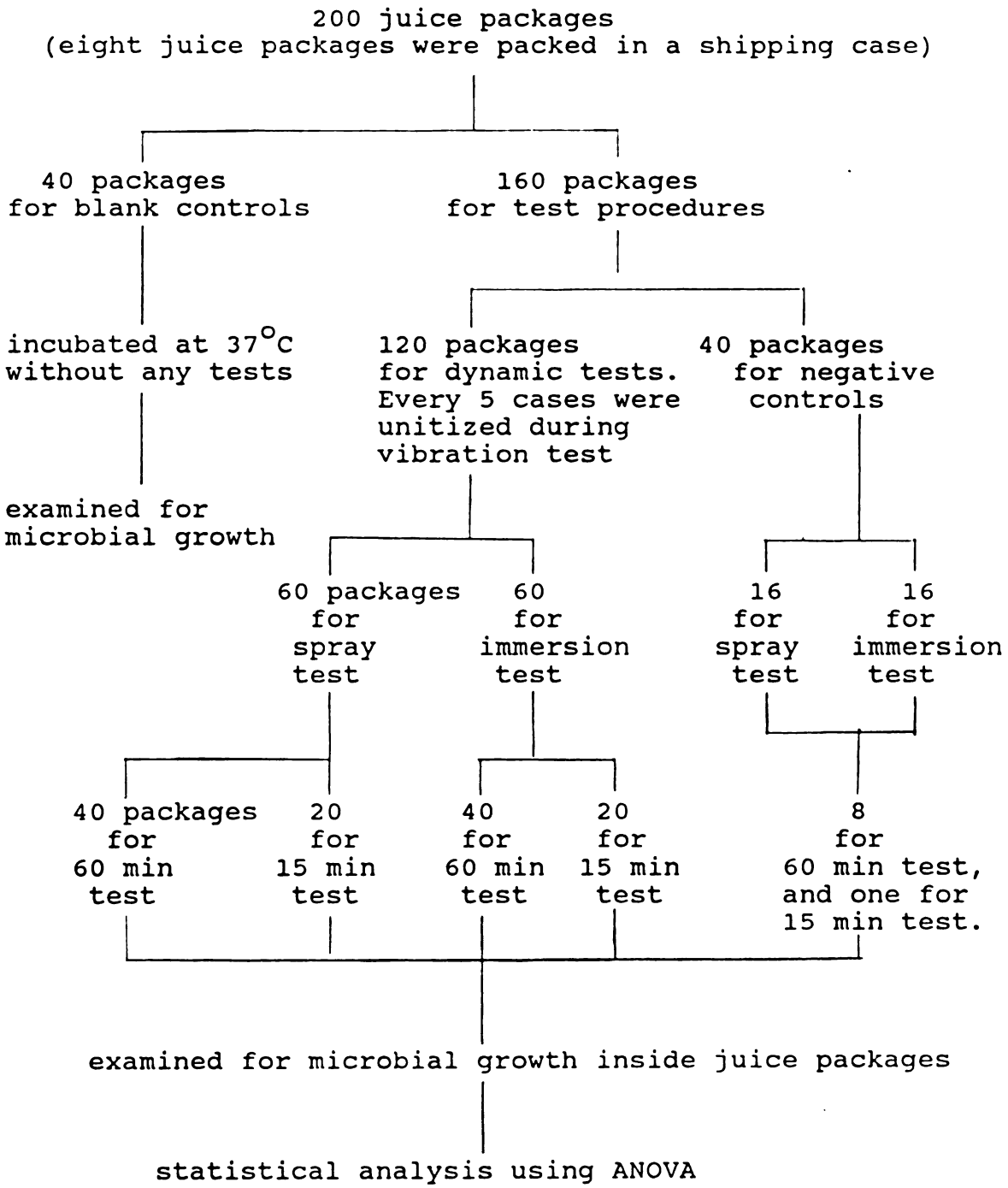


Fig. 6. Flow chart of experimental design for the procedures of dynamic and biotests.

the different pinhole sizes and test durations. A three-factor model was used to analyze the relationship of dynamic damage on stacking level and location in a shipping container (See Appendix D for the identification of stack levels and locations of packages), and microbial contamination tested using the two different biotests.

RESULTS AND DISCUSSION.

Development of the Spray Cabinet Technique.

The spray cabinet was designed to test package integrity based on microbial penetration. In this procedure, test microorganisms penetrate through package defects such as pinholes and/or improper seal areas, and then grow inside the package. This results in a change in the package appearance (swelling) and/or product (e.g., pH) which indicates a violation of the package integrity.

The first priority in developing the test was to have a method which provided for accomplishing complete package coverage. The spray cabinet was originally constructed using eight spray nozzles mounted on the top of the cabinet. The nozzles were assembled 10 cm from each other on a line in the middle of the top of the cabinet. Using this construction, packages were only sprayed with test culture on their top surface. Since package damage often occurs at the bottom and along the side seams, this spray pattern was inadequate. Therefore, the spray cabinet was redesigned to obtain complete coverage of the test packages. The spray pattern was extended by installing 32 spray nozzles positioned on the top, bottom and two main sides of the inner cabinet (Fig. 1.). Eight spray nozzles were assembled on a line (10 cm from each other, 10 cm from the edge) in the middle part of a side parallel with

the long edges, in order to cover with spray the entire surface of the test packages.

The capacity of the cabinet was determined by inspection of the coverage pattern. Based on this, four packages were selected as the number to be tested at a time. Each package was placed on a PVC stand (23.5 cm high) between two nozzles per side (eight nozzles on a side were divided into four sets, each containing two nozzles). Since there were four sides containing nozzles, every package was located in the center of eight nozzles, thus four packages could be simultaneously tested with complete coverage (Fig. 3.)

To obtain the flow pressure required to force the test microbial suspension through the nozzles and onto the packages, two pumps were installed. Package coverage was achieved by creating a mist effect inside the cabinet during the test. In all tests done thereafter with the spray cabinet, no package failures were observed, due to wicking of moisture by the raw edges associated with the package seams.

In a preliminary study, the growth characteristics of Lactobacillus cellobiosus in the MRS medium were determined. Ninety ml of MRS medium were filled into a 250 ml flask sealed with a rubber stopper, and sterilized using an autoclave. Ten ml of cell culture were aseptically inoculated into the medium in a laminar flow

cabinet, and the flask was placed into an incubator at 37°C. An initial decrease in pH was observed. The pH stabilized after reaching 4.0 (Fig. 7.). The percent carbon dioxide in the headspace of the flask increased with incubation time (Fig. 8.). An increased amount of CO_2 in the package headspace could lead to swelling of the container. The growth of Lactobacillus cellobiosus in apple juice was also determined using nine 250 ml brik packages which were inoculated with a 0.1 ml microbial suspension (10^3 cells/ml), and placed in a 37°C incubator. Swelling of the packages was observed after 72 hours, 3 negative controls which were not inoculated did not show swelling. A decrease in pH of the apple juice was also obtained (Fig. 9.). Production of CO_2 increased after 30 hours of incubation (Fig. 10). Thus, Lactobacillus cellobiosus was shown to be active in apple juice.

Efficiency of Biotests.

The efficiency of biotests was determined by measuring the percent microbial penetration of test vials containing the orifices. Percent penetration was defined as: the number of vials containing MRS medium, in which microbial growth was observed after testing, divided by the total test vials.

The equation is as follows:

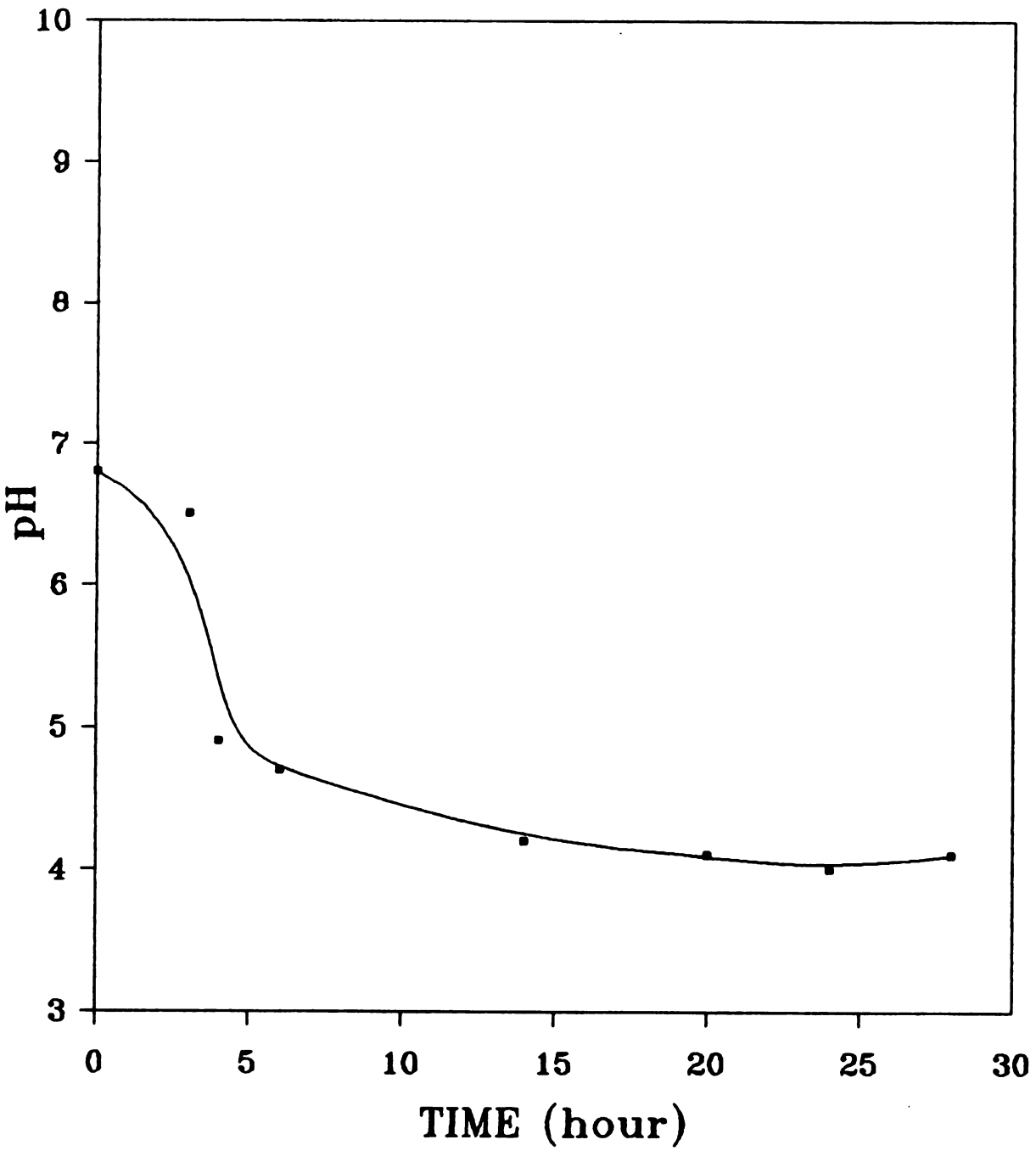


Fig. 7. Change in pH during incubation of Lactobacillus cellobiosus in Lactobacilli MRS medium at 37°C.

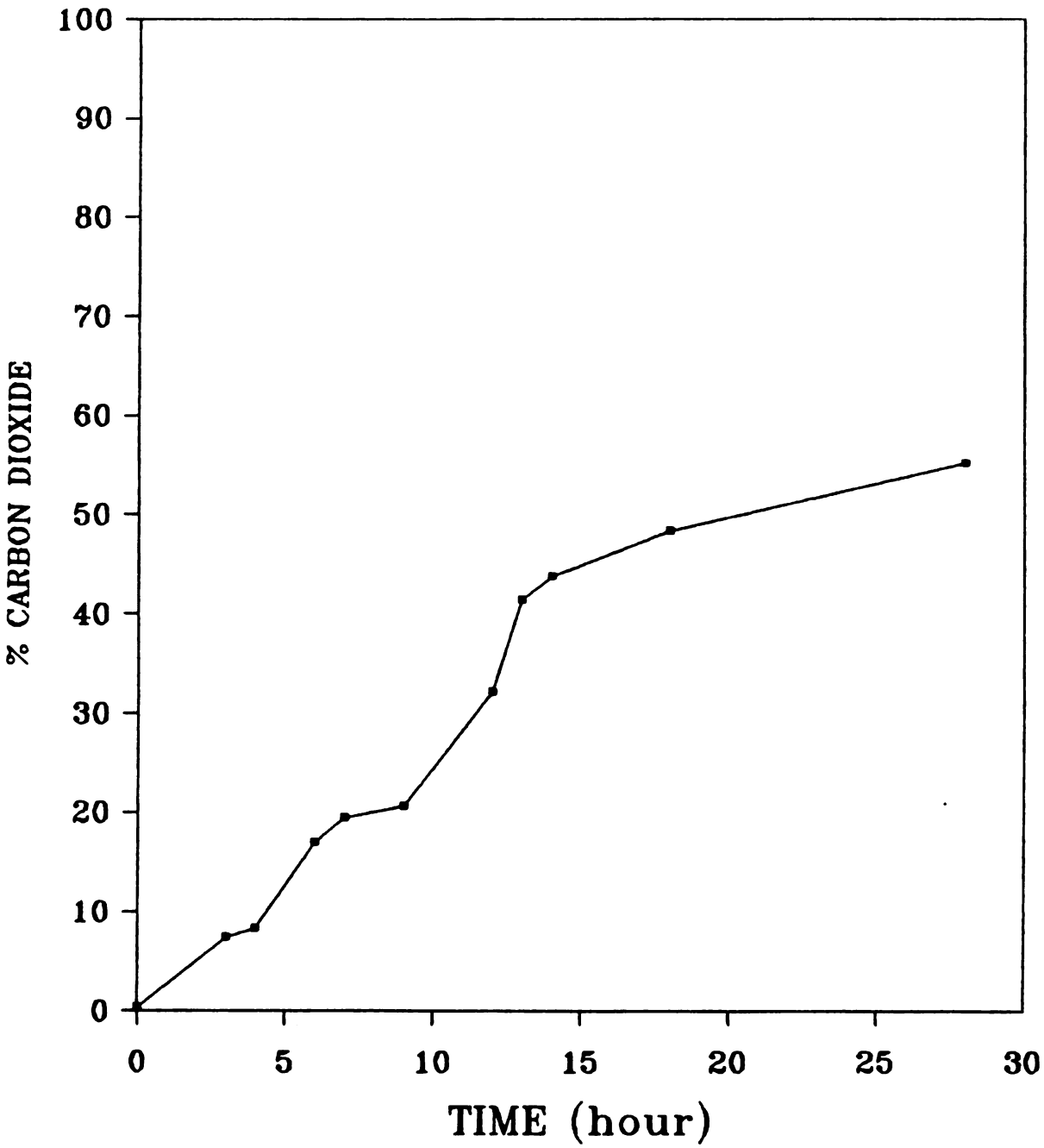


Fig. 8. Percent carbon-dioxide in the headspace of 250 ml flask during incubation of Lactobacillus cellobiosus in Lactobacilli MRS medium at 37°C.

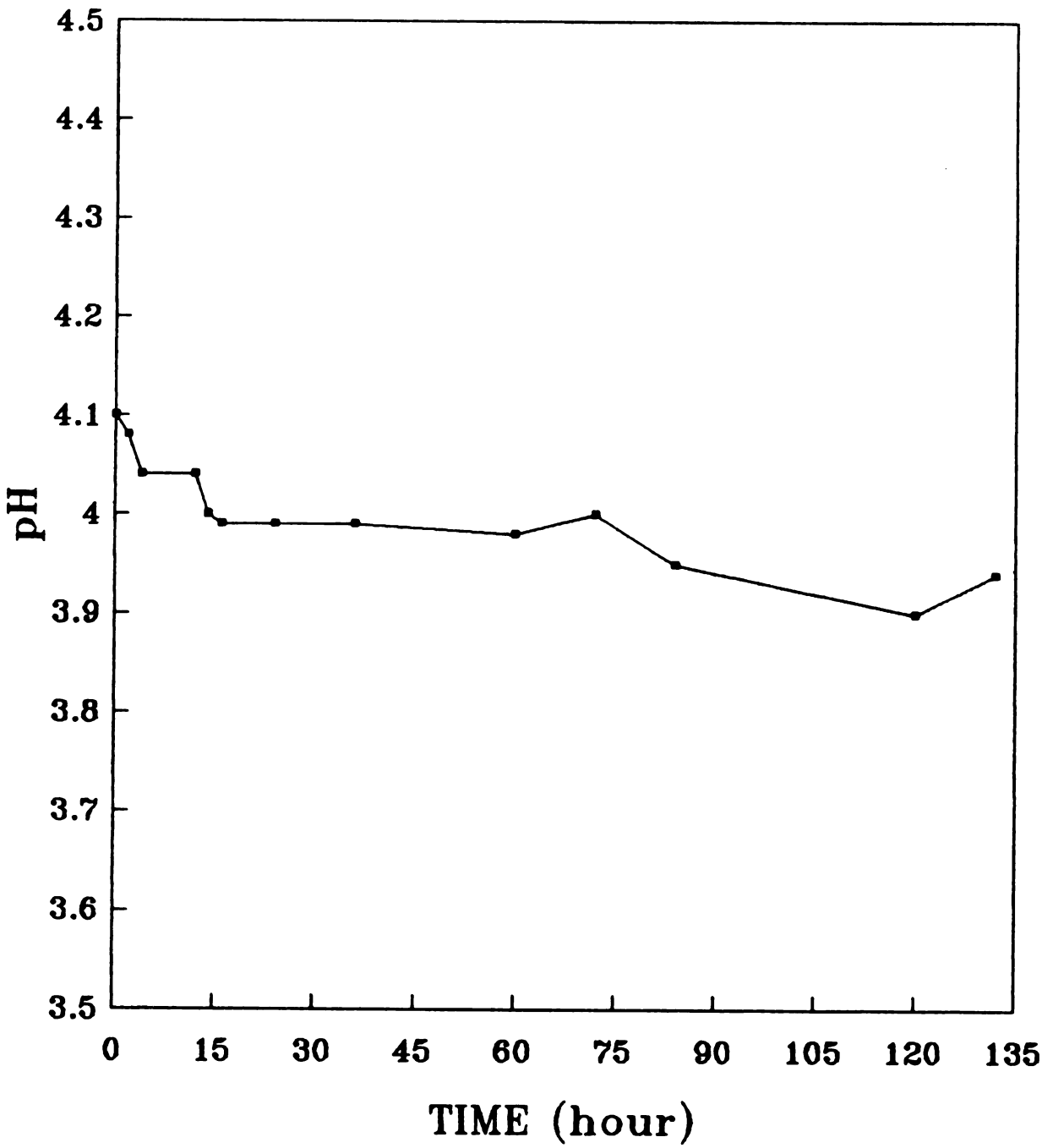


Fig. 9. Change in pH during incubation of Lactobacillus cellobiosus in aseptic apple juice package at 37°C.

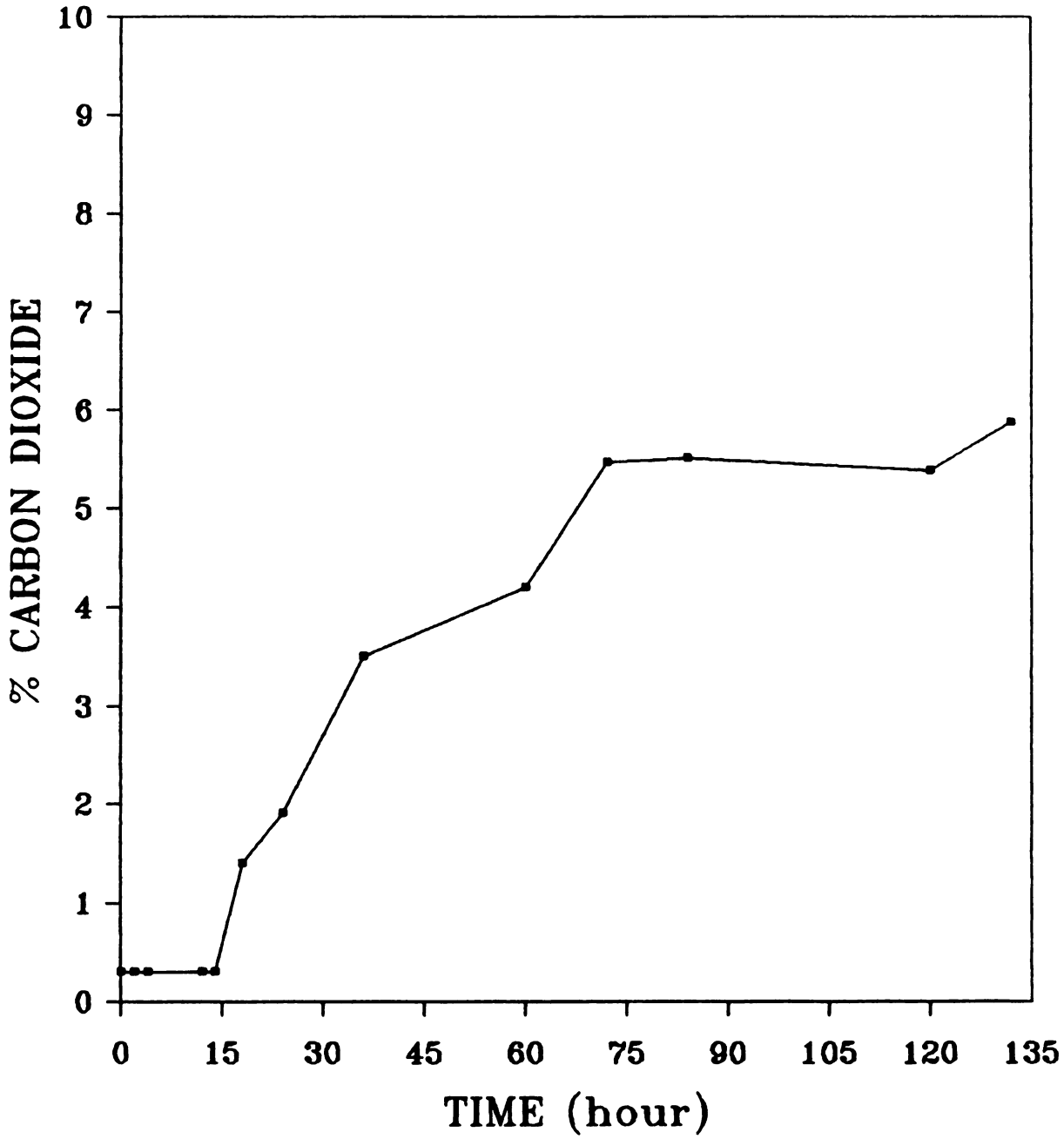


Fig. 10. Percent carbon dioxide in the headspace of aseptic package during incubation of Lactobacillus cellobiosus in apple juice package at 37°C.

$$\% \text{ microbial penetration} = \frac{\text{number of contaminated vials}}{\text{total test vials}} \times 100\%$$

Using the spray cabinet, the minimum detectable pinhole size was found to be 10 micron after 15 minutes of spraying, a 5 micron pinhole was detected after 30 minutes of spraying (Fig. 11). Four vials (no orifices) were used as negative controls and tested simultaneously with the orifices, no microbial growth was detected in any of the controls (three replications for each specific testing duration in both biotests). The amount of penetration increased with size of hole, and test duration up to 60 minutes of spraying. The maximum penetration level detectable for any of the pinhole sizes was 62.5% (Table 6.). Therefore, the test duration of 60 minutes to determine package integrity was used.

Two factors, hole size and test duration, were statistically analyzed to determine their effect on microbial penetration. Only hole size (Table 7.) was significant at a confidence level of 99.5%. Thus percent penetration increased with the larger pinholes. Test duration results did not provide sufficient evidence to indicate a significant difference within the various test periods. No statistically significant interactions between hole size and duration occurred in the spray cabinet test

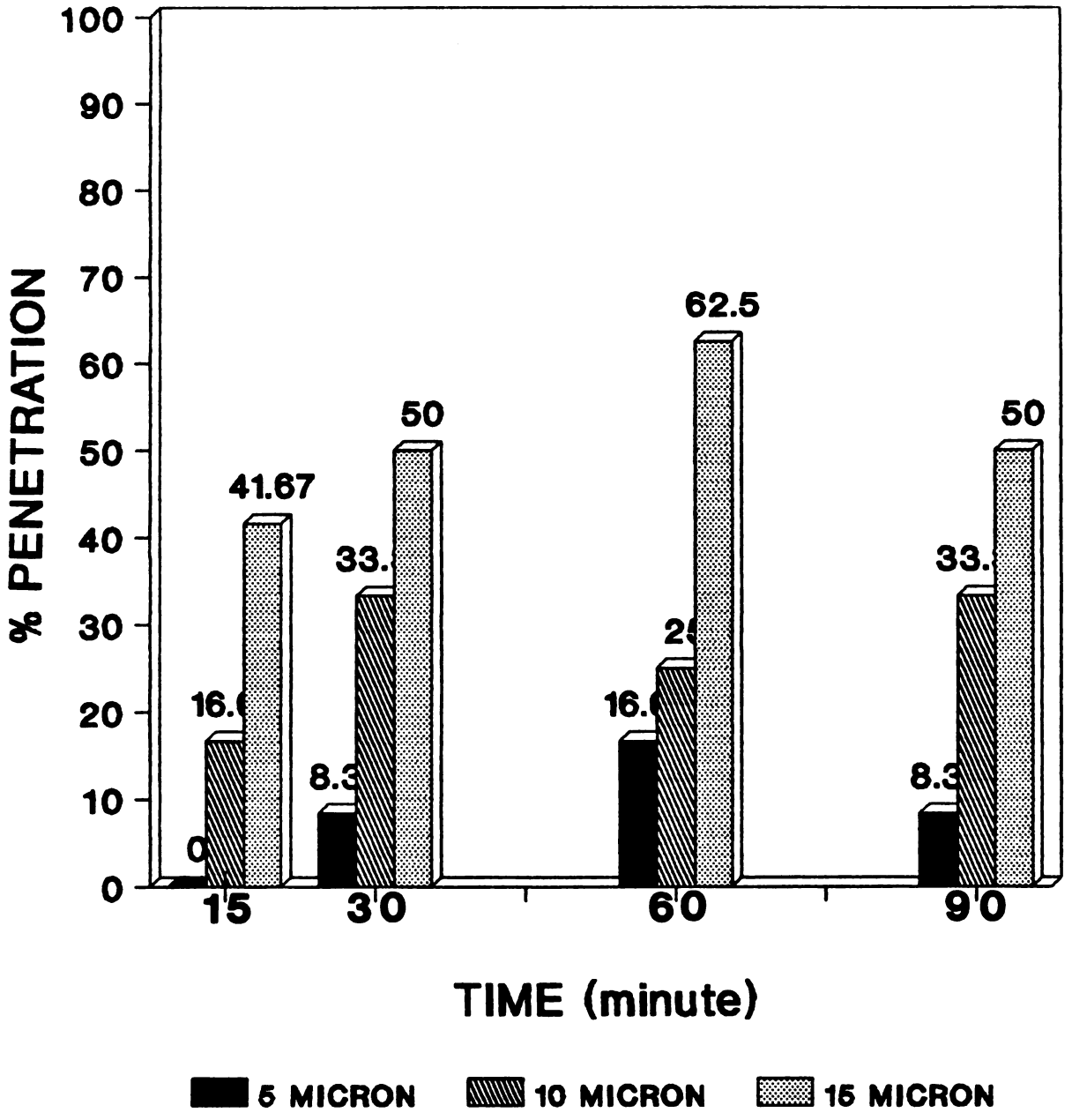


Fig. 11. Percent microbial penetration of test vials containing 5, 10 and 15 microns orifices using the spray cabinet technique.

Table 6. Percent microbial penetration through several sizes of pinhole using the spray cabinet.

	PINHOLE SIZE (micron)		
DURATION (minute)	5	10	15
15	0.00%	16.67%	41.67%
30	8.33%	33.33%	50.00%
60	16.67%	25.00%	62.50%
90	8.33%	33.33%	50.00%

Table 7. ANOVA table of analysis of factoral effect on the efficiency of the spray cabinet technique.

SOURCE	df	SS	MS	F	f-ratio
Hole size	2	0.80	0.400	8.42	$f_{2,15,0.005} = 7.70$
Duration	3	0.10	0.033	0.71	$f_{3,15,0.5} = 0.83$
Interaction	6	0.03	0.005	0.11	$f_{6,15,0.5} = 0.93$
Error	15	0.66	0.044		

(see Appendix E.).

Using the immersion test, no microbial penetration was observed after 60 minutes of immersion, 10 and 15 micron size holes were detected after 90 minutes of immersion. Five micron size holes were not detectable even after 90 minutes of testing (Fig. 12). No microbial growth was observed for any of the six negative controls used with the immersion method. The maximum detectable level was 50% which was observed with the 15 micron (Table 8).

Table 8. Percent microbial penetration through several sizes of pinhole using the immersion method.

	PINHOLE SIZE (micron)		
DURATION (minute)	5	10	15
15	0.00%	0.00%	0.00%
30	0.00%	0.00%	0.00%
60	0.00%	0.00%	0.00%
90	0.00%	16.67%	50.00%

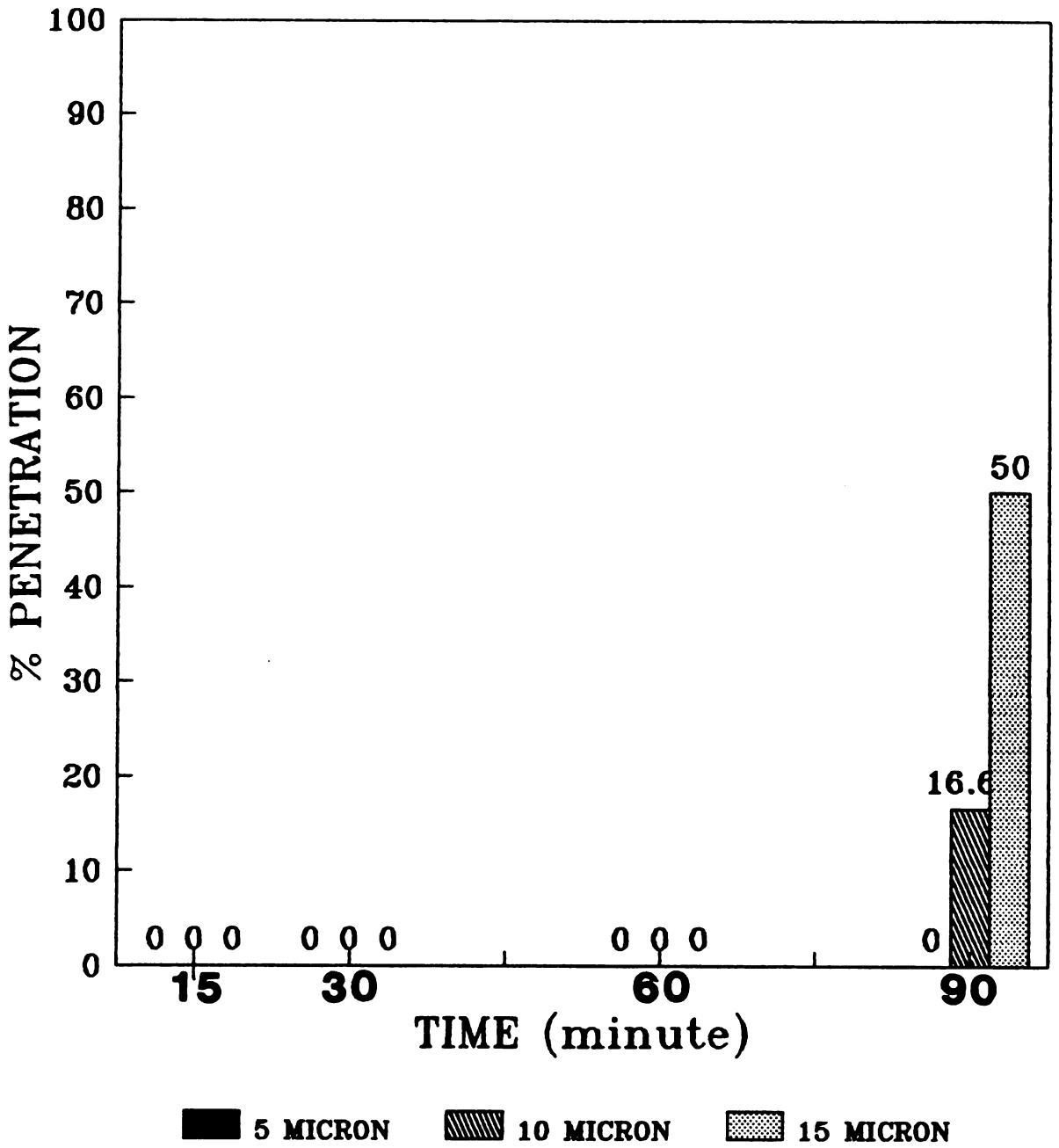


Fig. 12. Percent microbial penetration of test vials containing 5, 10 and 15 micron size orifices using the immersion test.

The spray cabinet technique was better able to detect pinholes than the immersion method (Fig. 13). Ten micron size defects could be detected after 15 minutes using the spray cabinet technique, however, were not detectable until after 90 minutes using the immersion procedure, also a high percent penetration was gained using the spray cabinet technique (Table 9). The difference between the results from the spray cabinet technique and immersion method was highly significant at a 99.5% confidence level for the 10 micron size orifices. Therefore, the spray cabinet technique was better able to detect pinholes of 5 - 15 micron. However, the test duration results did not provide enough evidence to indicate a significant difference (Table 10.).

The spray cabinet technique provided better detectability than the immersion method, probably due to the high fluid pressure was offered by two pumps in the spray cabinet. A 30 psi was generated in each pump to force cell culture sprayed onto the test samples. However, less pressure was performed in the immersion method, because it required to immerse the samples into a 30 meters depth to obtain such a high pressure.

Probability of detection (Placencia, 1986a), was defined as the chance to detect a pinhole using a microorganisms, using the equation as follows:

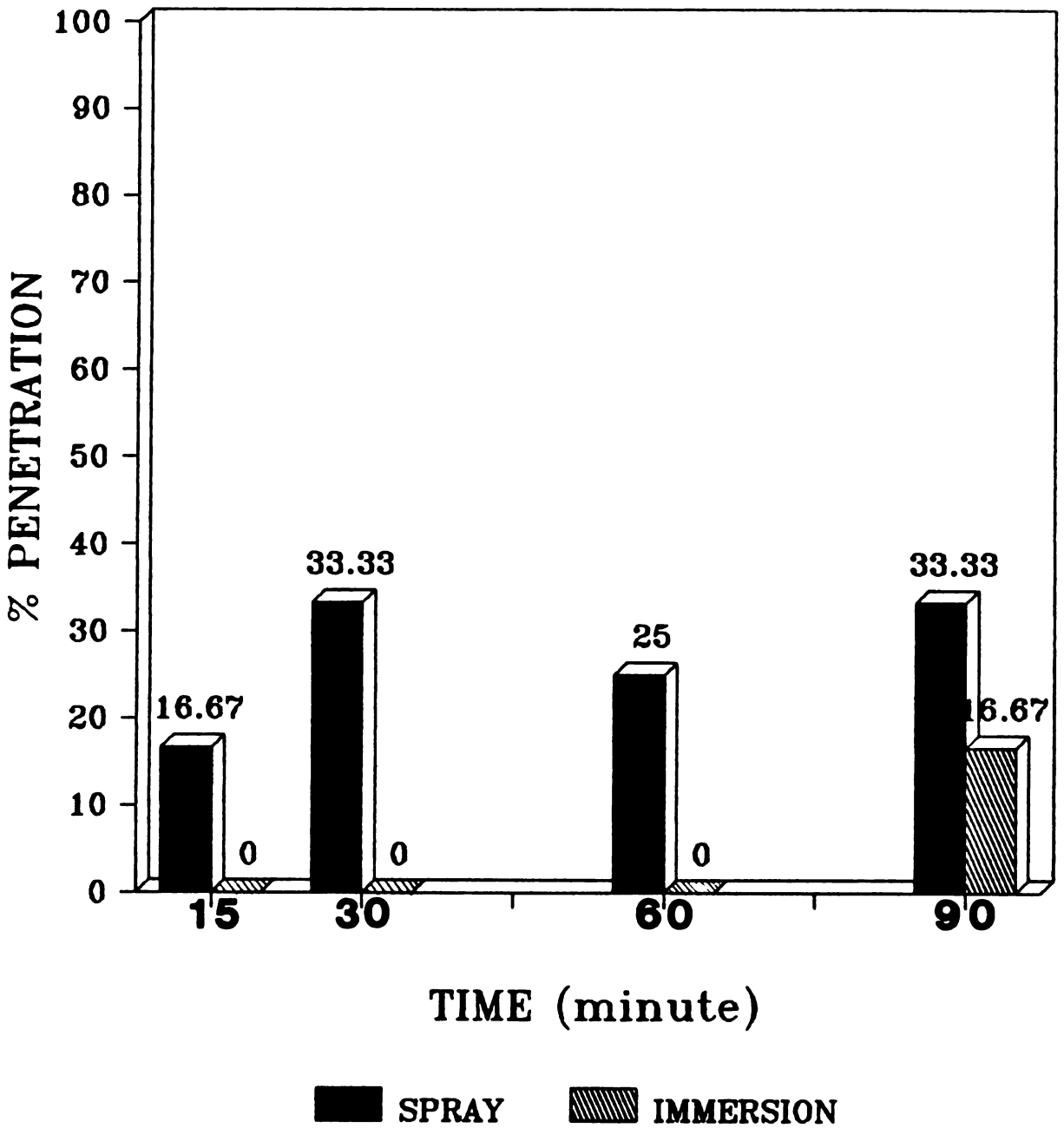


Fig. 13. Percent microbial penetration of test vials containing 10 micron size orifices using spray cabinet and immersion biotests.

Table 9. Comparison of percent penetration of ten micron size orifices using the spray cabinet and immersion tests.

DURATION (minute)	SPRAY TEST	IMMERSION TEST
15	16.67%	0.00%
30	33.33%	0.00%
60	25.00%	0.00%
90	33.33%	16.67%

Table 10. ANOVA table of comparing the efficiency of the spray cabinet and immersion techniques using 10 micron size orifices.

SOURCE	df	SS	MS	F	f
Biotest	1	0.1050	0.1050	32.81	$f_{1,3,0.05} = 10.13$
Duration	3	0.0304	0.0101	3.15	$f_{3,3,0.05} = 19.20$
Error	3	0.0096	0.0032		$f_{3,3.0.10} = 5.39$

$$\text{probability} = \frac{\text{percent defect was detected}}{\text{number of spores (or cells) in test suspension} * \text{number of holes/area}}$$

was calculated from data generated using the Membrane Agar Plate Strike-through (Placencia, 1986a) and FDA Exposure Chamber (Placecia, 1986b) methods to detect defects in packaging materials. The probability of a 5 micron size defect being detected was 10^{-9} for the membrane agar method, and 10^{-6} for the exposure chamber. For the spray cabinet technique, the probability was 10^{-8}. The results showed that the spray cabinet technique had better detectability than the membrane agar method, but not as good as the exposure chamber. Spores were used in the former research, which are smaller than vegetative cells, thus the detection level should be higher.

Microbial Integrity of Aseptic Juice Packages.

Eighteen packages (15% of total test packages) were observed leaking after exposure to specific dynamic tests (Table 11.), These packages were removed from subsequent biotesting. Fifteen packages were leaking at the bottom corner of the packages, the rest were ruptured in the side seal area. Fourteen packages were caused to leak by the

Table 11. Number of package leaking after exposure to dynamic testing[1].

		TEST TYPE						
		NUMBER OF LEAKING						
STACK LEVEL	LOCATION	18"[2] DROP	COMPRESSION	VIBRATION	12" DROP	RANDOM VIBRA.	SUM	TOTAL
1	C[3]					1	1	2
	M[4]					1	1	
2	C			1	1		2	2
	M							
3	C			1			1	1
	M							
4	C			1		2	3	3
	M							

Table 11. Number of package leaking after exposure to dynamic testing (continued).

		TEST TYPE						
		NUMBER OF LEAKING						
STACK LEVEL	LOCATION	18"[2] DROP	COMPRESSION	VIBRATION	12" DROP	RANDOM VIBRA.	SUM	TOTAL
5	C			3		3	6	7
	M					1	1	
SUM		3	0	6	1	8		18

1. As soon as a leaking package was discovered, it was removed from the shipping carton.

2. Packages were individually tested at an 18" drop and compression tested, no stack level was indicated during these tests.

3. The letter " C " indicates that the packages were located at the corner part of the shipping container during testing (See Appendix D for detail).

4. The letter " M " indicates that the packages were located at the middle part of the shipping container during testing.

vibration tests (Table 11.), no packages were found to be leaking after the compression test. Packages at stack level 5 suffered more than at any other level, because of the bouncing effect during vibration. More leaking packages were located at the corner in the shipping containers than in the middle of the cartons.

Package integrity was assessed by determining the percent microbial penetration through the packages. Percent penetration was defined as: the number of packages which contained product supporting the growth of Lactobacillus cellobiosus divided by the total number of test packages, as the following equation:

$$\% \text{ penetration} = \frac{\text{microbially contaminated juice packages}}{\text{total test juice packages}} \times 100\%$$

Production of CO_2, and change in product pH and package dimension (swelling) were used as positive indicators of microbial growth following each biotest. More microbial penetration was observed using the spray test than the immersion test. All corner juice packages at level 5 and 1 had viable growth of microorganisms after incubation at $37^{o}C$ for two weeks (Fig. 14.). Using the spray cabinet technique, 70% of total packages

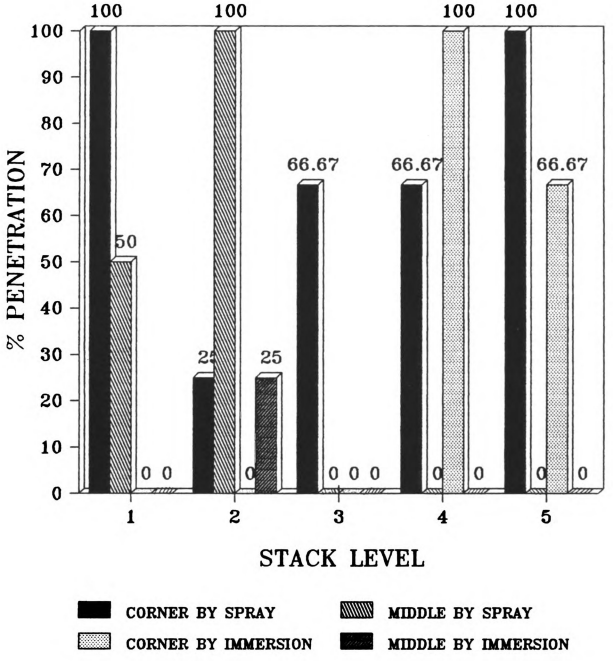


Fig. 14. Percent microbial penetration through packages tested for 60 minutes using the spray and immersion biotests.

demonstrated loss of microbial integrity in the corner areas. The packages which lost integrity were swollen (8.4 cm initial to a final depth of 9.1 cm). The carbon dioxide content in the headspace of these packages was as high as 7% compared to 2% initially. Thus, packages with an increase of the depth more than 0.3 cm, pH less than 3.74 and/or percent CO_2 greater than 3.5% were suspected to be penetrated by test microorganisms, which were tested using direct cell count to confirm the growth of microorganisms (Appendix F). Juice packages located in the middle of the shipping cases did not show any microbial contamination at levels 3, 4 and 5. However, severe spoilage was observed for middle packages at level 2 using the spray cabinet technique (Fig. 14.).

Only corner packages at level 4 were penetrated by the test microorganisms using the immersion method. Packages at levels 1, 2, and 3 did not show microbial growth after 60 minutes of immersion (Table 12.). Using the immersion test, it was found that more corner packages had loss microbial integrity than middle packages. However, not as great a number were detected as with the spray technique.

A three-factor model was used to statistically analyze the data including the different biotests, package location and stack level (Table 13). Test methods and location of packages had a significant impact (p = 90%).

Table 12. Percent microbial penetration through aseptic juice packages using the spray cabinet technique and immersion method[1].

STACK LEVEL	SPRAY TEST		IMMERSION TEST	
	CORNER	MIDDLE	CORNER	MIDDLE
1	100.00%	50.00%	0.00%	0.00%
2	25.00%	100.00%	0.00%	25.00%
3	66.67%	0.00%	0.00%	0.00%
4	66.67%	0.00%	100.00%	0.00%
5	100.00%	0.00%	66.67%	0.00%

1. Both biotests were conducted for 60 minutes.

Table 13. Anova table of analysis of percent penetration through package using the spray cabinet technique and the immerson method.

SOURCE	df	SS	MS	F	f
A. Biotest	1	0.50	0.50	5.00	$f_{1,4,0.10} = 4.54$
B. Location	1	0.62	0.62	6.20	$f_{1,4,0.05} = 7.71$
C. Stack Level	4	0.17	0.04	0.40	$f_{4,4,0.10} = 4.11$
INTERACTION					
AB	1	0.02	0.02	0.20	
AC	4	0.38	0.09	0.90	
BC	4	1.20	0.03	0.30	
Error	4	0.40	0.10		

Difference between stack levels was not significant. No significant interactions between factors was observed (Table 13).

Comparison of Various Spray Durations using the Spray Cabinet Technique.

From the results of the orifice test, the minimum detectable defect was found to be 10 micron after 15 minutes of spraying. An optimum spray time of 60 minutes was found with no further advantage gained after this period (Fig. 11). A comparison of different package spraying durations was made to determine loss of integrity in packages which were exposed to dynamic hazard testing at 60 minutes. No significant effect was found for the test durations (Table 14). Microbial penetration was not observed in the middle packages after 15 minutes of spraying (Table 14), which indicated that there were probably not any defects larger than ten micron after exposure to the dynamic tests. Corner samples showed more microbial penetration than packages located in the middle for two durations (Fig. 15). Stack level did not result in any significant difference in microbial penetration. However, package location affected the results at a confidence level of 90% (Table 15.). Results coincided with previous analysis (Table 7, 13).

Table 14. Comparison of microbial penetration (%) at two durations using the spray cabinet technique.

STACK LEVEL	60 minutes		15 minutes	
	CORNER	MIDDLE	CORNER	MIDDLE
1	100.00%	50.00%	50.00%	0.00%
2	25.00%	100.00%	50.00%	0.00%
3	66.67%	0.00%	0.00%	0.00%
4	66.67%	0.00%	50.00%	0.00%
5	100.00%	0.00%	50.00%	0.00%

Table 15. ANOVA table of analysis of the comparison of percent penetration at two duration using the spray cabinet technique.

SOURCE	df	SS	MS	F	f-ratio
A.Duration	1	0.48	0.48	3.43	$f_{1,4,0.10} = 4.54$
B.Location	1	0.83	0.83	5.93	$f_{1,4,0.05} = 7.71$
C.Stack level	4	0.28	0.07	0.50	$f_{4,4,0.01} = 4.11$
INTERACTION					
AB	1	0.0004	0.0004	0.002	
AC	4	0.10	0.03	0.21	
BC	4	0.45	0.11	0.79	
Error	4	0.57	0.14		

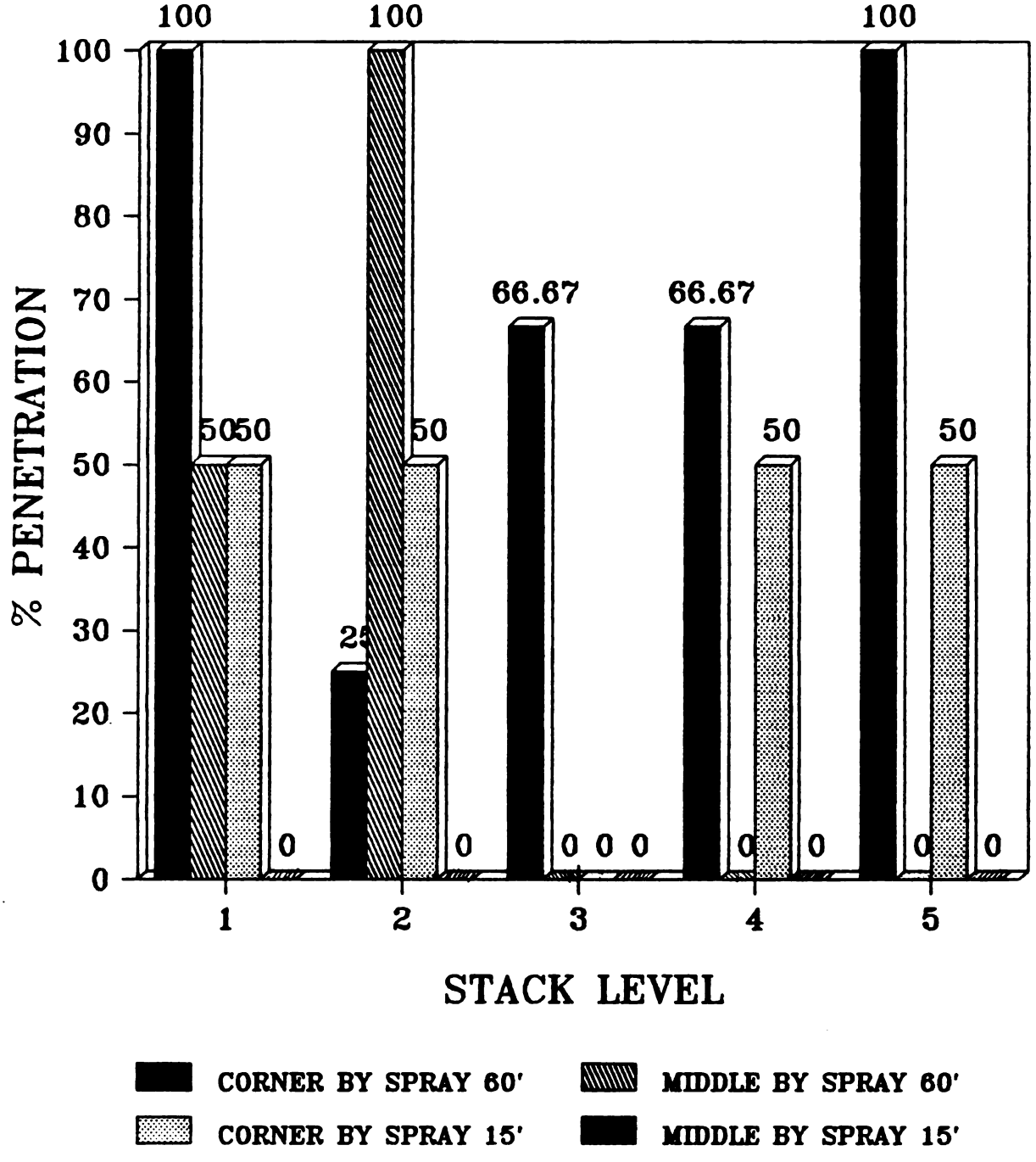


Fig. 15. Percent microbial penetration through packages tested using the spray cabinet technique for 15 and 60 minutes.

Measurement of Package Damage Due to Dynamic Tests.

Total package damage which included leakage and/or microbial penetration through the packages (Fig. 16), was determined by three methods: 1.) Obvious leakage found after dynamic tests. 2.) Microbial growth in the packages as a result of spray cabinet testing. 3.) Microbial growth in the packages as a result of immersion testing. Thus, the percent violation of package integrity was determined by totally the number of packages leaking after the dynamic tests and the number showing microbial penetration after the two biotests, divided by total test packages subjected to the dynamic tests.

The most damage was observed in the corner of the cartons at level 5, followed by damage in the corners at level 4. No loss of package integrity was observed in the middle packages at level 3 and 4 (Table 16). Results of statistical analysis showed that both location and stacking level effected loss of package integrity at a confidence level of 99.5%. A significant results from the interaction of location and stack level was also obtained (p=99.5%) (Table 17).

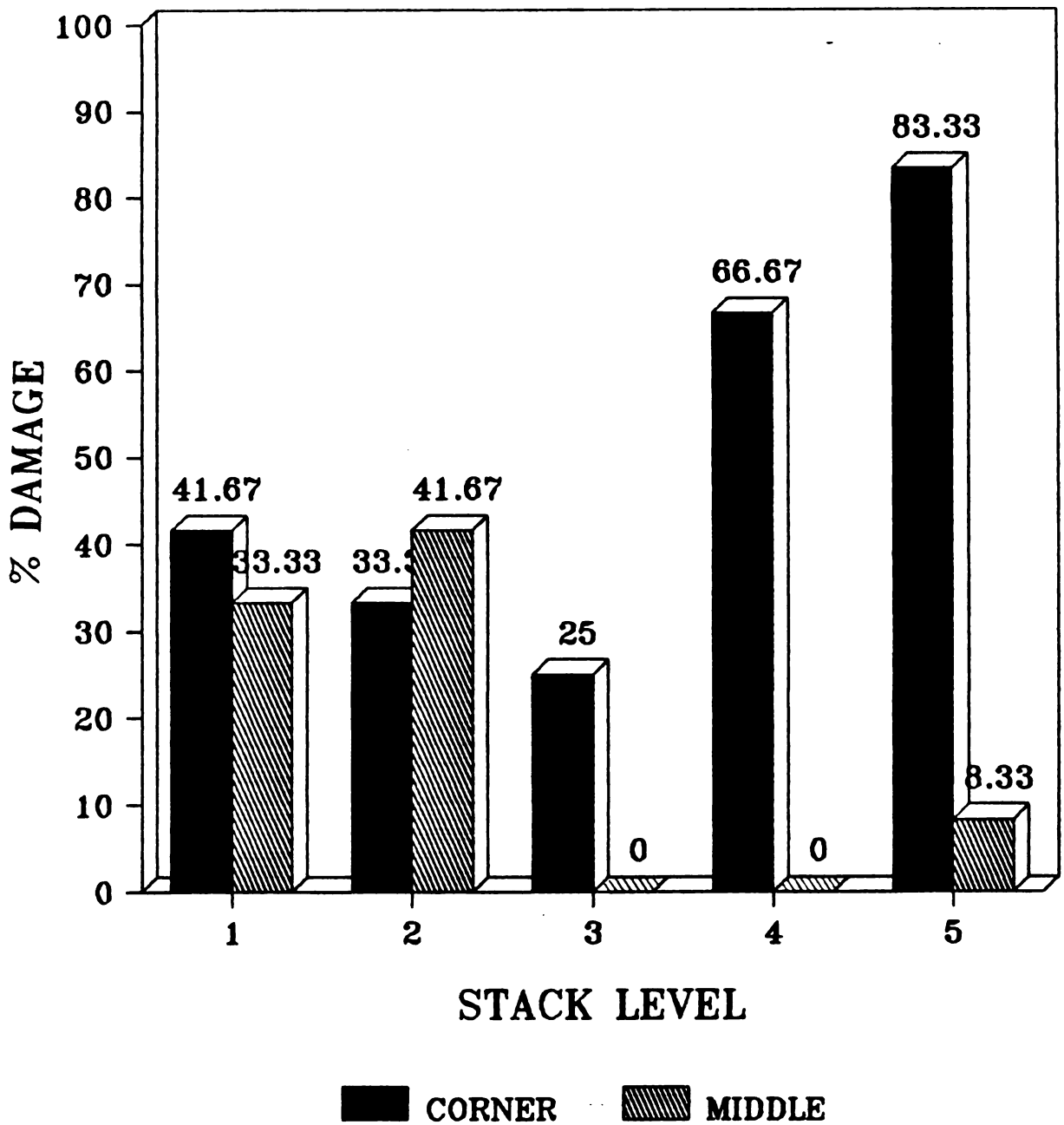


Fig. 16. Total package damage due to exposure to dynamic testing.

Table 16. Total loss of package integrity as a result of subjecting the packages to dynamic test.

STACK LEVEL	LOCATION	
	CORNER	MIDDLE
1	41.67%	33.33%
2	33.33%	41.67%
3	25.00%	0.00%
4	66.67%	0.00%
5	83.33%	8.33%

Table 17. ANOVA table of analysis of total loss of package integrity due to dynamic testing.

SOURCE	df	SS	MS	F	f
LOCATION	1	3.33	3.33	26.50	$f_{1.110,0.005} = 8.25$
STACK LEVEL	4	1.50	0.75	5.96	$f_{4,110,0.005} = 4.10$
INTERACTION	4	8.00	2.00	15.90	
ERROR	110	13.83	0.13		

CONCLUSION

A spray cabinet technique was developed to determine the package integrity of 1.89 L aseptic paperboard juice packages after exposure to dynamic testing. Two pumps and thirty-two nozzles were installed in the cabinet, in order to achieve complete coverage over all surfaces of the test packages. To determine package integrity, a cell culture containing 10^7 cells / ml of Lactobacillus cellobiosus was sprayed onto the packages through the nozzles. Four packages were simultaneously tested with complete coverage. No package failures were observed due to wicking of moisture by raw edges associated with the package seams. Therefore, the spray cabinet technique was satisfactorily used to determine package integrity.

A preliminary test was devised to determine the efficiency of the spray cabinet technique to detect pinholes using 5, 10 and 15 micron size orifices. The minimum pinhole size detectable after 15 minutes of spraying was 10 micron, a 5 micron defect was detected after 30 minutes of spraying. The percent defects increased as the size pinhole increased, no higher level of penetration was achieved after 60 minutes of spraying. None of the negative controls were found to contain viable microorganisms at any of the various test durations. The

efficiency of the spray cabinet technique was compared to an immersion method using the standard orifices. The results indicated that the spray cabinet provided better detectability than the immersion method.

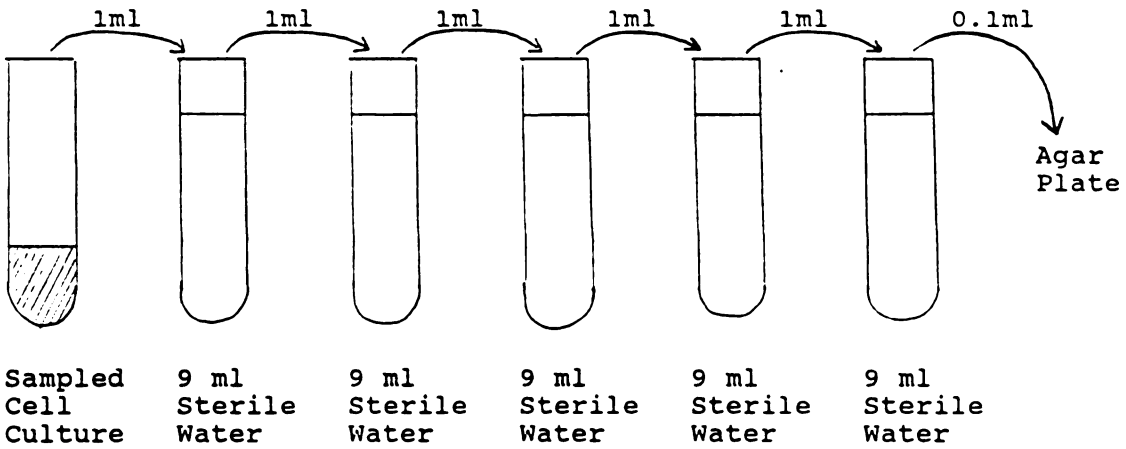
The integrity of aseptic juice packages was assessed using the spray cabinet and immersion methods. Juice packages were exposed to dynamic testing prior to the biotests. Loss of package integrity was characterized by pH change in the product (apple juice), production of CO_2, swelling of the package and by cell numbers in the product. A high level of detection was found using the spray cabinet technique. A significant violation of integrity was observed for the packages located in the corner of the shipping cartons. No microbial penetration was found for the middle packages in the shipping cartons at stack levels 3,4 and 5. Corner packages suffered more damage than those located in the middle, and the most damage occurred to those at stack level 5. The spray cabinet technique resulted in greater detectability than the immersion method, probably due to the pressure (30 psi) associated with the spray cabinet technique.

APPENDICES.

Appendix A. Ingredients in Lactobacilli MRS Medium

Chemicals	gram/liter broth
Bacto-Pepteose Peptone #3	10.00
Bacto-beef extract	10.00
Bacto-Yeast extract	10.00
Dextrose	20.00
Tween 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulfate	0.10
Manganese sulfate	0.05
Disodium phosphate	2.00

Appendix B. Example of Calculation of Cell Concentration in the Culture.



If 40 colonies were counted in a plate from the final diluent which was obtained by diluting the cell culture five times, the cell concentration of the cell culture was measured as:

$$\text{cell conc.} = \text{no. of colonies} \times 10^{-5} \times 10$$
$$= 40 \times 10^{-5} \times 10$$
$$= 4 \times 10^{7} \text{ cells / ml cell culture.}$$

Appendix C. Determination of an Adequate Concentration of Chlorine Solution for Decontamination of the Samples and Spray Cabinet after Biotesting.

Procedures:

One ml (10^7 cells / ml) of cell culture of Lactobacilus cellobiosus was placed on agar plate of Lactobacilli MRS broth, and mixed with one ml of hypochlorite solution. The mixture was equally spread over the agar plate, and then incubated at 37°C for 48 hours to inspect microbial colonies occurred.

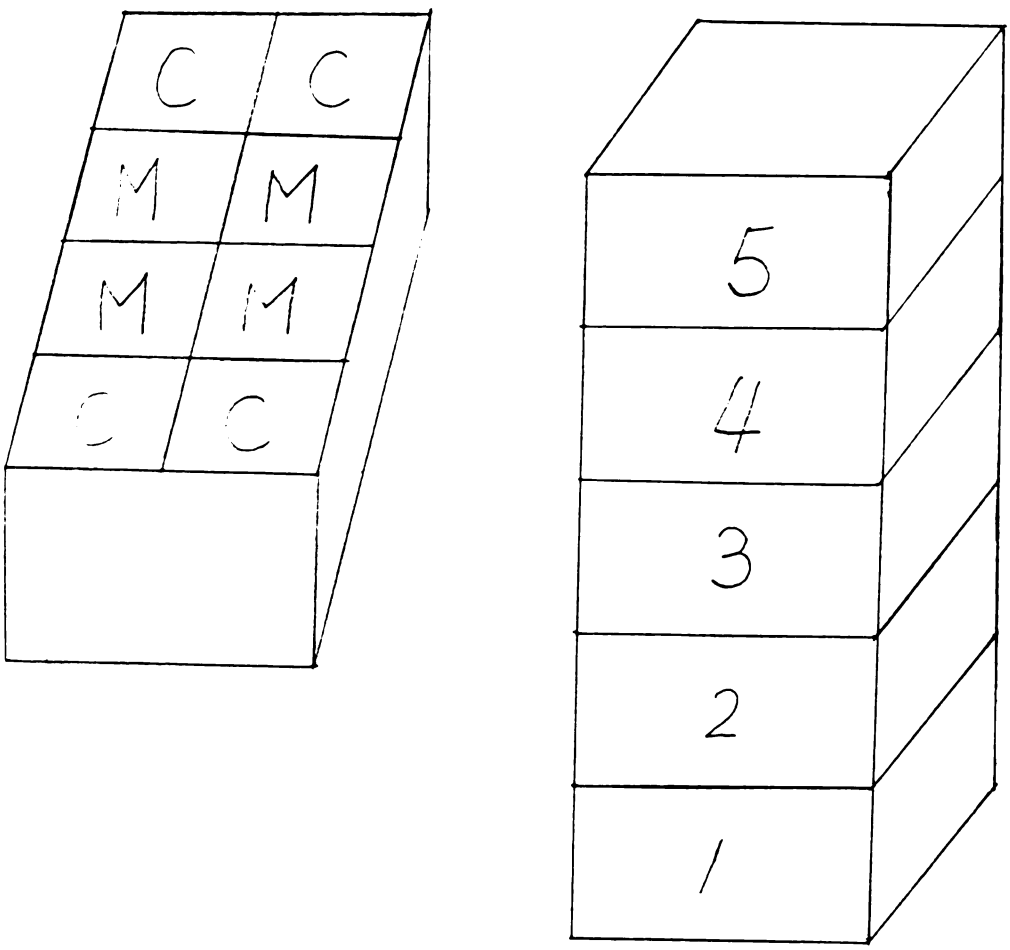
Results:

CONCENTRATION (ppm)		MICROBIAL GROWTH ON THE AGAR PLATE (three replications)
INITIAL	AFTER MIXING	
1000	500	+ + +
2000	1000	- + +
4000	2000	- + +
5000	2500	- - -
6000	3000	- - -
negative controls (distilled water)		
0	0	+ + +

1. The "+" sign means microbial colonies were observed after incubation, "-" means no microbial growth was observed.

The minimum concentration of hypochlorite solution to inhibit microbial growth was 2500 ppm, therefore, this concentration was selected to decontaminate the system in this study.

Appendix D. Diagram of Identification of the Location in the Shipping Cartons and Stack Level during Vibration Testing.



The code "c" indicates the packages placed at the corners of the shipping cartons, "m" is referred as the packages placed in the middle of the shipping cartons.

The stack level is defined the bottom case as the level 1 and the top as level 5.

Appendix E. Calculation of Two-Factor Model to Determine the Confidence of the Test Efficiency of Different Pinhole Sizes and Durations for Orifice Test.

Unbalanced factorial data.

Y = Percent of microbial contamination resulted by biotests.

A = Pinhole sizes (5, 10, 15 microns). (a=3)

B = Test durations (15, 30, 60, 90 minutes). (b=4)

Cell means ($\bar{Y}_{ij}$) and number of replication (r_{ij}):

	A1	A2	A3	$\bar{Y}_{.j.}$
B1	0.00% (3)	16.67% (3)	41.67% (3)	0.1945
B2	8.33% (2)	33.33% (2)	50.00% (2)	0.3055
B3	16.67% (2)	25.00% (2)	62.50% (2)	0.3472
B4	8.33% (2)	33.33% (2)	50.00% (2)	0.3055
$\bar{Y}_{i..}$	0.0833	0.2718	0.5104	$\bar{Y}$=0.2882

SS_E = sum of Y^2_{ijk} - sum of $Y^2_{ij.}/r_{ij}$

= 3.6945 - 3.0382 = 0.6563

(individual data not shown)

Weights: $W_{i.}$ = (t/a)/(sum of $1/r_{ij}$) t=ab

$W_{.j}$ = (t/b)/(sum of $1/r_{ij}$)

$W_{1.}$ = (12/3)/(1/3+1/2+1/2+1/2) = 2.1818
$W_{2.}$ = (12/3)/(1/3+1/2+1/2+1/2) = 2.1818
$W_{3.}$ = (12/3)/(1/3+1/2+1/2+1/2) = 2.1818
sum of $W_{i.}$ = 6.5454

$W_{.1}$ = (12/4)/(1/3+1/3+1/3) = 3.0000
$W_{.2}$ = (12/4)/(1/2+1/2+1/2) = 2.0000
$W_{.3}$ = (12/4)/(1/2+1/2+1/2) = 2.0000
$W_{.4}$ = (12/4)/(1/2+1/2+1/2) = 2.0000
sum of $W_{.j}$ = 9.0000

Estimates of parameters:

$a_i = \bar{Y}_{i.} - \bar{Y}$

$a_1 = 0.0833 - 0.2882 = -0.2049$
$a_2 = 0.2708 - 0.2882 = -0.0174$
$a_3 = 0.5104 - 0.2882 = 0.2222$

$b_j = \bar{Y}_{.j} - \bar{Y}$

$b_1 = 0.1945 - 0.2882 = -0.0937$
$b_2 = 0.3055 - 0.2882 = 0.0173$
$b_3 = 0.3472 - 0.2882 = 0.0590$
$b_4 = 0.3055 - 0.2882 = 0.0173$

$(ab)_{ij} = \bar{Y}_{ij} - \bar{Y}i - \bar{Y}_{.j} - \bar{Y}$

$(ab)_{11} = 0.0000 - 0.0833 - 0.1945 + 0.2882 = 0.0104$
$(ab)_{12} = 0.0833 - 0.0833 - 0.3055 + 0.2882 = -0.0173$
$(ab)_{13} = 0.1667 - 0.0833 - 0.3472 + 0.2882 = 0.0244$
$(ab)_{14} = 0.0833 - 0.0833 - 0.3055 + 0.2882 = -0.0173$
$(ab)_{21} = 0.1667 - 0.2708 - 0.1945 + 0.2882 = -0.0104$
$(ab)_{22} = 0.3333 - 0.2708 - 0.3055 + 0.2882 = 0.0452$
$(ab)_{23} = 0.2500 - 0.2708 - 0.3472 + 0.2882 = -0.0798$
$(ab)_{24} = 0.3333 - 0.2708 - 0.3055 + 0.2882 = 0.0452$
$(ab)_{31} = 0.4167 - 0.5104 - 0.1945 + 0.2882 = 0.0000$
$(ab)_{32} = 0.5000 - 0.5104 - 0.3055 + 0.2882 = -0.0277$
$(ab)_{33} = 0.6250 - 0.5104 - 0.3472 + 0.2882 = 0.0556$
$(ab)_{34} = 0.5000 - 0.5104 - 0.3055 + 0.2882 = -0.0277$

Sum of squares:

$$SS_A = (t/a)\left\{\left[\text{sum of } W_{i.}(a_i)^2\right] - \frac{\left[\text{sum of } (W_{i.})(a_i)\right]^2}{\text{sum of } W_{i.}}\right\}$$

$$SS_A = (12/3)\{[2.1818x(-0.2049)^2 + 2.1818x(-0.0174)^2 + 2.1818x(0.2222)^2] - [2.1818x(-0.2049) + 2.1818x(-0.0174) + 2.1818x0.2222]^2 / 6.5454$$
$$= 4 \times \{ 0.2000 - 0.000000007 \} = 0.8000$$

$$SS_B = (t/b)\left\{\left[\text{sum of } W_{.j}(b_j)^2\right] - \frac{\left[\text{sum of } (W_{.j})(b_j)\right]^2}{\text{sum of } W_{.j}}\right\}$$

$$SS_B = (12/4)\{[3x(-0.0937)^2 + 2x(0.0173)^2 + 2x(0.0590)^2 + 2x(0.0173)^2] - [3x(-0.0937) + 2x0.0173 + 2x0.0590 + 2x0.0173]^2 / 9$$
$$= 3 \times \{ 0.0345 - 0.0010 \} = 0.1005$$

$$SS_{AB} = \sum_{i=1}^{a} \{ \sum_{j=1}^{b} r_{ij}(ab)^2_{ij} - [\sum_{j=1}^{b} r_{ij}(ab)_{ij}]^2 \text{ / sum of } r_{ij} \}$$

$$SS_{AB} = \{ [3x(0.0104)^2 + 2x(-0.0173)^2 + 2x(0.0244)^2 + 2x(-0.0173)^2] - [3x0.0104 + 2x(-0.0173) + 2x0.0244 + 2x(-0.0173)]^2/9 \} + \{ [3x(-0.0104)^2 + 2x(0.0452)^2 + 2x(-0.0798)^2 + 2x(0.0452)^2] - [3x(-0.0104) + 2x0.0452 + 2x(-0.0798) + 2x0.0452]^2 /9 \} + \{ [3x(0.000)^2 + 2x(-0.0277)^2 + 2x(0.0556)^2 + 2x(-0.0277)^2] - [3x0.0000 + 2x(-0.0277) + 2x0.0556 + 2x(-0.0277)]^2 /9 \}$$

$$= \{ 0.0027 - 0.00001 \} + \{ 0.0212 - 0.00001 \} + \{ 0.0093 - 0.000000018 \}$$

$$= 0.0332$$

ANOVA Table for analysis of factorial effect.

Source	df	SS	MS	F	f-ratio
Pinhole size (A)	2	0.8000	0.4000	8.42	$f_{2,15,0.005} = 7.70$
Duration (B)	3	0.1005	0.0335	0.71	$f_{3,15,0.5} = 0.826$
Interaction (AB)	6	0.0332	0.0055	0.11	$f_{6,15,0.5} = 0.933$
Error (E)	15	0.6563	0.0475		

From the ANOVA table, only treatment A (pinhole size) shows significant evidence during the test, the confidence level was more than 99.5%, it means the spray test could provide a very good efficiency to detect various sizes of pinhole. The time factor does not provide sufficient results to show significant evidence between different period in the test. Not any interaction for pinhole sizes and time periods shown in the spray method.

Appendix F. Determination of Microbial Penetration by the Characteristics of the Growth of Lactobacillus cellobiosus

The characteristics of microbial growth in the apple juice were determined using the blanks (the packages were exposed neither dynamic tests, nor biotests, and measured the depth, pH and CO_2% during incubation at 37°C for 14 days) as the standards. Ten packages were used to determined the initial conditions of the apple juice, and then fourty control packages were determined the change of conditions during incubation, no microbial growth was found in these packages. The data of standard is shown in Table A.

Table A. Conditions of initial packages and controls after incubation at 37°C for 14 days.

CONDITIONS OF THE INITIAL PACKAGES		
DEPTH	pH	CO_2%
8.5 ± 0.11	3.76 ± 0.02	2.33 ± 0.25
CONDITIONS OF THE CONTROLS AFTER INCUBATION AT 37°C		
INCREASE OF DEPTH	pH	CO_2%
0.2 ± 0.07	3.78 ± 0.01	2.45 ± 0.12

The change of controls were used to determine the microbial growth in the apple juice. If the increase of

the depth was more than 0.3; pH less than 3.74 and/or the percent of CO_2 reached 3.5% of the packages, packages were suspected to be penetrated by microorganisms, which would be tested by the cell count to confirm if the microbial growth occurred.

The raw data of packages to determine the microbial penetration occurred was shown in Table B.

Table B. The conditions of the packages after incubation at 37°C for 14 days.

STACK LEVEL	LOCATION	NO.	DEPTH CHANGE	pH	CO2%	CELL COUNT	COMMENT
1	CORNER	1	0.9*[1]	3.75	1.71	+[2]	
		2	0.8*	3.74	4.15	+	
		3	1.0	3.74	7.27*	+	
		4	0.2	3.90	2.40		
		5	0.1	3.72*	2.79	-	
		6	0.4*	3.75	2.30	-	
		7	0.7*	3.71*	2.60	+	
		8	0.1	3.76	2.45		
		9	0.2	3.74	2.39		
	MIDDLE	1	0.3	3.76	2.34		
		2	0.7*	3.73*	2.61	+	
		3	0.3	3.76	2.22		
		4	0.9*	3.74	2.84	+	
		5	0.1	3.75	2.34		
		6	0.1	3.77	2.41		
		7	0.2	3.78	2.60		
		8	0.4*	3.76	2.30	-	
		9	0.1	3.77	2.82		
		10	0.0	3.76	2.83		
		11	0.1	3.75	6.36*	+	

Table B. Conditions of the packages after incubation at 37°C for 14 days. (Continued)

STACK LEVEL	LOCATION	NO.	DEPTH CHANGE	pH	CO2%	CELL COUNT	COMMENT
2	CORNER	1	0.5*	3.75	2.32	-	
		2	0.3	3.74	2.68		
		3	0.2	3.75	2.12		
		4	0.5*	3.73*	2.99*	+	
		5	0.3	3.78	2.08		
		6	0.4*	3.75	2.52	-	
		7	0.4*	3.77	2.54	-	
		8	0.2	3.78	5.12*	+	leaking at botttom
		9	0.4*	3.78	2.26	-	
	MIDDLE	1	0.7*	3.72*	2.74	+	
		2	1.1*	3.73*	2.35	+	
		3	1.0*	3.74	2.63	+	
		4	1.1*	3.74	2.38	+	
		5	0.4*	3.76	2.21	-	
		6	0.1	3.78	2.16		
		7	0.1	3.79	2.05		
		8	0.8*	3.73*	5.78	+	
		9	0.1	3.76	2.56		
		10	0.1	3.75	2.30		
		11	0.3	3.76	2.37		
		12	0.2	3.74	2.31		

Table B. Conditions of the packages after incubation at 37°C for 14 days. (Continued)

STACK LEVEL	LOCATION	NO.	DEPTH CHANGE	pH	CO2%	CELL COUNT	COMMENT
3	CORNER	1	0.7*	3.75	2.33	+	
		2	0.5*	3.76	2.41	-	
		3	0.1	3.75	2.52		
		4	0.2	3.78	2.62		
		5	0.3	3.75	2.55		
		6	0.2	3.77	2.08		
		7	0.1	3.74	2.30		
		8	0.2	3.78	2.25		
		9	0.4*	3.77	1.98	-	
	MIDDLE	1	0.4*	3.76	2.03	-	
		2	0.1	3.75	2.34		
		3	0.3	3.75	2.51		
		4	0.1	3.75	2.63		
		5	0.1	3.71*	2.35	-	
		6	0.1	3.75	2.47		
		7	0.0	3.71*	2.11	-	
		8	0.1	3.71*	2.35	-	
		9	0.0	3.72*	2.38	-	
		10	0.3	3.77	2.46		
		11	0.4*	3.75	2.46	-	
		12	0.1	3.74	2.27		

Table B. Conditions of the packages after incubation at 37°C for 14 days. (Continued)

STACK LEVEL	LOCATION	NO.	DEPTH CHANGE	pH	CO2%	CELL COUNT	COMMENT
4	CORNER	1	0.5*	3.73*	2.41	-	
		2	0.6*	3.73*	2.52	+	
		3	0.6*	3.74	2.36	+	
		4	0.3	3.81	2.32		
		5	0.4*	3.74	3.85	-	
		6	0.1	3.72*	5.25	+	
		7	0.0	3.74	9.62*	+	leaking at bottom
		8	0.1	3.75	2.15		
	MIDDLE	1	0.1	3.72*	2.33	-	
		2	0.1	3.72*	2.46	-	
		3	0.2	3.72*	2.34	-	
		4	0.3	3.73*	2.33	-	
		5	0.1	3.80	2.76		
		6	0.0	3.80	2.45		
		7	0.1	3.85	2.51		
		8	0.1	3.78	2.26		
		9	0.2	3.74	2.66		
		10	0.2	3.75	2.75		
		11	0.1	3.75	2.31		
		12	0.1	3.75	2.40		

Table B. Conditions of the packages after incubation at 37°C for 14 days. (Continued)

STACK LEVEL	LOCATION	NO.	DEPTH CHANGE	pH	CO2%	CELL COUNT	COMMENT
5	CORNER	1	0.7*	3.73*	2.12	+	
		2	0.0	3.74	4.33*	+	leaking at bottom
		3	0.1	3.75	2.54		
		4	0.3	3.74	5.44*	+	
		5	0.0	3.71*	7.37	+	leaking at bottom
		6	0.1	3.74	2.01		
	MIDDLE	1	0.4*	3.75	2.04	-	
		2	0.4*	3.75	2.11	-	
		3	0.4*	3.76	2.06	-	
		4	0.3	3.76	2.18		
		5	0.3	3.78	2.48		
		6	0.3	3.80	2.01		
		7	0.1	3.75	2.25		
		8	0.1	3.74	3.32*	-	
		9	0.2	3.75	2.54		

1. The "*" indicates the package was suspected to penetated by microorgainsms.

2. The "+" indicates that positive result was observed in the direct cell count which meant there were microorganisms existed in the package.

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BIBLIOGRAPHY

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