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TECHNICAL REPORT
66-44-FD

# INCIDENCE OF CLOSTRIDIUM BOTULINUM IN RAW MEATS

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## FOREWORD

A radiation dose of 4.5 Mrad is commonly accepted as the minimum sterilizing dose for meats. This belief is based upon the assumption that it is necessary to destroy $6X10^{10}$ spores of C. botulinum to make the food safe for human consumption. No definitive published evidence exists of the actual numbers of botulinal spores occurring in raw meats. The small amount of published material and indirect unpublished data indicate that the total indigenous mesophilic, putrefactive, anaerobic spore population is very low, and among these, the presence of C. botulinum is even lower. Hence, a thorough survey for clostridial spores, particularly C. botulinum, in raw foods, is essential, so that correct radio-sterilization processes can be established.

This contract consisted of two phases. The first phase was concerned with the development of a sensitive method for detecting and enumerating botulinal spores overwhelmed by competitive clostridial spores and heat tolerant vegetative bacteria. This was successfully accomplished. The next phase involved the application of this method to the most extensive survey reported to date on the natural incidence of mesophilic clostridial spores, including C. botulinum, in raw beef, chicken and pork, taken during the four seasons of the year, at seven different geographic locations in the U.S.A. and Canada, and under the worst possible handling and processing conditions in slaughtering plants.

Results indicate that mesophilic clostridial spores are naturally present in very small numbers, and that the presence of botulinal spores is a relatively rare event.

The activities under this contract was monitored by Mr. Abe Anellis as the Project Officer, and by Dr. Durwood B. Rowley as the Alternate Project Officer.

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## ABSTRACT

Two thousand three hundred and fifty eight (2,358) raw meat samples were analyzed for putrefactive and botulinal spores. Of 19,727 P.A. isolates, only one was botulinal (Type C). 77% of the samples had 3 P.A.'s/g or less. The overall mean was 2.82 P.A.'s/g.

## SUMMARY

The anerobic film pouch was demonstrated to be an effective device for the primary isolation of Clostridium botulinum types A and B spores from inoculated raw pork, beef, and chicken. Optimal pasteurization of these meats (for reduction of non-spore microflora without affecting indigenous putrefactive anaerobic spore levels) was 50 min. at 60C. Clostridium botulinum spores were recovered with good precision from meat samples inoculated with mixtures of C. botulinum and putrefactive anaerobe 3679 at 1:1 and at 1:99 ratios.

The anaerobic film pouch was used in a survey to quantitate and isolate the naturally occurring clostridial and botulinal spores in 2,358 samples of raw meat (k,078 chicken, 624 beef, 656 pork). One of the 19,727 putrefactive anaerobic sporeformers isolated was confirmed by the mouse protection test to be Clostridium botulinum type C. This isolate was from a posterior sample of chicken from Western Canada which contained 5.33 clostridia per g. These data indicate a very low incidence of botulinal contamination in raw meats (0.042% of 2,358 samples) and suggest a twenty thousand-to-one ratio of nonbotulinal putrefactive anaerobes to mesophilic C. botulinum spores.

Seventy-seven percent of the 2,358 samples had only three or less putrefactive anaerobes (P.A.'s)/g. The mean P.A. contamination levels for beef, pork, and chicken were 3.03, 3.03, and 2.05/g, respectively. Samples from the bloody neck area had higher levels of P.A. contamination than did samples of trimmings of beef and pork. Posterior chicken samples had higher levels of P.A.'s than did anterior chicken samples or giblets. Statistical analysis of all the beef, pork, and chicken samples combined indicated a significant seasonal effect. Autumn, winter, summer, and spring were the highest to lowest levels of P.A. contamination, respectively. The order of P.A. contamination by geographic regions was, from highest to lowest: far western U.S., southwest central U.S., western Canada, southern U.S., eastern Canada, eastern U.S., and north central U.S.

## INTRODUCTION

Investigators who have attempted to quantify putrefactive anaerobic spores in meats have all commented on the relative scarcity of these organisms. Harriman, Del Giudice, Shinn, and Hansen (1948) reported an average of 2-4 putrefactive anaerobic spores per g in pork sampled in a Chicago packing plant. Burke, Steinkraus, and Ayres (1950), in a similar study in Iowa, found the average putrefactive spore level to be less than 1/g. Comparable results were reported with beef (Ayers and Adams, 1953; Ayers, 1954). Schack, Greenberg, Blodgett and Silliker (1958), found less than 1 putrefactive anaerobic spore per g in 70 raw hams sampled at plants in Minnesota and Alberta. Recently, Steinkraus and Ayres (1964) conducted another survey in Iowa and, again, found very low putrefactive anaerobic spore populations in pork and beef.

No attempt was made to isolate botulinal spores in most of these surveys. While this omission was predicated usually on the very low total putrefactive anaerobic spore load actually found, it must be admitted that the surveys have been far too limited, both geographically and in numbers of samples, to permit generalization on the actual occurrence of C. botulinum spores in meats. Also, isolation techniques in these surveys have varied in such basic considerations as pasteurization protocol, establishment of anaerobiosis, culture medium, and requisites for declaring a culture or colony a putrefactive anaerobe.

Wheaton and Pratt (1961) reported that media exerted a profound influence on the recoverability of severely heated putrefactive anaerobic spores. Freshly prepared media were much superior to dehydrated media for this purpose. However, this difference was not as apparent with mildly heated spore suspensions, (Wheaton, Pratt and Jackson, 1959). The "severe" treatment was defined as 150 second exposure at 121 C; the "mild" as 27 second at 106.5 C. Greenberg, Silliker, and Basa (1958) suggested that dehydrated media such as Peptone Colloid Broth (Difco) might be superior to "home made" media in recovering putrefactive anaerobes from foods likely to contain thermoduric streptococci.

The research reported herein consists of: (a) the selection of a procedure for the enumeration and isolation of mesophilic putrefactive anaerobic sporeformers, particularly C. botulinum types A, B, and C, in raw meats and (b) a comprehensive survey of raw beef, pork, and chicken using the selected method.

## MATERIALS AND METHODS

### Phase I: Selection of Procedure

#### Pasteurization

The following protocol was employed in establishing optimal pasteurization time-temperature relationships:

Approximately 1 liter of a 1:10 suspension of ground raw meat in 0.015 M phosphate buffered dilution water was blended in a 2 liter Erlenmayer flask. The flask was then stoppered with a cork, pierced with a centigrade thermometer so that the bulb of the thermometer was at the center of the meat blend during the heating process. The flask was constantly agitated and maintained within $\pm$ 1 C of the desired temperature. Ten ml samples of the blend (approximately 1 g meat) were removed as soon as the desired pasteurization temperature was reached and at 10 or 15 min intervals thereafter. Bacteriological analyses were run immediately upon sampling. In each experiment a controlled reference sample was obtained as the flask contents reached 50C.

The following three analyses were made:

A. Total Plate Counts

Samples were plated with Tryptone Glucose Yeast Extract Agar (Difco). Plates were counted after 48 hr incubation at 37 C.

B. Enterococci

Samples were placed in Azide Dextrose Broth (Difco) and incubated 24 hr at 37 C. Growth was considered presumptive for enterococci. Positive tubes were confirmed by placing a 0.1 ml aliquot into Trypticase Soy Broth (Baltimore Biological Laboratories) fortified with a 6.5% sodium chloride. After 24 hr incubation at 45 C, those tubes showing growth were examined by gram stain for the presence of streptococci. Catalase production was demonstrated by placing 0.5 ml of the culture on a spot plate and adding a few drops of 5% hydrogen peroxide. Lack of bubbles after 3 min was considered negative for catalase. Confirmed enterococci were considered to be streptococci capable of growth in Azide Dextrose Broth and at 45 C in 6.5% sodium chloride, but incapable of producing catalase.

C. Putrefactive Anaerobes

Putrefactive anaerobic spore formers were detected in Peptone Colloid Broth (Difco) modified by the addition of 1 g of dextrose, 0.2 g $FeSO_4$, and 0.3 g sodium thiosulfate per liter. Tubes were incubated 7 days at 37 C and examined for odor, hydrogen sulfide production (blackening), and bacterial growth. All tubes showing growth, whether or not they were putrid or hydrogen sulfide positive, where pasteurized 15 min at 70 C. One-tenth ml was then transferred to fresh peptone colloid tubes which were subsequently incubated 48 hr at 37 C.

## Pouch Technique

Pouches were prepared as described by Bladel and Greenberg (1965). Media used in pouch tests were:

A. Brewer's Anaerobic Agar (Difco).

B. Angelotti Agar. A modification of SPS Agar (Angelotti, Hall, Foter, and Lewis, 1962) consisting of 1.5% Bacto-Tryptone (Difco), 1% Bacto Yeast Extract (Difco), 3% Bacto-Agar (Difco), 0.06% sodium thioglycollate, and 0.01% L-cystine. The medium was adjust[illegible] to pH 7.0 ± 0.2 and sterilized 15 min at 121 C. Freshly prepar[illegible]d, seitz filtered solutions of $FeSO_4 \cdot 7H_2O$ and $SO_3$ (anhydrous) were each added to the medium at 0.025% fin[illegible]ncentration. Large quantities of the basal medium were prepared [illegible] advance. Sufficient medium was melted each day and the Seitz filtered solutions were added while the medium was at 60C in a water bath.

C. Peptone Colloid Agar was prepared by adding 3.0% Bacto-Agar to modified Peptone Colloid Broth.

Following pasteurization (50 min, 60C), aliquots of 1:10 suspensions of raw meat in buffered dilution water were pipetted into pouches. Agar (tempered to 60C in a water bath) was then added and the pouch flexed to mix its contents.

After 72 hr incubation at 37C, colonies were counted and picked from the pouches as described by Bladel and Greenberg (1965) into tubes of modified Peptone Colloid Broth and incubated.

## Botulinal Toxin Evaluation

Presumptive evidence of toxicity was established by means of intraperitoneal injection of 0.5 ml of the peptone colloid subculture, originating from colonies picked from the pouch. Swiss strain white mice (15-20g) were used. Cultures causing death within four days were confirmed as containing botulinal toxin by protection testing against trivalent ABC anti-toxin (Fort Dodge Laboratories, Fort Dodge, Iowa). When both protected and unprotected animals died within four days, the cultures were further evaluated by dilution and by pasteurization at 99C for 15 min. Heat stable toxins and those which were indistinguishable in activity in protected and unprotected mice, were considered non-specific and non-botulinal.

## Phase II: Incidence of C. botulinum

### Sample Collection

Beef, pork, and poultry samples were received during the summer, autumn, winter, and spring from plants in the following geographical regions: Eastern Seaboard, South, North Central, Central and Southwest Central, Far West, Eastern Canada, and Western Canada. Wherever possible, each region was represented by two plants, preferably one old and one new plant.

The samples were collected by in-plant personnel, packaged in clean jars or plastic bags, and shipped in dry ice to the laboratory. Upon receipt, the samples were inspected and held frozen until analyzed.

Each area and season was represented by 12 one pound samples of the following meat items: lean beef from the bloody neck area, beef trimmings for dry sausage, lean-fat pork mixture from the bloody neck area, and pork trimmings for sausage. Likewise, each area and season was represented by 36 chicken samples taken from "parts missing" birds after dressing, chilling, and before boxing. The samples consisted of 12 anterior portions (wing and breast), 12 posterior portions (leg and thigh) and one pound of giblets representing approximately 12 birds. Whenever possible, the samples consisted of 90% broilers, 5% roasters, and 5% fowl.

Sample Preparation

Each of the beef and pork samples were hand-chopped separately while frozen until a texture of hamburger was reached. Chicken samples were boned prior to chopping. Eleven g from each sample was placed into a sterile four ounce screw-capped bottle containing broken glass chips. The chopping knife, uscolite cutting board, and rubber gloves were sanitized between samples by scrubbing with hot water, followed by 200 ppm hypochlorite solution, and a final hot water rinse.

Enumeration and Isolation of Putrefactive Anaerobes

Ninety-nine ml sterile dilution solution (0.0003M $KH_2PO_4$) were added to each sample bottle. The samples were rapidly shaken 5 minutes on a reciprocating mechanical shaker (Mechanical Shaker, Miracle Paint Rejuvenator Co., St. Paul, Minn.). The meat suspension was heated in a water bath for 50 min at 60C. Pasteurization temperature was verified by a glass thermometer situated at the geometric center of a bottle containing a corresponding sample. Five ml was pipetted into each of six plastic pouches, thereby providing 0.5 g meat per pouch and a total of three g meat per sample analyzed. The pouches used were twice the area of a petri dish.

Approximately 45 ml modified Angelotti's medium was added to each pouch. The contents of the pouches were mixed and the pouches were placed between narrowly spaced wooden slats in a forming device. After solidification of the agar, the pouches were removed from the form and incubated at 37C for 72 hr. The pouches were examined and all black, sulfide producing colonies were transferred into test tubes (20 x 150 mm) containing 20-25 ml modified peptone colloid medium. The colonies were transferred from the pouches by cutting away one side of the pouch and removing individual colonies with an alcohol-flamed scalpel. The peptone colloid tubes were incubated at 37C for 72 hr; after which, all presumptive cultures turning the medium black and/or having a putrid odor were subjected to mouse inoculation tests. Each peptone colloid culture was coded so that a complete history of its source and toxicological analysis could be correlated.

Botulinal Toxin Evaluation

Refer to Phase I.

## RESULTS

One of the important considerations in this study was selection of an optimum pasteurization procedure for the meat samples. The objective was to reduce non-spore contamination within a reasonably short time period, without destroying indigenous mesophilic putrefactive anaerobic spores. A review of the literature found the lowest temperature in routine use to be 80C.

The data presented in Table 1 suggested that 50 and 55C were below the temperatures required for effective reduction of non-sporing contaminants within 60 min. Fifty degree pasteurization, for example, reduced the total count of raw pork only 70% in 60 min. In another typical result chicken, pasteurized at 55C, still retained about 4% of its original population after 60 min exposure. About 99.99% reduction was obtained in 15-45 min at 60C.

Enterococci, the most prevalent non-sporing thermoduric bacteria in raw meats, appeared to withstand 55C treatment quite readily. In beef, no enterococcus reduction occurred within 45 min at the temperature. In chicken and pork, 60 min exposure at 55C resulted in only 90% reduction. At 60C the enterococcus population was reduced in beef at least 99%. Forty-five min at that temperature reduced the enterococcus levels in raw chicken 99.99% and the same treatment reduced the enterococcus count in pork 99.9%. These data are presented in Table 2.

The enterococcus and total aerobic count results demonstrated that a pasteurization temperature of at least 60C would be required to effect meaningful reductions in extraneous microflora. Thirty min at 60C has been used routinely for pasteurization isolation of Type E C. botulinum spores from marine materials. Since Type E spores are appreciably less heat resistant than are types A, B, and C, it should follow that a 60C treatment would not be excessively injurious to mesophilic botulinal spores in meats. In order to determine a "cut-off" point, beyond which indigenous spores would be expected to be lost, several uninoculated raw meat-buffer blends were sampled at 15 min intervals at 50, 55, 60, 65, and 70C. The results shown in Table 3 are derived from single one g meat samples. While the data do not establish a minimum non-injurious [illegible] pasteurization process, it is evident that 70C is unacceptable. Putrefactive anaerobes could be recovered from beef held 45 min and from chicken 60 min at 65C, but not from either product after it reached 70C.

Taking all factors into consideration, 50 min at 60C was selected as the "optimal" meat pasteurization protocol for subsequent work.

Peptone Colloid Broth (Difco), supplemented with dextrose, ferrous sulfate and sodium thiosulfate, has been advocated as an ideal method for quantification of small numbers of non-injured putrefactive anaerobic spores (Greenberg, Silliker, and Basa, 1958). Detection of putrefactive anaerobic 3679 spore inocula from beef, pork, and chicken by means of Peptone Colloid Broth most probable number determinations was evaluated as a possible reference technique. The results of six experiments (2 each with chicken,

pork, and beef, pasteurized 50 min at 60C) using a 3 tube MPN system, are summarized in Table 4. The indigenous PA level averaged 0.37 per g. Thus, populations of 0.47, 1.37, 10.4 and 100 would be expected from samples inoculated with 0.1, 1.0, 10 and 100 per g. The actual mean recovery values for these systems were 0.82, 1.32, 10.8 and 76, respectively. These data demonstrated the Peptone Colloid MPN procedure to be a reasonably accurate method for determining putrefactive anaerobic spore populations in raw meats.

The peptone colloid system was next tested for detection of C. botulinum spores in mixed culture with putrefactive anaerobes in beef, pork, and chicken. Approximately equal number of spores of C. botulinum type 62 A and P.A. 3679 were added to beef, pork, or chicken and pasteurized at 60C for 50 min. Positive peptone colloid tubes were tested for botulinal toxin. The results, listed in Table 5, again showed good overall precision. The figures shown in the "Total P.A." columns include both P.A. 3679 and C. botulinum recoveries, since both organisms appear as putrefactive anaerobes in peptone colloid tubes. These values should be roughly one-half those of the corresponding "Total Putrefactive Anaerobe" figures. Where one P.A. 3679 and one C. botulinum spore were added per g, the total MPN was 2.3 and confirmed C. botulinum, 1.8. Where 10 of each were added, 14 were detected, with 7.6 toxic. The 100-100 inoculation averaged out at 54 total P.A.'s and 21 botulinal spores.

The anaerobic film pouch technique was compared with the Peptone Colloid MPN system for ability to detect indigenous putrefactive anaerobic spores in beef, pork, and chicken. Counts obtained in pouches containing Angelotti agar, Brewer's agar and Peptone Colloid agar were compared with MPN values obtained with Peptone Colloid broth. The data, listed in Table 6, show that the pouch system, using all three media, was at least as sensitive as was the MPN procedure. Angelotti agar gave consistently higher counts than the other two media, but the difference was not statistically significant at the 95% level.

The methods were then compared by testing beef, pork, and chicken which had been inoculated with P.A. 3679, C. botulinum 33 A and C. botulinum 113B. The inoculum consisted of two P.A. 3679 spores per g of meat and one spore per g of each of the two C. botulinum strains. Three experiments were run on each type of meat. The data demonstrated again that the pouch system was as sensitive as the most probable number technique. Again, the Angelotti agar showed a tendency to produce higher counts. The summarized data show a mean total peptone colloid MPN of 2.9 per g, 1.3 of which (or 45%) were confirmed C. botulinum. Angelotti agar pouches averaged 5.8 P.A. spores per g with 2.7 (47%) as C. botulinum, (Table 7).

C. botulinum spores undoubtedly constitute an exceedingly small percentage of the total putrefactive anaerobic spore population. It was, therefore, essential to confirm the utility of the Bladel pouch-Angelotti agar system in recovering small numbers of C. botulinum spores from chicken, beef, and pork bearing a heavy P.A. spore load. A series of tests were conducted, utilizing inocula consisting of a 99-1 ratio of P.A. 3679 to C. botulinum. Six individual tests were conducted on all three meat substrates. The data are listed for the individual meats in Tables 8, 9, and 10, and are

summarized in Table 11. A total of almost 4,200 individual colonies were evaluated in these experiments, 103 of which were identified as C. botulinum. The approximate 2.5% recovery, when compared to the expected 1.0%, is exceedingly precise for a microbiological procedure.

On the basis of the preceeding results, the anaerobic plastic film pouch was employed in the phase II survey to analyze 2,358 samples of beef, pork, and chicken. The results in Table 12 show that 19,727 mesophilic putrefactive anaerobic sporeformers (P.A.'s) were isolated. The overall mean concentration of P.A. spores was 2.82 per g of meat. The variation in the levels of P.A. contamination of the meat samples is given in Table 13. Seventy-seven per cent of the 2,358 samples had three P.A. spores per g or less. The most heavily contaminated sample had 115 P.A. spores/g.

One C. botulinum type C spore was detected among the 19,727 P.A. isolates. This was isolated from a posterior sample of chicken from Western Canada containing 5.33 clostridia per g. The data suggests therefore, that the incidence of botulinal contamination in raw meats is very low (0.042% of 2,358 samples). The results further indicate an approximate ratio of twenty thousand to one of P.A. to mesophilic C. botulinum spores in raw meats.

These data were analyzed statistically and the following differences were detected at the 95% or better confidence level using chi-square and multiple regression analyses:

1. The level of P.A. spores was significantly lower in chicken than in beef or pork, while the levels of P.A. spores in beef and pork were equal.
2. Samples from the bloody neck area had significantly higher P.A. spore levels than did the trimmings from the beef and pork samples.
3. The posterior chicken samples had significantly higher levels of P.A. spores than the anterior chicken samples or the giblets.
4. The level of P.A. spores varied with the season (Table 15). The order of P.A. spore contamination from highest to lowest was autumn, winter, summer, and spring, respectively. The seasonal differences were significant at the 99% confidence level.
5. There were significant differences in the levels of P.A. spore contamination of meats from the various geographic areas surveyed. The order of contamination, from highest to lowest was: far western U.S., southwest central U.S., western Canada, southern U.S., eastern Canada, eastern U.S., and north central U.S.

## DISCUSSION

The phase I data support the utility of commercially available dehydrated media (as illustrated by modified Peptone Colloid Broth) in recovering putrefactive anaerobic spores from mildly pasteurized raw meats. In addition, it was found feasible to detect C. botulinum spores present in P.A. 3679-inoculated meats by using Peptone Colloid Broth in a 3 tube most probable number system and subjecting positive tubes to toxin assay in mice

Two attributes of the most probable number approach tend to discourage its use for routine botulinal spore assay. First, the probability of a



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single C. botulinum spore entering an individual culture tube is extremely remote, particularly at the low levels expected in fresh meats. Mixed cultures would be the rule. Crisley and Helz (1961) have reported inhibition of C. botulinum spore germination by filtrates of S. faecalis. The question of "missed" positives would thus consistently cloud results obtained by any MPN procedure. The second problem is the tremendous glassware and incubation space requirements involved in conducting any large scale studies. Indeed, space and equipment requirements tend to be prohibitive for the typical laboratory in all conventional anaerobic isolation techniques.

The anaerobic pouch procedure is not only convenient but also was demonstrated to be a highly efficient device for isolation of C. botulinum type A and B spores from fresh pork, beef, and chicken inoculated with at least 100-fold greater numbers of saprophytic putrefactive anaerobic spores.

The most obvious conclusion resulting from the survey is that the level of P.A. spore contamination in raw meat is very low at the plant level. Furthermore, botulinal contamination rarely occurs. These results assume greater significance when it is considered that the bloody neck area and trimmings were selected as those portions of beef and pork which would most likely have the greatest contamination. These results, therefore, are in agreement with the low levels of P.A. contamination previously reported in the literature (Harriman et al, 1948; Burke, et al, 1958; and Steinkraus and Ayers, 1964). The fact that one botulinal spore was detected from among the 19,727 P.A.'s isolated emphasized the exceedingly low probability of detecting botulinal spores in anything but an extensive survey such as described herein.

The large number of samples permitted the demonstration of statistically significant differences in levels of P.A. spore contamination. However, while it may be of academic interest that beef and pork, for example, had higher levels of P.A. contamination than chicken, the differences were so small as to be of no commercial importance.

It must be emphasized that the survey was directed specifically toward the enumeration and isolation of mesophilic putrefactive anaerobic spores, particularly those of C. botulinum. Vegetative cells of P.A.'s would have been destroyed during the pasteurization of the samples. However, it is also true that vegetative cells of P.A.'s would be readily destroyed during a commercial thermal or radiation process applied to meat products

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Table 1. Reduction of standard plate count microflora in raw meats at various pasteurization temperatures

| Temp °C | Time min | Product sample | Log reduction | Product sample | Log reduction | Mean log reduction |
|---|---|---|---|---|---|---|
| 50 | 0 | Pork A | - | Beef C | - | - |
| | 15 | | 0.5 | | 0.1 | 0.30 |
| | 30 | | 1.2 | | 0.5 | 0.85 |
| | 45 | | 0.9 | | 0.9 | 0.90 |
| | 60 | | 0.5 | | 1.2 | 0.85 |
| 55 | 0 | Pork B | - | Chicken A | - | - |
| | 15 | | 0.3 | | 0.3 | 0.30 |
| | 30 | | 2.5 | | 1.1 | 1.80 |
| | 45 | | 2.3 | | 1.0 | 1.65 |
| | 60 | | 2.7 | | 1.4 | 2.05 |
| 60 | 0 | Beef A | - | Chicken B | - | - |
| | 15 | | 4.5 | | 3.7 | 4.10 |
| | 30 | | 4.4 | | 4.3 | 4.35 |
| | 45 | | 3.4 | | 4.2 | 3.80 |
| | 60 | | 4.7 | | 6.3 | 5.50 |
| 65 | 0 | Beef B | - | Chicken C | - | - |
| | 15 | | 7.3 | | 4.7 | 6.00 |
| | 30 | | 7.9 | | 5.7 | 6.80 |
| | 45 | | 7.5 | | 5.7 | 6.60 |
| | 60 | | 8.2 | | 5.3 | 6.75 |

Table 2. Enterococcus survival in uninoculated meats at 55 and 60 C

| | Time min. | Log reduction enterococci 55C | 60C |
|---|---|---|---|
| **Beef** | 0 | - | - |
| | 15 | -1 | >2 |
| | 30 | 0 | >2 |
| | 45 | 0 | >2 |
| | 60 | 2 | >2 |
| **Chicken** | 0 | - | - |
| | 15 | 1 | 1 |
| | 30 | 1 | 2 |
| | 45 | 2 | 3 |
| | 60 | 1 | 4 |
| **Pork** | 0 | - | - |
| | 15 | 1 | 1 |
| | 30 | 0 | 1 |
| | 45 | 1 | 2 |
| | 60 | 1 | 3 |

Table 3. Putrefactive Anaerobic Spore Survival in Uninoculated Meats

| | Time Minutes | Temperature (°C) 50 | 55 | 60 | 65 | 70 |
|---|---|---|---|---|---|---|
| Pork | 0 | P | P | P | P | P |
| | 15 | P | P | P | P | P |
| | 30 | P | P | P | A | P |
| | 45 | P | P | P | A | A |
| | 60 | P | P | P | A | A |
| Beef | 0 | P | P | P | P | A |
| | 15 | P | P | P | P | A |
| | 30 | P | P | P | P | A |
| | 45 | P | P | P | P | A |
| | 60 | P | A | A | A | A |
| Chicken | 0 | P | P | P | P | A |
| | 15 | P | P | P | P | A |
| | 30 | P | P | P | P | A |
| | 45 | P | P | P | P | A |
| | 60 | P | A | P | P | A |

P = Present

A = Absent

Table 4. Detection of P.A. 3679 spores by the Peptone Colloid MPN method

| P.A. spore inoculum/g | Calculated spores/g | Actually detected spores/g* |
|---|---|---|
| 0 (indigenous) | | 0.37 |
| 0.1 | 0.47 | 0.82 |
| 1.0 | 1.37 | 1.32 |
| 10 | 10.37 | 10.8 |
| 100 | 100 | 76 |

* Geometric mean of 6 experiments in meat

Table 5. Detection of C. botulinum 62A spores in meats inoculated with P.A. 3679 spores by the Peptone Colloid MPN method.

| Inoculum | | MPN total | MPN confirmed |
|---|---|---|---|
| P.A.3679 | C.botulinum 62A | P.A.'s/g* | C.botulinum/g* |
| 0(indigenous) | 0 | 0.62 | 0.3 |
| 0.1 | 0.1 | 0.98 | 0.69 |
| 1.0 | 1.0 | 2.3 | 1.8 |
| 10 | 10 | 14 | 7.6 |
| 100 | 100 | 54 | 21 |

* Geometric mean of 6 experiments in meat

Table 6. Comparison of pouch and MPN recovery methods for detection of indigenous P.A. spores in raw meats.

| Method | | P.A. spores/g | | | |
|---|---|---|---|---|---|
| System | Method | Beef | Pork | Chicken | Geometric Mean |
| Pouch | Angelotti Agar | 5.00 | 1.66 | 3.00 | 3.03 |
| Pouch | Brewer Agar | 1.36 | 0.41 | 2.73 | 1.23 |
| Pouch | Peptone Colloid Agar | 3.95 | 0.41 | 3.33 | 1.73 |
| MPN | Peptone Colloid Broth | 0.53 | 0.35 | 4.30 | 0.93 |

Table 7. Comparison of pouch and MPN recovery methods for detection of *C. botulinum* spores in raw meats.[a]

| Method | Beef[b] | | | Pork[b] | | |
|---|---|---|---|---|---|---|
| | Spores /g | confirmed botulinal/g | % botulinal | Spores /g | confirmed botulinal | % botulinal/g |
| Pouch: Angelotti | 3.6 | 2.2 | 61 | 6.7 | 3.1 | 42 |
| Pouch: Brewer | 6.5 | 2.6 | 40 | 0.43 | - | - |
| Pouch: P.C. agar | 5.1 | 2.0 | 39 | 1.4 | 0.38 | 27 |
| MPN: P.C. broth | 2.3 | 0.58 | 25 | 3.5 | 2.3 | 66 |
| | **Chicken[b]** | | | **Summation[c]** | | |
| Pouch: Angelotti | 7.9 | 2.[illegible] | 34 | 5.8 | 2.7 | 47 |
| Pouch: Brewer | 7.5 | 2.4 | 32 | 3.0 | 0.97 | 32 |
| Pouch: P.C. agar | 5.8 | 2.0 | 35 | 3.5 | 1.2 | 33 |
| MPN: P.C. broth | 3.0 | 0.91 | 30 | 2.9 | 1.3 | 45 |

a = Inoculum contained P.A. 3679, *C. botulinum* 33A, and *C. botulinum* 113B

b = Geometric mean of 3 experiments

c = Geometric mean of 9 experiments

Table 8. Recovery of C. botulinum from chicken by pouch method*

| Test | C.botulinum strain | P.A. spores | | C. botulinum spores | | |
|---|---|---|---|---|---|---|
| | | Total picked | Per g chicken | Total confirmed | Per g chicken | % detected |
| 1 | 41B | 264 | 110.0 | 2 | 0.83 | 0.76 |
| 2 | 41B | 168 | 70.0 | 3 | 1.25 | 1.79 |
| 3 | 41B | 214 | 89.2 | 0 | 0 | 0 |
| 4 | 33A | 246 | 102.5 | 2 | 0.83 | 0.81 |
| 5 | 33A | 213 | 88.7 | 2 | 0.83 | 0.93 |
| 6 | 33A | 192 | 80.0 | 3 | 1.25 | 1.56 |
| Mean | | 216 | 90.1 | 2 | 0.83 | 0.92 |

*Inoculum contained P.A. 3679 and C. botulinum spores at 99:1 ratio.

Table 9. Recovery of C. botulinum from pork by pouch method*

| Test | C.botulinum strain | P.A. Spores | | C. botulinum spores | | |
|---|---|---|---|---|---|---|
| | | Total Picked | Per g pork | Total Confirmed | Per g pork | % Detected |
| 1 | 33A | 145 | 60 | 5 | 2.08 | 3.47 |
| 2 | 33A | 452 | 188 | 23 | 9.62 | 5.12 |
| 3 | 33A | 288 | 120 | 14 | 5.83 | 4.86 |
| 4 | 41B | 218 | 95 | 9 | 3.75 | 3.94 |
| 5 | 41B | 316 | 132 | 16 | 6.67 | 5.05 |
| 6 | 41B | 411 | 171 | 4 | 1.67 | 0.91 |
| Mean | | 305 | 128 | 11.5 | 4.94 | 3.86 |

*Inoculum contained P.A. 3679 and C. botulinum spores at 99:1 ratio.

Table 10. Recovery of *C. botulinum* from beef by pouch method.*

| | | P.A. spores | | C.botulinum spores | | |
|---|---|---|---|---|---|---|
| Test | C.botulinum strain | Total Picked | Per g Beef | Total Confirmed | Per g Beef | % detected |
| 1 | 33A | 150 | 63 | 3 | 1.25 | 2.0 |
| 2 | 33A | 83 | 35 | 5 | 2.09 | 6.0 |
| 3 | 33A | 160 | 67 | 1 | 0.42 | 0.67 |
| 4 | 41B | 112 | 47 | 3 | 1.25 | 2.7 |
| 5 | 41B | 264 | 110 | 6 | 2.50 | 2.3 |
| 6 | 41B | 295 | 123 | 2 | 0.83 | 0.76 |
| Mean | | 178 | 74 | 3.3 | 1.38 | 1.87 |

*Inoculum contained P.A. 3679 and *C. botulinum* spores at 99:1 ratio.

Table 11. Summation of C. botulinum detection results by pouch method*

| Meat | Number of experiments | P.A. spores/g | Confirmed C. botulinum spores/g | % C.botulinum spores detected |
|---|---|---|---|---|
| Chicken | 6 | 90 | 0.83 | 0.92 |
| Pork | 6 | 128 | 4.94 | 3.86 |
| Beef | 6 | 74 | 1.38 | 1.87 |
| Mean | | 97 | 2.38 | 2.45 |

*Inoculum contained P.A. 3679 and C. botulinum spores at 99:1 ratio.

Table 12. Incidence of mesophilic putrefactive anaerobic spores, including *C. botulinum*, in raw beef, pork and chicken

| Meat | No. of samples | Total no. P.A.'s isolated | Mean P.A.'s/g | No. botulinal isolates |
|---|---|---|---|---|
| Beef, bloody neck area | 298 | 2929 | 3.277 | 0 |
| Beef trimmings | 326 | 2742 | 2.803 | 0 |
| Pork, bloody neck area | 319 | 3655 | 3.820 | 0 |
| Pork trimmings | 337 | 2308 | 2.317 | 0 |
| Chicken, anterior | 373 | 2673 | 2.390 | 0 |
| Chicken, posterior | 379 | 3071 | 2.700 | 1 |
| Chicken, giblets | 326 | 2349 | 2.403 | 0 |
| TOTALS | 2,358 | 19,727 | 2.816 | 1 |

Table 13. Distribution of raw beef, pork and chicken samples according to level of contamination with putrefactive anaerobes.

| | No. samples at each P.A. concentration | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Beef | | Pork | | Chicken | | | Total no. samples at each P.A. conc. |
| P.A.'s/g | Bl. neck | Trmgs. | Bl. neck | Trmgs. | Anterior | Posterior | Giblets | |
| <0.33 | 15 | 41 | 16 | 32 | 27 | 31 | 26 | 188 |
| 0.33 | 20 | 32 | 19 | 51 | 40 | 35 | 32 | 236 |
| 0.66 | 18 | 15 | 26 | 20 | 39 | 30 | 38 | 186 |
| 1.00 | 16 | 16 | 17 | 24 | 15 | 23 | 16 | 127 |
| 1.33 | 13 | 29 | 20 | 23 | 20 | 21 | 26 | 153 |
| 1.66 | 24 | 29 | 18 | 23 | 15 | 25 | 29 | 163 |
| 2.00 | 33 | 42 | 34 | 35 | 31 | 45 | 31 | 272 |
| 2.33 | 33 | 23 | 28 | 34 | 41 | 31 | 20 | 209 |
| 2.66 | 15 | 19 | 19 | 30 | 31 | 30 | 14 | 159 |
| 3.00 | 18 | 16 | 24 | 11 | 16 | 16 | 17 | 117 |
| 3.33 | 14 | 8 | 13 | 10 | 21 | 12 | 9 | 88 |
| 3.66 | 9 | 2 | 7 | 6 | 8 | 12 | 9 | 53 |
| 4.00 | 9 | 4 | 10 | 4 | 3 | 8 | 8 | 47 |
| 4.33 | 9 | 3 | 7 | 5 | 5 | 9 | 7 | 45 |
| 4.66 | 8 | 2 | 9 | 3 | 2 | 6 | 3 | 34 |
| 5.00 | 4 | 3 | 6 | 4 | 8 | 1 | 4 | 30 |
| 5.33 | 3 | 5 | 5 | 1 | 7 | 3 | 5 | 29 |
| 5.66 | 3 | 3 | 3 | | 3 | 2 | 1 | 15 |
| 6.00 | 2 | 2 | 4 | 1 | 5 | 7 | 3 | 24 |
| 6.33 | 3 | 2 | 1 | 2 | 3 | 2 | 1 | 14 |
| 6.66 | | 2 | 3 | 3 | | | 1 | 9 |

Table 13 (continued)

No. samples at each P.A. concentration

| P.A.'s/g | Beef | | Pork | | Chicken | | | Total no. samples at each P.A. conc. |
|---|---|---|---|---|---|---|---|---|
| | Bl. neck | Trmgs. | Bl. neck | Trmgs. | Anterior | Posterior | Giblets | |
| 7.00 | 2 | 2 | 4 | | 2 | 4 | 1 | 15 |
| 7.33 | 2 | | 2 | | 2 | 5 | 2 | 13 |
| 7.66 | 1 | 1 | 2 | 3 | | 1 | 1 | 9 |
| 8.00 | 1 | 2 | 3 | | | 1 | 2 | 9 |
| 8.33 | 3 | 3 | 1 | 2 | 1 | | 2 | 12 |
| 8.66 | 2 | 2 | | | 1 | 5 | | 10 |
| 9.00 | 1 | | | 1 | 1 | 2 | | 5 |
| 9.33 | | 1 | 1 | | | | | 2 |
| 9.66 | | 1 | | | | 1 | | 2 |
| 10.00 | | 2 | 2 | 1 | 1 | | 1 | 7 |
| 10.33 | 2 | 1 | 1 | | 1 | | | 5 |
| 10.66 | | 1 | | | | 2 | | 3 |
| 11.00 | 3 | 2 | 2 | | | | 1 | 8 |
| 11.33 | 1 | 1 | | | | 1 | | 3 |
| 11.66 | | | | | | 1 | 1 | 2 |
| 12.00 | | 1 | | | | | 1 | 2 |
| 12.33 | 2 | | | | | 1 | 1 | 4 |
| 12.66 | | 1 | 1 | | | | 1 | 3 |
| 13.00 | | 1 | | | | | | 1 |
| 13.33 | | | | 1 | 1 | 1 | | 3 |
| 13.66 | | | 1 | 1 | | | | 2 |
| 14.00 | 1 | | | 1 | 1 | | | 3 |

Table 13 (continued)

| P.A.'s/g | Beef | | Pork | | Chicken | | | Total no. samples at each P.A. conc. |
|---|---|---|---|---|---|---|---|---|
| | Bl. Neck | Trmgs. | Bl. Neck | Trmgs. | An-terior | Pos-terior | Giblets | |
| 14.33 | 1 | 2 | 1 | | | | 1 | 5 |
| 14.66 | | | | | | | | |
| 15.00 | | 1 | | 1 | 1 | 1 | | 4 |
| 15.33 | | 1 | | | | | | 1 |
| 15.66 | | | | | | | | |
| 16.00 | 1 | | | | | | | 1 |
| 16.33 | | | | | | | | |
| 16.66 | 1 | | | | | 1 | | 2 |
| 17.00 | | | 1 | | | | | 1 |
| 17.33 | | | | | | | | |
| 17.66 | | | | | | 1 | | 1 |
| 18.00 | | | | 1 | | | 1 | 2 |
| 18.33 | | | | | | | | |
| 18.66 | | 1 | | | | | | 1 |
| 19.00 | 1 | | | | | | | 1 |
| 19.33 | | | | | | | 1 | 1 |
| 19.66 | 1 | | 1 | | | | | 2 |
| 20.00 | 1 | | | | | | 1 | 2 |
| 20.33 | | | | | | | | |
| 20.66 | | | | | | | 1 | 1 |
| 21.00 | | | 1 | 1 | | | | 2 |
| 21.33 | | | 1 | | | | | 1 |
| 21.66 | | | 1 | | | | | 1 |

Table 13 (continued)

| P.A.'s/g | Beef Bl. neck | Beef Trmgs. | Pork Bl. neck | Pork Trmgs. | Chicken An-terior | Chicken Pos-terior | Chicken Giblets | Total no. samples at each P.A. conc. |
|---|---|---|---|---|---|---|---|---|
| 22.00 | | | | | | | | |
| 22.33 | | | | | | | | |
| 22.66 | | 1 | | | | | | 1 |
| 23.00 | | | | | | | | |
| 25.66 | | | | 1 | | 1 | | 2 |
| 31.33 | 1 | | | | | | | 1 |
| 32.00 | | | | | 1 | | | 1 |
| 35.66 | | | | 1 | | | | 1 |
| 21.00 | | | | | | 1 | | 1 |
| 51.66 | 1 | | | | | | | 1 |
| 67.66 | | | 1 | | | | | 1 |
| 69.00 | | 1 | | | | | | 1 |
| 71.33 | | | 1 | | | | | 1 |
| 115.00 | | | 1 | | | | | 1 |
| Total no. samples | 298 | 326 | 319 | 337 | 373 | 379 | 326 | 2358 |
| % samples 1 P.A./gm or less | 43.19 | 31.90 | 24.45 | 37.69 | 32.44 | 31.40 | 36.50 | 31.26 |
| % samples 3 P.A.'s/gm or less | 68.79 | 80.37 | 69.28 | 83.90 | 79.09 | 75.73 | 78.53 | 76.76 |

*C. botulinum type C spore isolated

TABLE 14

Incidence of putrefactive anaerobes

in raw beef, pork, and chicken

| | No. samples | Total P.A.'s | Mean P.A.'s |
|---|---|---|---|
| Beef | 624 | 5671 | 3.03 |
| Pork | 656 | 5963 | 3.03 |
| Chicken | 1078 | 8093 | 2.50 |

TABLE 15

Distribution of samples and concentrations of putrefactive anaerobes according to seasons

| | No. of samples | Total no. P.A.'s isolated | Mean P.A.'s/g | No. botulinal isolates |
|---|---|---|---|---|
| Spring | 585 | 2499 | 1.422 | 0 |
| Summer | 624 | 4382 | 2.338 | 0 |
| Autumn | 563 | 7015 | 4.149 | 0 |
| Winter | 586 | 5831 | 3.314 | 1 |

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Two thousand three hundred fifty eight (2,358) raw meat samples were analyzed for putrefactive and botulinal spores. Of 19,727 P.A. isolates, only one was botulinal (Type C). 77% of the samples had 3 P.A.'s/g or less. The overall mean was 2.82 P.A.'s/g.

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| KEY WORDS | LINK A ROLE | LINK A WT | LINK B ROLE | LINK B WT | LINK C ROLE | LINK C WT |
|---|---|---|---|---|---|---|
| Isolation | 8. | | 8 | | | |
| Clostridium Botulinum | 1,2 | | 1,2 | | | |
| Meat | 1 | | 5 | | | |
| Raw | 0 | | 0 | | | |
| Counting | 8 | | 8 | | | |
| Ratios | | | 8 | | | |
| Anaerobic spores | 1,2 | | 1 | | | |
| Putrefactive | 0 | | 0 | | | |

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