T H E  U N I V E R S I T Y  O F  M I C H I G A N

COLLEGE OF ENGINEERING
Department of Chemical Engineering

Final Report

# EFFECT OF BACTERIAL ECOLOGY ON CANNED FOOD SPOILAGE

L. L. Kempe
J. T. Graikoski

ORA Project 04200

under contract with:

DEPARTMENT OF HEALTH, EDUCATION AND WELFARE
DIVISION OF ENVIRONMENTAL ENGINEERING AND FOOD PROTECTION
PUBLIC HEALTH SERVICE
CONTRACT NO. EF-127 (R-1)
WASHINGTON 25, D. C.

administered through:

OFFICE OF RESEARCH ADMINISTRATION  ANN ARBOR

September 1962

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## SUMMARY

Growth of Clostridium botulinum was prevented in trypticase broth when the preservative chemicals used in Pork Luncheon Meat (PLM) were incorporated in the same concentrations used in the meat. It was also found that metallic Mn slightly reduced the antiseptic effects of these chemicals and that Fe powder was even more effective in this regard.

Irradiation of PLM was found to impair the preservative qualities of the chemicals in this product. Pasteurization of PLM with gamma radiations reduced the stability of the meat. This could be dangerous since it was found that C. botulinum cultures developed and produced toxin under these conditions. Moreover, the saprophytic bacteria had been killed, so they were not present to produce gas and swell the can before development of the C. bolutinum cultures.

PLM containing powdered metals spoiled in progressively shorter times as metals having greater electrochemical activity were used. This was true for both unheated and autoclaved PLM. The activity series for these metals in PLM was that which could be expected from the Electromotive Series except that Fe was somewhat more and Al somewhat less reactive than the latter Series predicts.

The occasional finding of limited amounts of botulinus toxin in non-heat-processed PLM inoculated with C. botulinum and containing added metals is disquieting. It indicates that botulinus toxin could form in PLM during storage if the meat is not properly refrigerated. This is particularly likely if the steel in the can is exposed or if aluminum cans are used.

# PART A

# INOCULATED PACK STUDIES

# I. SUMMARY

Pork Luncheon Meat (PLM) was reground, mixed with powdered metals, repacked in small tin cans, and then heat sterilized by a technique that prevented condensation of steam in the meat. These metals included Mg, Fe, Mn, Al, Zn, Ni, Sn, and Co. The PLM was then inoculated with spores of Clostridium botulinum, The cans sealed under vacuum, and incubated at 30°C. Suitable controls without powdered metals, heat processing, or inoculation were also prepared.

When hard swells developed, the cans were opened, examined subjectively as well as microbiologically, and assayed for botulinus toxin with the following results:

(1) PLM containing powdered metals spoiled in progressively shorter times as metals that had greater electrochemical activity were incorporated. This is true for both unheated and autoclaved cans of PLM. The relative activities of these metals was that which could be expected from their positions in the Electromotive Series except that Fe was somewhat more and Al somewhat less reactive than the Series predicts.

(2) When non-heat-processed cans of spoiled PLM were examined microbiologically, they were found to contain mixed cultures. Gram positive cocci were almost always represented in this culture, as were Gram negative bacteria. The presence of C. botulinum spores from the inoculum did not change the nature of the culture causing the spoilage except in Run LM-2, where a few Gram positive bacteria were also found.

(3) Ordinarily, no toxin was found when inoculated, non-heat-processed PLM spoiled. In Runs LM-2 and LM-4, however, a small amount of toxin was present when electrochemically active metals had previously been mixed into the meat. This finding, which is considered to be normal for a limited number of the spoiled cans in these experiments, is compared with a similar finding reported by Jensen (1954) after studying spoilage of spiced ham by C. sporogenes. The occurrence of even limited amounts of toxin in these experiments suggests the possibility of toxin formation in commercial PLM during storage if the meat is not kept refrigerated. The possibility of finding toxin in even more likely in cans where electrolytic activity exists, such as in the presence of normal corrosion of Fe or, possibly, in the new Al or Al anode cans.

# II. INTRODUCTION

Canned foods (canned meats in particular), are usually heat-processed to such an extent that few if any viable bacteria remain. The bacteria surviving such treatment are heat-resistant spores. Consequently, when such products spoil, the spoilage is usually due to a pure culture formed from the outgrowth of a bacterial spore. But a different situation exists in canned, preserved meats of the substerile variety. These meats include canned ham, bacon, and Pork Luncheon Meat (PLM). The latter is marked "Perishable—Keep Under Refrigeration" (Halvorson, 1955).

In the preparation of these products, reasonable care is exercised to use high-quality raw meats. The products are pickled or cured and are then heated to pasteurization schedules or somewhat higher, but no attempt is made to sterilize them. Thus the stability of these meats depends upon the preservative ingredients used in pickling or curing. When bacterial spoilage occurs it results from the normal bacterial flora of the meats that develop when conditions become suitable for their growth. Since the native flora consist of a large number of bacteria, it might be expected that spoilage would result from any of them. This is not the case, however. Spoiled PLM usually contains cultures of large Gram positive cocci or Gram negative bacteria. When anaerobic bacteria are present, as when they are inoculated into the meat, they too may be found growing in the spoiled product. Some time ago we (Kempe and Graikoski, 1959) observed that metallic iron accelerated the spoilage of PLM. These observations led us to ask a number of questions and review others from the literature as follows:

(1) Does metallic iron act primarily by decomposing $NO_3^-$ and $NO_2^-$ in the PLM, followed by a reduction of the redox potential--or is another mechanism involved?

(2) Does the redox potential in the meat establish a hierarchy of bacteria based upon the $E_h$ at which they will develop cultures in canned PLM?

(3) Could C. botulinum spores develop during spoilage of canned PLM along with the other bacteria when electrolytically active metals are present?

A great deal of research has been conducted over the past three years on the factors involved in spoilage of canned, preserved meats. The results of these studies are reviewed in a book by L. B. Jensen entitled, Microbiology of Meats (1954) and also in a paper by H. O. Halvorson entitled "Factors Determining Survival and Development of Clostridium botulinum in Semi-Preserved Meats" (1955). It seems, however, that little attention has been given to the possible influence that metals in the containers could have on microbial spoilage. This is surprising since the effect of redox potential on preservation of

PLM has been well recognized and electrochemical corrosion is a series of redox reactions. Research workers in the food industry have written about the latter (Neish and Donelson, 1960; Frankenthal, et al., 1959) and have even developed cans with sacrificial aluminum anodes to protect the steel (McKernan, et al., 1957), but apparently they have not associated this with microbiological safety of the product. The advent of aluminum cans could prove to be a mixed blessing, since Al is electrochemically very active under certain conditions. It would really seem better to develop containers which resemble those made of nonmetallic substances in having no electrochemical activity.

The remarks that Halvorson (1955) made seven years ago were based on results of studies in tin cans. They may not apply to aluminum cans or those with anodic protection. Halvorson pointed out that "the curing ingredients, such as salt, sodium nitrate, and sodium nitrite, in the concentrations ordinarily used in the curing of such items as luncheon meat will inhibit growth and toxin production of C. botulinum... To the best of my knowledge, there has been no case of botulinum poisoning traced to these products during this period of time." (He referred to the period from 1929 to the time of writing.)

Against this background, then, we have proceeded to devise experiments that would permit us to learn whether metals of the kinds used in containers would affect microbiological spoilage and possible toxin production by C. botulinum.

## III. MATERIALS AND METHODS

Commercially packed Pork Luncheon Meat (PLM) was obtained from the research group at Swift and Co., which prepared special lots for us. Although the lots were prepared according to Swift's standard mixture, this special procedure assured us of the composition and date of preparation. The formula used in the preparation of the mix was given to us as follows.

Meat

| | |
|---|---|
| Regular pork trimmings | 30% |
| Special lean prok trimmings | 60% |
| Ham trimmings | 10% |

Other Ingredients (per 100 lb of meat)

| | |
|---|---|
| Water | 3 lb |
| Salt | 3 lb 8 oz |
| Dextrose | 3 lb 2 oz |
| Corn syrup | 2 lb |
| Ascorbic acid | 1/3 oz |
| Sodium nitrate | 1/4 oz |
| Sodium nitrite | 1/8 oz |
| Coriander | 1/4 oz |
| White pepper | 1/4 oz |

This PLM is marked "Perishable—Keep Under Refrigeration." It was packed in 6-lb cans, which were cold when we received them in our laboratory. They were immediately placed at 40°F and kept at that temperature until used.

In preparation for an experimental pack, cans of PLM were opened and the meat was removed, ground in a hand grinder, and then placed in a loose condition in flat enameled pans. Dissolved gases were removed from the meat by placing it in an evacuated chamber. Otherwise these gases would have bloated the meat when it was later vacuum packed.

The ground, degassed PLM was then mixed with various metal powders and packed into tin cans. From this stage on, the size of the cans and the treatment they received varied somewhat, depending upon the particular experiment at hand. For some experiments, the PLM was used in this simple condition, the next step involving inoculation followed by closing the cans under vacuum in a commercial-type closing machine.

When sterile PLM was required, the degassed meat was packed into mushroom-

type (204 x 204, or 204 x 214) cans, leaving sufficient head space; the can lids were set in place and secured with a rubber binder. Then these small cans were placed in individual No. 2 tin cans and the No. 2 cans, with thin lids set loosely in place, were steamed in an autoclave at atmospheric pressure for 15 minutes in order to replace most of the air in the cans with steam. The No. 2 cans were then removed and quickly closed. Thus at this stage the packet consisted of a mushroom-type can with its cover held in place by a rubber binder and enclosed in a No. 2 can; and the extra space in both cans was filled with steam. The No. 2 cans were returned to the autoclave and the pressure brought to 15 psig. The time of autoclaving varied from 45 min in early experiments, to 90 minute in later experiments. The autoclave pressure was then slowly reduced to atmospheric and the cans removed. From experience it was found best to further cool the cans to room temperature and then place them in a refrigerator. When hot cans were opened, the meat and its juice in the small can exploded due to flash vaporization; when the small cans were removed from the No. 2 cans at room temperature, the juice tended to spill from the mushroom can. But when the whole system was at 40°F or below, little difficulty was encountered. After the small can had been removed, the rubber band was detached, the cover of the small can was aseptically lifted, and heat-shocked spores were injected into the meat. The cover on the small can was then sealed in a commercial-type closing machine and the canned meat was ready for incubation.

It should be pointed out that this procedure was developed to permit heat-sterilization of the meat without the addition of water. There are changes that occur regardless, but these are inherent in the system. Heat processing releases fat and denatures the protein, and probably also releases water that is present in the meat in a bound condition.

## IV. EXPERIMENTS

### 1. RUN LM-1: Growth of *Clostridium botulinum* 62A in Sterilized Pork Luncheon Meat (PLM) Containing Added Fe Powder

#### a. Organism

*C. botulinum* 62A spores grown in liver broth at 37°C; diluted to a concentration of 4,300,000 spores per ml; 1 ml injected into each can.

#### b. Procedure

Same as that described in Section III (Materials and Methods) except that the mushroom-size cans were sealed while hot rather than in a vacuum closing machine.

#### c. Data

See Table I.

#### d. Results and Conclusions

(1) Cans do not swell upon incubation when they contain PLM alone.

(2) Heat-processed PLM inoculated with 4,300,000 heat-shocked, *C. botulinum* 62A spores per can developed gas, gram-positive rods, and botulinum toxin in 13 days of incubation at 30°C.

(3) Heat-processed PLM containing 1 gm (gram) of iron powder and inoculated with 4,300,000 heat-shocked, *C. botulinum* 62A spores per can developed gas, gram-positive rods, and toxin within 6 days at 30°C.

(4) Heat-processed PLM containing 1 gm of iron powder but not inoculated, swelled in 6 to 12 days nevertheless. The microscopic data indicates that the meat was not sterile. Probably it was not heated sufficiently for complete sterilization.

In any event, this run demonstrates that iron powder hastens spoilage of PLM, as was previously found and reported by us (Kempe and Graikoski, 1959).

TABLE I

GROWTH AND TOXIN DEVELOPMENT BY C. BOTULINUM 62A SPORES AT 30°C
IN HEAT-PROCESSED PLM CONTAINING ADDED IRON POWDER (RUN LM-1)

| Description | No. of Days Until Swelling | Microscopic Examination, Can Contents | Subculture | Toxicity of Can Contents | Remarks |
|---|---|---|---|---|---|
| PLM (Non-Inoculated) | > 271 | | | | Withdrawn, unswollen, after 271 days |
| | > 271 | | | | |
| PLM (Inoculated) | 12 | Gm(+)rods | + | | |
| | 13 | Gm(+)rods | 0 | 4/4 | |
| | 13 | Gm(+)rods | + | 4/4 | |
| | 13 | Gm(+)rods | 0 | 4/4 | |
| | 14 | | | | |
| | 14 | | | | |
| PLM and Fe Dust (Inoculated) | 6 | Gm(+)rods | Gm(+)rods | 4/4 | Mice fed this PLM died in less than 24 hours in all cases; 1 gm Fe powder was used per 100 gm of meat |
| | 6 | Gm(+)rods | Gm(+)rods | 4/4 | |
| | 6 | Gm(+)rods | Gm(+)rods | 4/4 | |
| PLM and Fe Dust (Non-Inoculated) | 6 | 0 | 0 | 0/4 | 1 gm iron powder per 100 gm of meat |
| | 12 | few cocci | 0 | 0/4 | |

2. RUN LM-2: Growth of C. botulinum 62A in PLM Containing Added Metal Powders

a. Organism

Same as that used in Run LM-1.

b. Procedure

Same as that used in Run LM-1.

c. Data

See Table II.
Can code:

INS: Inoculated, not sterilized, no added iron

NINS: Non-inoculated, non-sterilized, no added iron

IS: Inoculated, sterilized, no added iron

NIS: Non-inoculated, sterilized, no added iron

FeS: Inoculated, sterilized, added iron 0.5%

FeNS: Inoculated, not sterilized, added iron 0.5%

ZnS: Inoculated, sterilized, added zinc dust 0.5%

ZnNS: Inoculated, not sterilized, added zinc dust 0.5%

d. Results and Conclusions

(1) INS: Inoculated, non-sterilized PLM was stable to incubation at 30°C for at least half a year.

(2) NINS: Non-inoculated, non-sterilized PLM was also stable for approximately half a year, with the one exception shown (IS), which developed gas in 39 days.

(3) IS: Inoculated, sterilized PLM was stable for approximately 39 days. Apparently the heat sterilization reduced the stability of PLM. (Note that INS was stable.) This could have been due to a changed condition in water in the pork from a bound to an unbound condition, thus diluting preservative salts present in the PLM.

TABLE II

SPOILAGE AND TOXIN DEVELOPMENT IN STERILIZED PLM CONTAINING ADDED METAL POWDER, INOCULATED WITH C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-2)

| Code | Can No. | No. of Days to Swelling | Microscopic Examination, Can Contents | Toxicity of Can Contents | Subculture in Casitone Broth | Remarks |
|---|---|---|---|---|---|---|
| INS | 1 | 190 | | | | |
| | 2 | 232 | | | | |
| | 3 | >660 | | | | |
| | 4 | >660 | | | | |
| | 5 | >660 | | | | |
| NINS | 1 | 39 | Gm(+)bacilli | No toxin | | Not putrid |
| | 2 | 189 | | | | |
| | 3 | 200 | | | | |
| | 4 | 706 | | | | |
| | 5 | Lost | | | | |
| IS | 1 | 39 | Gm(+)bacilli | | | All cans putrid |
| | 2 | 39 | Gm(+)bacilli | | | |
| | 3 | 39 | Gm(+)bacilli | | | |
| | 4 | 39 | Gm(+)bacilli | | | |
| | 5 | 55 | Gm(+)bacilli | | | |
| | 6 | 180 | | | | |
| NIS | 1 | >660 | | | | |
| | 2 | >660 | | | | |
| | 3 | >660 | | | | |
| | 4 | >660 | | | | |
| FeS | 1 | 9 | Gm(+)rods | 4/4,<24hr | 0 | |
| | 2 | 9 | Gm(+)rods | 4/4,<24hr | 0 | |
| | 3 | 9 | Gm(+)rods | 4/4,<24hr | + | |
| | 4 | 10 | Gm(+)rods | 4/4,<24hr | 0 | |
| | 5 | 17 | Gm(+)cocci | NC | + | NC=Not Checked |
| FeNS | 1 | 4 | Gm(+)cocci | 0/4 | + | |
| | 2 | 5 | Gm(+)cocci | 2/4 | + | |
| | 3 | 5 | Gm(+)cocci | 1/3 | + | |
| | 4 | 5 | Gm(+)cocci | 1/4 | + | |
| | 5 | 5 | Gm(+)cocci | NC | + | |
| | 6 | 5 | Gm(+)cocci | NC | + | |

TABLE II (Concluded)

| Code | Can No. | No. of Days to Swelling | Microscopic Examination, Can Contents | Toxicity of Can Contents | Subculture in Casitone Broth | Remarks |
|---|---|---|---|---|---|---|
| ZnS | 1 | 11 | Gm(+)cocci | 4/4,<20hr | + | |
| | 2 | 13 | Gm(+)cocci | 4/4,<20hr | + | |
| | 3 | 14 | Gm(+)cocci | 4/4,<20hr | + | |
| | 4 | 14 | Gm(+)cocci | 4/4,<20hr | + | |
| | 5 | 14 | Gm(+)cocci | 4/4,<20hr | + | |
| | 6 | 17 | Gm(+)cocci | 4/4,<20hr | + | |
| ZnNS | 1 | 7 | Gm+(cocci) | 1/4 | + | |
| | 2 | 9 | Gm+(cocci) | 3/4 | + | |
| | 3 | 9 | Gm+(cocci) | 1/4 | + | |
| | 4 | 9 | Gm+(cocci) | 1/4 | + | |
| | 5 | 9 | Gm+(cocci) | 1/4 | + | |
| | 6 | 10 | Gm+(cocci) | 0/4 | + | |

(4) NIS: These non-inoculated, sterilized samples were stable, as would be expected.

(5) FeS: Inoculated, sterilized, PLM with added iron spoils in 1 to 2 weeks. The spoilage must be due to C. botulinum, as confirmed by the presence of toxin.

(6) FeNS: Inoculated, non-sterilized, PLM with added iron spoils in 4 to 5 days, with development of no toxin or limited amounts of toxin. This more rapid gas production appears to be due to gram-positive cocci. The small amount of toxin indicated could be botulinus toxin, in which case it could have developed after a hard swell was rated. Toxin was not assayed immediately.

(7) ZnS: Inoculated, sterilized PLM with added zinc dust spoiled in two weeks. Gram-positive cocci were found but could have been present in the original PLM as well. The meat was also extremely toxic to mice, which indicates that although the cocci grew, apparently the C. botulinum did also. But what is more important, in this case apparently the gas swelling was caused by the C. botulinum rather than by the cocci.

Thus, the nicely balanced time of swelling found with iron is not operative here, and the zinc is probably dangerous in

that it allows formation of C. botulinum toxin without the protection of early gas formation by the cocci.

(8) ZnNS: Inoculated, non-sterilized PLM with added zinc dust spoiled in slightly more than a week with concomitant formation of small amounts of toxin. Like ZnS, this was similar to FeNS except that spoilage occurred 2 to 5 days later.

Spoilage occurred faster with added Zn dust than with Fe powder. As shown in Table III, this would be expected from the galvanic couples and positions in the electromotive series if reduction of the redox potential initiates the spoilage

TABLE III

SPOILAGE OF STERILIZED AND NON-STERILIZED PLM IN THE PRESENCE OF ADDED ZINC DUST OR IRON POWDER AND C. BOTULINUM 62A SPORES (RUN LM-2)

| Sample | No. of Cans Spoiled | No. of Days Until Spoilage | Toxicity |
|---|---|---|---|
| Sterilized (No Spores) | 0/5 | > 660 | |
| Sterilized + Spores | 5/5 | 39-180 | |
| Sterilized + Fe + Spores | 5/5 | 9-17 | 16/16 |
| Sterilized + Zn + Spores | 6/6 | 11-17 | 24/24 |
| Non-Sterilized (No Spores) | 3/5 | 39-706 | |
| Non-Sterilized + Spores | 2/5 | 190-232 | |
| Non-Sterilized + Fe + Spores | 6/6 | 4-5 | 4/15 |
| Non-Sterilized + Zn + Spores | 6/6 | 7-10 | 7/24 |

The data in Table III also indicate that non-sterilized PLM spoils faster than sterilized PLM when C. botulinum 62A spores are present. Moreover, toxin develops in the sterilized PLM but less rapidly in non-sterilized PLM.

3. RUN LM-3: Growth of C. botulinum 62A in Non-Sterilized PLM Packed with Various Metals

a. Organism

Same as that used in Run LM-1; 18,000 spores per can or 1,385 spores per gm.

b. Procedure

0.25 gm of each of the following powdered metals were added per 100 gm of

ground PLM and thoroughly mixed; Fe, Sn, Co, Ni, Al, Mn, and Mg. Four cans of each mixture were packed; however only three were inoculated, the fourth being a control. These cans of meat were not sterilized and were packed under vacuum.

c. Data

See Table IV.

TABLE IV

SPOILAGE AND TOXIN DEVELOPMENT IN PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-3)

| Metal | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Subculture in T.S.* Broth | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| Fe | 1 | 7 | Gm(+)cocci | C. botulinum | 0/3 | |
| | 2 | 7 | Gm(+)cocci | C. botulinum | 0/3 | |
| | 3 | 7 | Gm(+)cocci | C. botulinum | | |
| No Spores | 4 | 8 | | | 0/3 | |
| Sn | 1 | 195 | | | | |
| | 2 | > 575 | | | | |
| | 3 | > 90 | | | | |
| No Spores | 4 | > 575 | | | | |
| Co | 1 | 147 | | | | |
| | 2 | 147 | | | | |
| | 3 | 177 | | | | |
| No Spores | 4 | 147 | | | | |
| Ni | 1 | > 280 | | | | |
| | 2 | > 280 | | | | |
| | 3 | > 280 | | | | |
| | 4 | > 575 | | | | |
| Al | 1 | 19 | Gm(+)cocci | C. botulinum | 0/4 | Since the 1/4 was at 7 days |
| | 2 | 19 | Gm(+)cocci | C. botulinum | 1/4 | this 1 is really 0 |
| | 3 | 20 | Gm(+)cocci | C. botulinum | 0/4 | |
| No Spores | 4 | 19 | Gm(+)cocci | | | |
| Mn | 1 | 5 | Gm(+)cocci | C. botulinum | 0/3 | |
| | 2 | 5 | Gm(+)cocci | C. botulinum | 0/3 | |
| | 3 | 6 | Gm(+)cocci | C. botulinum | | |
| No Spores | 4 | 5 | Gm(+)cocci | | 0/3 | |
| Mg | 1 | 2 | Gm(+)cocci | C. botulinum | 0/4 | One mouse died at 7 days |
| | 2 | 2 | Gm(+)cocci | C. botulinum | 0/4 | |
| | 3 | 3 | Gm(+)cocci | C. botulinum | | |
| No Spores | 4 | 2 | Gm(+)cocci | | 0/4 | One mouse died at 7 days |
| No Metal | 1 | 135 | Gm(+)cocci | | 0/4 | |
| | 2 | 147 | | | | |
| | 3 | > 575 | | | | |
| No Spores | 4 | > 270 | | | | |

*T.S. = Trypticase - Soy Broth

By arranging the results according to the number of days meeded to develop gas, we have the data shown in Table V.

TABLE V

SPOILAGE OF NON-STERILIZED PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES (RUN LM-3)

| Sample | No. of Cans Spoiled | No. of Days Until Spoilage | Toxin Development |
|---|---|---|---|
| No Metal | 2/4 | 135-147 | 0/4 |
| Mg | 4/4 | 2-3 | 0/8 |
| Mn | 4/4 | 5-6 | 0/6 |
| Fe | 4/4 | 7-8 | 0/3 |
| Al | 4/4 | 19-20 | 0/12 |
| Co | 3/4 | 147 | |
| Ni | 0/4 | > 575 | |
| Sn | 0/4 | 195 | |

d. Results and Conclusions: Except for Al

The time required to produce spoilage followed the expected sequence for these metals based upon their position in the electromotive series. Aluminum is often erratic in galvanic couples due to the protective oxide film which usually coats it.

Since the PLM was not sterilized, the spoilage rates indicate relative rates at which spoilage could be expected to occur in cans made from these metals. Oddly enough, from the results of this experiment, aluminum would appear to be better than iron for cans containing PLM. This could be due to the effect of the oxide coating on aluminum, a coating which is not always reliable in the presence of organic acids.

4. RUN LM-4: Effect of the Presence of Various Metals on the Growth of C. botulinum in Non-Sterilized PLM

a. Organism

Same as that used in Run LM-1; 13,500 spores per ml; 1 ml of inoculum in each 204 x 214 can.

b. Procedure

Swift's PLM was reground, mixed with powdered metals, and then packed in 204 x 214 tin cans. These cans were not lined, only tinned. The PLM was not sterilized. Three cans out of 4 in each series were inoculated with 1 ml of the heat-shocked suspension, closed under vacuum, and then incubated at 30°C.

The concentration of powdered metals was 0.25 gm/100 gm PLM for Mg, Al, Mn, Zn, Fe, Co, and Ni; 0.5 gm of Sn were used per 100 gm of meat.

c. Data

See Table VI.

d. Results and Conclusions

(1) Irregular toxin production occurred; two mice died, one in the Mg and one in the Zn and Al sets. This too follows our published observation on spoilage of non-sterilized PLM containing Fe powder (Kempe and Graikoski, 1959). It suggests, however, that toxin formation may be fast enough in the presence of Zn and Al to be of importance.

(2) When spoilage occurred, gram-positive cocci were always found. These are large cocci; they may be diplococci or micrococci. No evidence of C. botulinum growth was found except for the minimal toxin production.

The order of spoilage is summarized in Table VII.

Table VII shows that gas develops first in the presence of Mg, that Fe causes early spoilage, and that Al, Mn, Zn and Sn incite spoilage. Of these, Al, Mn, and Zn are alike, whereas Sn acts more slowly. Co and Ni appear to be inactive. It is interesting to evaluate these results in the context of the electromotive series. Fe again appears out of place, as it does in LM-2 and LM-3, since its presence causes spoilage to occur before the more electrolytically active metals such as Zn and Mn. Sn was expected to be less active than Co and Ni, but this was not the case.

Even though the presence of Fe results in more rapid spoilage, however, it did not incite toxin formation at the time of spoilage as did Zn and Al.

TABLE VI

SPOILAGE AND TOXIN DEVELOPMENT IN PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-4)

| Metal | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Subculture in T.S* Broth or N.A.** Plates | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| No Metal | 1 | 18 | | | 0/4 | |
| | 2 | > 480 | | | | |
| | 3 | > 210 | | | | |
| | 4 | > 210 | | | | |
| Mg | 1 | 3 | Gm(+) cocci | Gm(+) cocci | 0/4 | |
| | 2 | 3 | Gm(+) cocci | Gm(+) cocci | 0/4 | |
| No Spores | 3 | 3 | Gm(+) cocci | Gm(+) cocci | 2/4 | |
| Al | 1 | 26 | Gm(+) cocci | Gm(+) cocci | 0/3 | |
| | 2 | 31 | | | - | |
| | 3 | 27 | Gm(+) cocci | Gm(+) cocci | 1/3 | |
| No Spores | 4 | 29 | | | | |
| Mn | 1 | 29 | | | | |
| | 2 | 23 | Gm(+) {cocci, bacilli | | 0/3 | |
| | 3 | 23 | Gm(+) cocci | | 0/3 | |
| No Spores | 4 | 29 | Gm(+) bacilli | Gm(+) cocci | | |
| Zn | 1 | 25 | Gm(+) cocci | | 1/3 | |
| | 2 | 25 | Gm(+) cocci | | 0/3 | |
| | 3 | 25 | Gm(+) cocci | | NC | NC = Not Checked |
| No Spores | 4 | 25 | Gm(+) cocci | | 1/3 | |
| Fe | 1 | 7 | Gm(+) cocci | Gm(+) cocci | 0/3 | |
| | 2 | 7 | Gm(+) cocci | Gm(+) cocci | 0/3 | |
| | 3 | 8 | NC | NC | NC | |
| No Spores | 4 | 8 | Gm(+) cocci | Gm(+) cocci | 0/3 | |
| Co | 1 | > 480 | | | | Still incubating, August 62 |
| | 2 | > 480 | | | | |
| | 3 | > 480 | | | | |
| No Spores | 4 | > 210 | | | | |
| Ni | 1 | > 480 | | | | |
| | 2 | > 480 | | | | |
| | 3 | > 210 | | | | |
| No Spores | 4 | > 480 | | | | |
| Sn | 1 | 35 | | | | |
| | 2 | 57 | | | | |
| | 3 | 43 | Gm(+) cocci | | 0/4 | |
| | 4 | 35 | Gm(+) cocci | | 0/4 | |

*T.S. = Trypticase - Soy Broth

**N.A. = Nutrient Agar

TABLE VII

SPOILAGE OF NON-STERILIZED PLM IN THE PRESENCE OF ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES (RUN LM-4)

| Metal | No. of Cans Spoiled | No. of Days Until Gas Developed | Toxicity |
|---|---|---|---|
| None | 1/4 | 18 | 0/4 |
| Mg | 3/3 | 3 | 0/8 |
| Fe | 4/4 | 7-8 | 0/6 |
| Al | 4/4 | 26-31 | 1/6 |
| Mn | 4/4 | 23-29 | 0/6 |
| Zn | 4/4 | 25 | 1/6 |
| Sn | 4/4 | 35-57 | 0/8 |
| Co | 0/4 | > 480 | |
| Ni | 0/4 | > 480 | |

5. RUN LM-5: Growth of C. botulinum 62A in Heat-Processed PLM Containing Various Metals

a. Organism

Same as that used in LM-4. The spore suspension contained 13,500 C. botulinum 62A spores per ml.

b. Procedure

PLM was ground, and aliquot portions were mixed with various metals and packed in 204 x 214 tin cans (unlined). The lids were secured with a rubber band, and each can was placed in a No. 2 can. Lids for the No. 2 cans were set loosely in place; these cans were placed in a steam-heated autoclave and steamed at atmospheric pressure for 15 minutes in order to replace the air in the cans with steam. The No. 2 cans were then withdrawn one at a time, quickly sealed, replaced in the autoclave, and cooked at 15 psig for 45 minutes. The cans were then removed and cooled to room temperature by standing overnight. After the No. 2 cans were opened, the 204 x 214 cans were removed and their covers aseptically lifted. One ml of the heat-shocked spore suspension was injected into the meat, and the 204 x 214 cans were again sealed. These cans of meat were then incubated at 30°C.

Metals used were added as follows: Fe, Al, Mg, and Zn were each used in the ratio of 0.25 gm, and Sn was used in the ratio of 0.5 gm per 100 gm of PLM.

The cans containing Zn and Sn were vacuum sealed; the others were sealed at atmospheric pressure. All of them really should have been vacuum packed since they were at room temperature when closed.

c. Data

See Table VIII.

TABLE VIII

SPOILAGE AND TOXIN DEVELOPMENT IN STERILIZED PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-5)

| Metal | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Subculture in T.S.* Broth or N.A.** Plates | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| Mg | 1 | 2 | Negative | Gm(+)bacillus | 0/4 | |
| | 2 | 2 | Gm(+)bacilli | Gm(+)bacillus | 1/4 | |
| | 3 | 2 | Gm(+)bacilli | Gm(+)bacillus | | |
| No Spores | 4 | 2 | Negative | Gm(+)bacillus | 0/4 | |
| Al | 1 | 18 | Gm(+)bacilli | | 0/3 | |
| | 2 | 22 | Gm(+)bacilli | Gm(+)bacilli | 0/3 | |
| | 3 | 25 | | | | |
| No Spores | 4 | 34 | | | | |
| Zn | 1 | 28 | Gm(+)bacilli | Gm(+)bacilli | 4/4 | Neutralization test with |
| | 2 | 28 | Gm(+)bacilli | Gm(+)bacilli | 4/4 | Type A antitoxin showed |
| | 3 | > 470 | | | | presence of Type A toxin |
| Fe | 1 | 11 | Gm(+)bacilli | Gm(+)bacilli | 1/3 | |
| | 2 | 10 | Gm(+)bacilli | Gm(+)bacilli | 0/3 | |
| | 3 | 11 | | | | |
| No Spores | 4 | 22 | Gm(+)bacilli | Gm(+)bacilli | 0/3 | |
| Sn | 1 | > 470 | | | | |
| | 2 | 42 | Gm(+)bacilli | Gm(+)bacilli | 3/4 | |
| | 3 | > 470 | | | | |
| No Metal | 1 | 29 | Negative | Gm(+)bacilli | 0/4 | |
| | 2 | 55 | | | | |
| | 3 | > 470 | | | | |
| No Spores | 4 | 55 | | | | |

*T.S. = Trypticase - Soy Broth
**N.A. = Nutrient Agar

Note: Spoilage of the non-inoculated meat shows that the autoclaving was not sufficient to sterilize the meat. A gram-positive, spore-forming bacterium from the Fe, non-inoculated, can was isolated.

d. Results and Conclusions

Unfortunately, the meat was not completely sterilized by the heat processing so firm conclusions cannot be made. It is evident from the limited toxin formation, however, that this run was comparable to Run LM-4. It could be of interest, therefore, to observe the order of spoilage and compare it with that found in Run LM-4. Table IX should be useful in this connection.

TABLE IX

SPOILAGE OF IMCOMPLETELY STERILIZED PLM IN THE PRESENCE OF ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES (RUN LM-5)

| Metal | No. of Cans Spoiled | No. of Days Until Gas Developed | Toxicity |
|---|---|---|---|
| None | 3/4 | 29-55 | 0/4 |
| Mg | 4/4 | 2 | 1/8 |
| Fe | 4/4 | 10-11 | 1/6 |
| Al | 4/4 | 18-25 | 0/6 |
| Zn | 2/3 | 28 | 8/8 |
| Sn | 1/3 | 42 | 3/4 |

These samples spoiled in the same sequence as did the non-sterilized cases of PLM in Run LM-4; all of the spoilage occurred somewhat later, however. Consequently these results do confirm the sequence of spoilage of non-sterilized PLM containing added metals as being Mg, Fe, Al, Zn and Sn, when incubated at 20°C.

6. RUN LM-6: Growth of C. botulinum 62A in Non-Sterilized PLM Containing Added Metal Powders

a. Organism

Suspension of C. botulinum 62A spores in distilled water containing 350,000 spores per ml.

b. Procedure

A new batch of PLM was obtained from Swift and Co. and prepared according to the formula described in Section III.

The meat was removed from the 6-lb tins in which it had been received, and was then ground, mixed with powdered metals, packed into 204 x 214 tin cans, inoculated with 1 ml of the spore suspension, closed under vacuum, and incubated at 30°C. The metals used were 0.25 gm of Zn, Fe, Al, Mg and Mn, and 0.50 gm of Sn per 100 gm of PLM.

c. Data

See Table X.

TABLE X

SPOILAGE AND TOXIN DEVELOPMENT IN PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES AND INOCULATED AT 30°C (RUN LM-6)

| Metal | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Subculture in T.S.*Broth or N.A.**Plates | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| No Metal | 1 | > 460 | | | | |
| | 2 | > 460 | | | | |
| | 3 | > 460 | | | | |
| | 4 | > 460 | | | | |
| Mg | 1 | 2 | | | | |
| | 2 | 2 | | | | |
| | 3 | 2 | | | | |
| | 4 | 2 | | | | |
| Mn | 1 | 21 | Gm(+)cocci | | 0/4 | |
| | 2 | 16 | Gm(+)cocci | | 0/4 | |
| | 3 | Lost | | | | |
| | 4 | 27 | | | | |
| Fe | 1 | 9 | | | | |
| | 2 | 9 | Gm(+)cocci | Gm(+)cocci | 0/4 | |
| | 3 | 8 | Gm(+)cocci | Gm(+)cocci | 0/4 | |
| | 4 | 8 | Gm(+)cocci | Gm(+)cocci | 0/4 | |
| Al | 1 | 27 | | | | |
| | 2 | 39 | | | | |
| | 3 | 41 | Gm(+) {cocci, bacilli | Gm(+)cocci | 0/4 | |
| | 4 | 39 | | | | |
| Sn | 1 | > 460 | | | | |
| | 2 | > 460 | | | | |
| | 3 | > 460 | | | | |
| | 4 | > 460 | | | | |
| Zn | 1 | 17 | Gm(+)cocci | Gm(+)cocci | | |
| | 2 | 36 | | | | |
| | 3 | 47 | | | | |
| | 4 | 20 | Gm(+)cocci | Gm(+)cocci | 0/4 | |

*T.S. = Trypticase-Soy Broth
**N.A. = Nutrient Agar

d. Results and Conclusions

These data for non-sterilized PLM confirm previous results by indicating that different added metal powders cause different rates of spoilage, and that no toxin or very little toxin forms by the time the cans have swollen. These results are summarized in Table XI.

TABLE XI

SPOILAGE OF PLM IN THE PRESENCE OF ADDED METAL POWDERS AND *C. BOTULINUM* 62A SPORES (RUN LM-6)

| Metal | No. of Cans Spoiled | No. of Days Until Gas Developed | Toxicity |
|---|---|---|---|
| None | 0/4 | > 460 | |
| Mg | 4/4 | 2 | |
| Fe | 4/4 | 8-9 | 0/12 |
| Mn | 2/4 | 16-27 | 0/8 |
| Al | 4/4 | 27-41 | 0/4 |
| Zn | 4/4 | 17-47 | 0/4 |
| Sn | 0/4 | > 460 | |

In Table XI the effect of added metal powders on the rate of spoilage is again shown in sequence. It should be noted that Mn and Al have shifted positions.

One question must be asked: Are these incidents of spoilage (gas production) caused by chemical or bacteriological gas formation? In answer:

(1) Microscopic examination of stained slides showed cultures of Gm(+)cocci and bacilli;

(2) Toxin was occasionally found, indicating that the *C. botulinum* spores had just begun to develop cultures;

(3) In sterilized runs, much toxin along with putrid meat developed in about the same sequence of metal activity as measured by position in the electromotive series (see Runs LM-2, LM-7, LM-9, and LM-10); and

(4) The gas development usually became evident within 24 to 48 hours—i.e., a can would be under vacuum and then within a period of 48 hours or less, develop a hard swell. This did not always occur but did so especially often for cans spoiling within 3 weeks.

There is some doubt that the spoilage with Mg present was always caused by bacterial action, since Mg would develop $H_2$ in the juice of PLM.

## 7. RUN LM-7: Growth of C. botulinum 62A in Heat-Processed PLM.

### a. Organisms

(1) C. botulinum 62A spores, in distilled water, containing 350,000 spores per ml.

(2) Cocci previously isolated from PLM; 1 ml of a 36-hr culture in trypticase-soy broth was used.

### b. Procedure

PLM was pre-ground. One set of 204 x 214 cans was packed with PLM containing 0.25 gm Al powder per 100 gm meat, a second set with 0.25 gm of Fe powder per 100 gm meat, and a third set with no metal. The cans of meat were covered with lids held in place with rubber bands, and were then sealed in No. 2 tin cans, after the cans had been exhausted with atmospheric steam. The cans were autoclaved at 16 psig (119°C) for 1 hr and 35 min and allowed to cool overnight. The No. 2 cans were then opened and the 204 x 214 cans removed; the covers of the latter were aseptically lifted and the meat inoculated with 1 ml of the spore suspension and also with 1 ml of the cocci culture. One set of cans was left uninoculated as a control. The small cans were then closed in at atmospheric pressure and incubated at 30°C.

The cans were not vacuum sealed because of spillage problems. If they had been placed in a refrigerator then they could have been more easily vacuum sealed because the juices, particularly fat, would not have been so fluid.

### c. Data

See Table XII.

### d. Results and Conclusions

The data are summarized in Table XIII.

(1) No Metals: As expected, meat in the non-inoculated cans was stable; with a spore inoculum, the meat spoiled and developed toxin in 3 - 4 weeks; and when cocci were present, spoilage occurred in one week without toxin

TABLE XII

SPOILAGE AND TOXIN DEVELOPMENT IN STERILIZED PLM CONTAINING ADDED METAL POWDERS AND C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-7)

| Treatment | Can No. | No. of Days to Spoilage | Microscopic Examination, Can Contents | Subculture in T.S.* Broth or N.A.** Spores | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| No metal, no inoculum | 1 | > 455 | | | | |
| | 2 | > 180 | | | | |
| | 3 | > 180 | | | | |
| No metal or cocci, but spores | 1 | 30 | Gm(+)bacilli | Gm(+)bacilli | 4/4 | With A. T. 0/3 |
| | 2 | 20 | | | | |
| | 3 | 21 | | | | |
| No metal or spores, but cocci | 1 | 7 | | | | |
| | 2 | 97 | | | | |
| | 3 | > 97 | | | | |
| Al but no inoculum | 1 | 70 | | | | |
| | 2 | > 460 | | | | |
| | 3 | > 460 | | | | |
| Al + spores | 1 | 25 | | | | |
| | 2 | 20 | Gm(+)bacilli | Gm(+)bacilli | 4/4 | |
| | 3 | 16 | | | | |
| Al + cocci | 1 | 30 | | | | |
| | 2 | 20 | | | | |
| | 3 | 8 | Few Gm(+)bacilli | Gm(+)bacilli | | |
| Al + cocci + spores | 1 | 7 | Gm(+)bacilli | cocci + rods | 0/4 | |
| | 2 | 7 | Gm(+)bacilli | Gm(+)bacilli | 0/3 | |
| | 3 | 7 | Gm(+)bacilli | Gm(+)bacilli | 0/3 | |
| Fe + cocci + spores | 1 | 4 | Negative | Gm(+)bacilli | 0/4 | |
| | 2 | 4 | Gm(+)bacilli | Gm(+)bacilli | 0/4 | |
| Fe + cocci | | 4 | Few Gm(+)bacilli | Gm(+)bacilli | | |
| | | 4 | | | | |
| | | 4 | | | | |
| Fe but no inoculum | | 8 | Gm(+)bacilli | Gm(+)bacilli | 0/4 | |
| | | 9 | | | | |
| | | 10 | | | | |
| Fe + spores | 1 | 7 | Gm(+)bacilli | Gm(+)bacilli | 0/4 | |
| | 2 | 7 | Gm(+)bacilli | Gm(+)bacilli | 0/4 | |
| | 3 | Lost | | | | |
| Cocci+spores | 1 | 8 | Gm(+)bacilli | Gm(+){cocci / bacilli | 0/4 | |
| but no metal | 2 | 10 | Gm(+)bacilli | Gm(+)bacilli | 0/4 | |
| | 3 | 11 | | | | |

*T.S. = Trypticase-Soy Broth
**N.A. = Nutrient Agar

TABLE XIII

COMPARISON OF Al AND Fe POWDERS AS STIMULANTS FOR SPOILAGE OF STERILIZED CANNED PLM (RUN LM-7)

| Treatment | No. of Cans Spoiled | No. of Days Until Gas Developed | Toxicity |
|---|---|---|---|
| No Metals | | | |
| Not inoculated | 0/3 | > 455 | |
| Spore inoculum | 3/3 | 20-30 | 4/4 |
| Cocci inoculum | 1/3 | 7-97 | |
| Spore + cocci inoculum | 3/3 | 8-11 | 0/8 |
| Aluminum Powder | | | |
| Not inoculated | 0/3 | 70 | |
| Spore inoculum | 3/3 | 16-25 | 4/4 |
| Cocci inoculum | 3/3 | 8-30 | |
| Spore + cocci inoculum | 1/3 | 7 | |
| Iron Powder | | | |
| Not inoculated | 3/3 | 8-10 | 0/4 |
| Spore inoculum | 2/2 | 7 | 0/8 |
| Cocci inoculum | 3/3 | 4 | |
| Spore + cocci inoculum | 2/2 | 4 | 0/8 |

formation. These data explain beautifully why sub-sterile PLM in cans (perhaps bacon, etc.) have not been involved in C. botulinum outbreaks.

(2) Aluminum Powder: The above comments apply here also, with one addition. Aluminum accelerated spoilage and toxin production when only C. botulinum spores were present; some acceleration of cocci growth also appears to have occurred.

(3) Iron Powder: Iron accelerated spoilage in every case. The lack of toxin cannot be explained except on the basis of presence of gas forming bacteria other than C. botulinum in the group inoculated with spores only. This could have resulted from incomplete sterilization or contamination during handling.

8. RUN LM-8: Growth of C. botulinum 62A in Sterilized PLM Together With a Bacillus Isolated from Spoiled PLM

a. Organisms

(1) One ml of a suspension of C. botulinum 62A spores, heat shocked and containing approximately $10^7$ spores per ml, were used per can.

(2) One ml of a 24-hr culture in trypticase-medium of a bacillus previously isolated from spoiled PLM.

b. Procedure

The preparation of the meat, and sterilization procedures were those described in Run LM-7; however, the final steps were facilitated by placing the No. 2 cans in a refrigerator overnight to solidify the contents of the 204 x 214 can. This prevented spillage during removal of the small cans from the No. 2 can and permitted the small cans to be closed in the vacuum-closing machine.

In the Fe series, 0.25 gm of iron powder were mixed with 100 gm of meat.

c. Data

See Table XIV.

d. Results and Conclusions

Apparently this PLM contained a very heat-resistant bacillus. In this run, the theoretically sterile PLM, containing added Fe, developed spoilage in 2 out of 3 cans, whereas the cans containing no Fe were stable, as expected. The "sterilized" PLM was also stable when bacilli were injected; this too was expected.

It was somewhat surprising that the sterile PLM inoculated with C. botulinum spores but not with Fe, spoiled and developed toxin in 4-5 weeks. It has been reported that nitrite is reduced to very low values in PLM in about this interval at 25°C (Hougham and Watts, 1958), also, in this case, a very heavy inoculum ($10^7$) of spores was used per can.

The important objective of this experiment was fulfilled, since it was demonstrated that the bacillus previously isolated from spoiled PLM produced rapid (4-day) spoilage of sterile PLM when added iron was present but did not when it was absent.

Other results worth noting are as follows: Powdered iron hastened the spoilage and toxin development by C. botulinum spores; Fe + spores + bacillus produced

some toxin in PLM in the 4 days required for spoilage; and in the absence of Fe, the spoilage occurring in 4-12 days did not produce measurable toxin.

TABLE XIV

SPOILAGE AND TOXIN DEVELOPMENT IN STERILIZED PLM CONTAINING ADDED Fe POWDER, C. BOTULINUM 62A SPORES, AND A BACILLUS, AND INCUBATED AT 30°C (RUN LM-8)

| Treatment | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Subculture in T.S.* Broth or N.A.** Plates | Toxicity | Remarks |
|---|---|---|---|---|---|---|
| No metal, no inoculum | 1 | > 400 | | | | |
| | 2 | > 400 | | | | |
| | 3 | > 400 | | | | |
| No metal, but spore inoculum | 1 | 21 | | | 4/4 | |
| | 2 | 31 | | | | |
| | 3 | 35 | | | | |
| No metal, but bacilli | 1 | > 400 | | | | |
| | 2 | > 101 | | | | |
| | 3 | > 101 | | | | |
| No metal, but bacilli+spores | 1 | 4 | Gm(±)bacilli | Gm(-)bacilli | 0/3 | |
| | 2 | 10 | Gm(±)bacilli | Gm(-)bacilli | 0/3 | |
| | 3 | 12 | | | | |
| Fe, non-inoculated | 1 | 10 | Gm(±)bacillus | Gm(-)bacilli | 0/3 | |
| | 2 | 10 | Gm(±)bacillus | Gm(-)bacilli | 0/3 | |
| | 3 | Lost | | | | |
| Fe + spores | 1 | 9 | Gm(±)bacilli | Gm(-)bacilli | 3/3 | |
| | 2 | 9 | | | | |
| | 3 | 9 | | | | |
| Fe + bacillus | 1 | 4 | Gm(+)bacilli | Gm(±)bacilli | | |
| | 2 | 4 | | | | |
| | 3 | 4 | | | | |
| Fe + spores + bacillus | 1 | 4 | Gm(+)cocci Gm(-)rods | Gm(-)bacilli | 1/3 | |

*T.S. = Trypticase-Soy Broth
**N.A. = Nutrient Agar

9. RUN LM-9: Growth and Toxin Development by C. botulinum 62A in Heat-Processed PLM Containing Added Metal Powders

a. Organism

C. botulinum 62A spore suspension containing approximately $10^6$ spores per ml in distilled water.

b. Procedure

The same as that used for Run LM-8 except that heat processing was carried out twice, first for 1/2 hr at 121°C, and again (the next day) for 1 hour at 121°C. Then the cans were refrigerated, inoculated, and vacuum packed.

Co, Zn, Fe, and Al powders were all added in a concentration of 0.25 gm per 100 gm of meat.

c. Data

See Table XV.

TABLE XV

SPOILAGE AND TOXIN DEVELOPMENT IN HEAT-PROCESSED PLM CONTAINING ADDED METAL POWDERS AND INOCULATED WITH C. BOTULINUM 62A SPORES (RUN LM-9)

| Treatment | Can No. | No. of Days Until Spoilage | Microscopic Examination, Can Contents | Toxicity |
|---|---|---|---|---|
| No metal | 1 | > 90 | | |
| | 2 | > 90 | | |
| | 3 | > 90 | | |
| No spores | 4 | > 90 | | |
| Al | 1 | 31 | | |
| | 2 | 35 | Gm(+)bacilli | 3/3 |
| No spores | 3 | > 90 | | |
| Zn | 1 | 17 | Gm(+)bacilli | 3/3 |
| | 2 | 17 | Gm(+)bacilli | |
| | 3 | 17 | Gm(+)bacilli | |
| No spores | 4 | > 90 | | |
| Fe | 1 | 18 | Gm(+)bacilli | 3/3 |
| | 2 | 18 | | |
| | 3 | 18 | | |
| No spores | 4 | > 90 | | |
| Co | 1 | > 90 | | |
| | 2 | > 90 | | |
| | 3 | > 90 | | |
| No spores | 4 | > 90 | | |

d. Results and Conclusions

A chronological sequence for PLM spoilage in the presence of various metals was again shown. In this instance the order of spoilage was Zn, Fe, and Al. Co showed no effect within the 390-day period of the experiment.

## 10. RUN LM-10: Growth of C. botulinum 62A in Sterilized PLM Inoculated With Spores of This Organism

a. Organism

C. botulinum 62A spore suspension containing approximately $10^6$ spores per ml in distilled water.

b. Procedure

Same as that used for Run LM-8 except that the meat was heat-processed for 1 1/2 hours at 121°C. Added metals were used in concentrations of 0.25 gm per 100 gm of meat.

c. Data

See Table XVI.

TABLE XVI

SPOILAGE OF HEAT-PROCESSED PLM CONTAINING ADDED METALS INOCULATED WITH C. BOTULINUM 62A SPORES AND INCUBATED AT 30°C (RUN LM-10)

| Treatment | Can No. | No. of Days Until Spoilage |
|---|---|---|
| Ni | 1 | 23-48 |
| | 2 | 23-48 |
| | 3 | 23-48 |
| Sn | 1 | 23-48 |
| | 2 | 23-48 |
| | 3 | 23-48 |
| Mg | 1 | 20 |
| | 2 | 20 |
| | 3 | 23 |

d. Results and Conclusions

Spoilage of sterilized PLM occurred faster in the presence of Mg than in the presence of Ni or Sn, although spoilage occurred in all cases.

## V. DISCUSSION

Data for the repacked, non-heat-processed PLM are summarized in Table XVII.

TABLE XVII

DAYS OF INCUBATION AT 30°C REQUIRED FOR SPOILAGE OF PLM CONTAINING ADDED METALS AND C. BOTULINUM 62A SPORES

| Metal | Run No. LM | | | | Average | Group No. | Metals Included in Groups |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 6 | | | |
| Mg | | 2-3 | 3 | 2 | 2.5 | I | Mg |
| Fe | 4-5 | 7 | 7-8 | 8-9 | 8 | II | Fe |
| Mn | | 5-6 | 23-29 | 16-27 | 5.5; 22 | III | Mn,Zn,Al |
| Al | | 19-20 | 26-31 | 27-41 | 27 | | |
| Zn | 7-10 | | 25 | | 25 | | |
| Co | | 147-177 | > 480 | | 162 | IV | Sn, Co |
| Sn | | 195 | 35-57 | > 460 | 120 | | |
| Ni | | > 575 | > 480 * | | > 480 | V | Ni, No Metal |
| No Metal | 190-232 | 135-147 | > 480 | > 460 | 140-210 | | |

*1 can produced spoilage in 18 days

Spoilage of the meat as stimulated by metals, generally followed the relative position of the metals in the Electromotive Series. Similar results were found for heat-processed PLM, as shown in Table XVIII. The metals used appear in the Electromotive Series, showing the oxidizing systems positive, as follows:

$Sn^{+2}$, $Sn^{\circ}$ -0.14

$Ni^{+2}$, $Ni^{\circ}$ -0.24

$Co^{+2}$, $Co^{\circ}$ -0.28

$Fe^{+2}$, $Fe^{\circ}$ -0.44

TABLE XVIII

DAYS OF INCUBATION AT 30°C REQUIRED FOR SPOILAGE OF HEAT-PROCESSED PLM CONTAINING ADDED METALS AND C. BOTULINUM 62A SPORES

| Metal | Run No. LM | | | | | | | Average | Group No. | Metals Included in Groups |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 5 | 7 | 8 | 9 | 10* | | | |
| Mg | | | 2 | | | | 20-23 | 2 | I | Mg |
| Fe | 6 | 9-17 | 10-11 | | 9 | 18 | | 10 | II | Fe |
| Al | | | 18-25 | 16-25 | | 31-35 | | 25 | III | Zn,Al,Ni |
| Zn | | 11-17 | 28 | | | 17 | | 20 | | |
| Ni | | | | | | | 23-48 | | | |
| Sn | | | 42 | | | | 23-48 | 42 | IV | Sn |
| Co | | | | | | > 90 | | > 90 | V | Co, No Metal |
| No Metal | 12-14 | 39-180 | 29 | 20-30 | 21-35 | > 90 | | | | |

*Run 10 was not included in average

| | |
|---|---|
| $Zn^{+2}$, $Zn^{\circ}$ | -0.76 |
| $Mn^{+2}$, $Mn^{\circ}$ | -1.10 |
| $Al^{+3}$, $Al^{\circ}$ | -1.70 |
| $Mg^{+2}$, $Mg^{\circ}$ | -2.40 |

These metals were arbitrarily separated into 5 groups, on the basis of the rate at which they stimulated the spoilage of PLM.

Mg caused the most rapid spoilage, hard swells developing in every case within 3 days. Mg is so reactive chemically that visible gas could be detected when Mg powder was mixed with PLM and water in a test tube. Even so, this chemical reaction does not appear to be the only mechanism by which gas formed when PLM and Mg powder were mixed. Apparently the preservative salts in the PLM were partially neutralized since cultures developed during the 3-day spoilage period, when bacteria were present.

Next to Mg, Fe was found to be the most reactive metal tested. Actually its reactivity is greater than would be expected solely upon the basis of its position in the Electromotive Series. Metallic Fe has long been recoginized as a good stimulatory substance in bacteriological culture media for anaerobes. It may have additional stimulation effects for bacteria beyond reduction of the $E_h$. This is rather important since steel is commonly used for can bodies, and when corrosion occurs Fe is exposed to the food. In our experiments Fe unquestionably stimulated bacterial growth. For example, in Run LM-8, spoilage with toxin production occurred in heat-processed PLM within 9 days when Fe was present, whereas 21-35 days were required when it was absent. Similarly, the data in Run LM-6 show that for non-heat-processed PLM, spoilage occurred in 8-9 days with development of cocci cultures when Fe was present; when no metals were used the PLM did not spoil within 480 days.

Mn, Zn, and Al stimulated spoilage of PLM within about three weeks of incubation at 30°C. Zn and Mn are close together and appear after Al in the Electromotive Series. These metals should therefore be somewhat less reactive than Al, but the latter's oxide coating is known to reduce its electrochemical activity. Thus it is not surprising that all three metals had similar effects on the rate of spoilage of PLM. These metals also stimulated bacterial growth, as shown by the presence of cultures of gram-positive cocci and bacilli in the spoiled meats.

Sn demonstrated some activity by increasing the rate of PLM spoilage in both the heat-processed and non-heat-processed samples. Co had little or no effect and Ni appears to have had a preservative action. This latter effect was not verified by special studies but could be interesting since Ni salts are not usually considered to be toxic.

The nature of the action of reducing metals in stimulating the spoilage of PLM is not clearly understood. When Jensen (1954, p. 33-5) discusses the way in which $NO_3^-$ and $NO_2^-$ act to preserve meats, he points out that aerobic organisms can develop in nearly anaerobic conditions when $KNO_3$ and other oxidizable substances are present: and that anaerobes can grow under aerobic conditions if enough reducing substances are present. Halvorson (Jensen, 1954, p. 33-4) points out that $KNO_3$ and $KNO_2$ are beneficial in controlling food spoilage because they poise the redox potential at a high value, thus controlling the growth of many bacteria and of anaerobes in particular. Jensen (1954, p. 35) quotes a number of workers as suggesting that hydroxylamine could be involved in the preservative action of $NO_3^-$. It could inactivate catalase that is produced by most bacteria and thereby permit the accumulation of $H_2O_2$, which can inhibit and kill anaerobes. Quastel and Stephenson (Jensen, 1954, p. 35) suggest that the inhibitory action of $H_2O_2$ may be due to its effect on the redox potential rather than to a direct toxic effect; Stephenson (Jensen, 1954, p. 35) further indicates that $NO_3^-$ actually introduces $O_2$ into the substrate.

In another kind of experiment Hess (Jensen, 1954, p. 37) tested canned spiced ham inoculated with C. sporogenes and prepared with different cures. In the absence of $NO_3^-$, the meat was stable for the usual 30-day period with no gas formation; with $NO_3^-$ in the cure, some cans swelled. This demonstrates the gas-inducing properites of $NO_3^-$.

In another experiment, Hess was reported (Jensen, 1954, p. 39) to have found that after 32 cans of spiced ham prepared with $NO_3^-$, $NO_2^-$, salt, spices, and an inoculum of C. sporogenes had been incubated at 99°F for 30 days, 20 cans were sound, five had swollen due to bacillus, and 7 were putrid. The 7 cans were presumably putrid because of the growth of C. sporogenes.

Jensen (1954, p. 39) points out, as did Kempe and Graikoski later (1959), that such swelling should serve to warn the consumer. Jensen's conclusion was based upon work with C. sporogenes; Kempe and Graikoski worked with C. botulinum. Let us briefly examine our results in the light of these views.

It is probable that metals like Mg, Fe, Zn, etc., react with chemicals in the pickle to produce $E_h$ values similar to those quoted in the Electromotive Series. Thus, in a zone surrounding the metal particles, the $E_h$ may be locally quite low. This would allow bacterial growth to begin; once initiated, cultures often produce low $E_h$ potentials by virtue of their proliferation. Then the culture could quickly permeate the whole can of meat. This hypothesis leads to two questions: (1) Why do metals developing different $E_h$ values react at different rates in developing bacterial cultures; and (2) Do different metals produce different cultures from the galaxy of bacteria that are present as indigenous flora along with the C. botulinum inoculum?

Let us consider these questions together, since both the chemical reactions and microbial growth occur in the same place at the same time. Possibly both the chemical reactivity of the metals and the actual $E_h$ values produced are in-

volved. A highly reactive metal such as Zn or Fe would tend to quickly reduce the $E_h$ in its vicinity, while diffusion of oxidized substances to this site would tend to maintain the high $E_h$ value prevailing in the main portion of the PLM. When enough of the oxidizing substances has been reduced, a zone of sufficiently low $E_h$ would develop to allow initiation of growth of that bacterium present which has the highest $E_h$ tolerance. Development of this culture should further reduce the $E_h$ and stimulate the growth of organisms which have lower tolerances to oxidizing systems, with the eventual development of anaerobes. This process is well recognized in the bacteriological laboratory, where E. coli cultures, etc., can be used to initiate growth of anaerobes in a mixed inoculum. A delay in development of the anaerobe, until the facultative organism is established, is inherent in the process.

It must also be remembered that each culture starts from resting cells. In the case of anaerobic spore formers, the spores need to germinate before growth can occur. With laboratory cultures, this type of commensal activity between E. coli and C. sporogenes requires 3 to 5 days at 37°C: somewhat longer times would be involved at 30°C. It should be noted that $NO_3^-$ and particularly $NO_2^-$ gradually disappear from PLM during normal storage (Hougham and Watts, 1958). From these considerations, the following results would be anticipated for non-heat-processed PLM:

(1) Spoilage should occur in shorter and shorter times when metals are used that have greater reactivity as indicated by lower $E_h$ positions in the Electromotive Series.

Comment: This occurred in our experiments.

(2) Development of mixed cultures during the spoilage should occur.

Comment: Gram-positive cocci and gram-positive bacilli usually developed together, but the cocci predominated.

(3) Since $NO_2^-$ gradually disappears from meat during storage (Hougham and Watts, 1958) and $NO_2^-$ is an important preservative, some of the non-heat-processed PLM containing no added metals should spoil during incubation, due to bacterial growth.

Comment: This occurred in our experiments.

(4) Cultures of anaerobes might occasionally be found in cans of spoiled PLM that have not been heat-processed, when the $E_h$ drops quickly to levels that allow the growth of these cultures. Contrarywise, if the $E_h$ drops slowly, gas should be produced by the facultative bacteria that initiate growth at moderately low $E_h$ values; the cans would then swell and be removed from incubation before anaerobes developed. Since C. botulinum spores were injected, it should be possible to discover even limited anaerobic growth by toxin assay.

Comment: In Run LM-2, spoilage with predominance of a culture of gram-positive cocci occurred along with the formation of small amounts of botulinus toxin in non-heat-processed PLM when Fe and then again when Zn were mixed into the meat. In this same run, when the PLM was heat-processed and either Zn or Fe had been used, all of the spoiled meat showed large amounts of botulinus toxin. Additionally, in Runs LM-3, LM-4, and LM-6, toxin was not found in spoiled cans of non-heat-processed PLM containing Fe or other electrochemically active metals. None of the non-heat-processed, spoiled, control cans in any run showed botulinus toxin in the absence of active metals.

This indicates that the formation of toxin by C. botulinum 62A in PLM occurs later than the growth of the gram-positive cocci and other such bacteria. Hence the protection offered potential consumers of spoiled PLM is principally based upon the relative rates of growth of the cultures as motivated by some condition such as that postulated above. In the presence of pronounced electrolytic activity, this protection does not appear to be sufficient.

# PART B

# MICROBIOLOGY

# I. SUMMARY

The strain of Clostridium botulinum 62A that was used in our experiments was tested for its sensitivity to the preservative chemicals used in Pork Luncheon Meat (PLM). For these tests, the preservative chemicals were added to trypticase broth and the growth of C. botulinum was evaluated in these media. Our results generally confirmed those previously reported by Jensen (1954). In these media, vegetative inocula developed cultures more quickly than spores inocula but the resulting cultures of C. botulinum 62A were inhibited by essentially the same concentration of the preservative chemicals, regardless of whether vegetative or spore inocula were used.

In addition it was found that when all of the preservative chemicals used in PLM were combined and added to trypticase broth in the concentrations used in PLM, the growth of C. botulinum was effectively inhibited. This inhibitory effect, however, was somewhat reduced by metallic Mn and even more effectively counteracted by metallic Fe. The latter also stimulated gas production by this bacterium.

When the metals Fe, Co, Ni, Al, Mn, Mg, Zn and Sn were present in the trypticase broth, they did not inhibit the growth of C. botulinum 62A. In trypticase broth containing added nitrite, the inhibitory effect of this chemical for C. botulinum was reduced by the presence of Zn.

A bacterium isolated from spoiled PLM was tentatively identified as Escherichia intermedium. It is a good gas producer in this meat, a property that could be useful in distending cans and thus warning of spoilage. The coccus, which was usually present in PLM, was tentatively identified as Staphylococcus albus although it would also have been Pediococcus cerevisiae.

PLM was packed in tin cans, irradiated with gamma rays from cobalt 60, inoculated with C. botulinum 62A spores, and incubated at 30°C to confirm some tentative data previously obtained. It was found that increasing amounts of irradiation caused spoilage and toxin production to occur more quickly in PLM; the unirradiated controls did not spoil. It can be concluded, therefore, that irradiation Pasteurization of PLM is not only useless but dangerous. The preservative quality of the curing chemicals is impaired by irradiation and the saprophytic, vegetative bacteria are killed. When these latter bacteria are present, they can act as warning agents since they are usually able to form gas and swell the cans before C. botulinum can grow and develop toxin (Kempe and Graikoski, 1959).

## II. INTRODUCTION

The PLM used in this study was preserved with nitrate, nitrite, salt, spices, and ascorbic acid. It was desirable, therefore, to investigate effects of these substances on the ability of the spores of our strain of Clostridium botulinum 62A to develop cultures in the PLM. It was also of interest to evaluate the effects of the powdered metals in the same way. Aside from the experiments developed for these purposes, similar experiments were conducted with vegetative bacteria isolated from cultures occurring in cans of spoiled PLM. These experiments will be described individually in Part B, Section IV of this report.

## III. EFFECT OF CHEMICAL CURING INGREDIENTS IN PLM ON THE GROWTH OF BACTERIA

### 1. EXPERIMENT 1: Combined Effects of Metals and the Chemical Curing Ingredients in PLM on the Growth of Bacteria

#### a. Object

To test the growth of C. botulinum, in the presence of other bacteria, when inoculated into a culture medium containing the chemicals used in curing PLM.

#### b. Organism

C. botulinum 62A spores grown in liver extract medium, a coccus isolated from PLM, and a bacillus isolated from PLM. For identification see Experiment 10.

#### c. Medium

| | gms |
|---|---|
| Distilled water (substituted for pork) | 453.59 |
| NaCl | 15.88 |
| Dextros | 14.27 |
| Corn Syrup (Karo) | 9.07 |
| Ascorbic Acid | 0.094 |
| $NaNO_3$ | 0.071 |
| $NaNO_2$ | 0.0454 |
| (Spices were omitted) | |

This medium was dispensed into 20-ml screw-cap tubes and a plug of PLM added. Some of the tubes also received a little Fe or Zn powder. The tubes were then autoclaved for 15 min at 121°C and inoculated.

Each tube was inoculated with 1 ml of a spore suspension containing $4.3 \times 10^6$ C. botulinum 62A spores per ml. Additionally, 1 ml of a 36-hr culture of the coccus or bacillus grown in trypticase broth were used as indicated.

#### d. Data

See Table XIX.

TABLE XIX

COMBINED EFFECTS OF METALS AND CHEMICAL CURING INGREDIENTS IN PLM ON THE GROWTH OF BACTERIA (EXPERIMENT 1)

| Tube No. | Contents | Growth 20 hr | Gas 40 hr | Growth 3 days | Gas 3 days |
|---|---|---|---|---|---|
| 1 | Fe + spores | 0 | B* | 3+ | 4+ |
| 2 | Fe + coccus | 2+ | B | 3+ | 1+ |
| 3 | Fe + bacterium | 4+ | 4+ | 4+ | 3+ |
| 4 | Fe + spores + bacterium | 4+ | 4+ | 4+ | 3+ |
| 5 | Fe + spores + coccus | 2+ | B* | 4+ | 3+ |
| 1 | Zn + spores | 0 | B* | 0 | 0 |
| 2 | Zn + coccus | 2+ | B* | 1+ | 0 |
| 3 | Zn + bacterium | 4+ | 3+ | 4+ | 4+ |
| 4 | Zn + spores + bacterium | 4+ | 5+ | 4+ | 3+ |
| 5 | Zn + spores + coccus | 4+ | B* | 1+ | 0 |
| 1 | Spores | 0 | 0 | 0 | 0 |
| 2 | Spores + bacterium | 4+ | 4+ | 4+ | 4+ |
| 3 | Spores + coccus | 3+ | 0 | 2+ | 0 |
| 4 | Coccus | 2+ | 0 | 2+ | 0 |
| 5 | Bacterium | 4+ | 4+ | 4+ | 4+ |

*B indicates bubbles from chemical action.

e. Results and Conclusions

(1) Gas was produced by C. botulinum 62A spores in this medium when Fe was present but not when Zn or no added metal was used.

(2) The bacterium, probably Escherichia intermedium, produced large amounts of gas within 40 hours under all conditions.

(3) The coccus, probably Staphylococcus albus, required Fe to produce gas although it would grow without any metal present and also with Zn present.

(4) The chemical preservatives in PLM, when added to casitone broth, prevented growth of C. botulinum in the broth.

(5) Fe stimulates the development of C. botulinum 62A cultures from spores in this medium; it is required for gas production when only C. botulinum is present.

(6) The bacterium, isolated from PLM, is a good gas producer; this could be useful for warning of spoilage of PLM since the gas causes the containers to swell.

## 2. EXPERIMENT 2: Comparison of the Effect of Nitrite on the Growth of Bacteria Isolated from PLM with Its Effect on C. Botulinum 62A Spores

### a. Object

Examine the effects of various concentrations of $NaNO_2$ in 4% trypticase media on C. botulinum 62A spores, and on two bacteria isolated from PLM.

### b. Organism

C. botulinum 62A spores, grown in liver extract medium; a gram-positive coccus (probably Staphylococcus albus), and a gram-negative bacterium (Escherichia intermedium), grown in 4% trypticase broth

### c. Medium

4% trypticase broth containing $NaNO_2$ was used. The broth was dispensed in 15-ml portions into 15-cm x 15-mm screw-cap culture tubes, autoclaved, and cooled. The proper amount of a $NaNO_2$ solution was then added and the media inoculated with the bacteria; 0.1 ml of inoculum was used. Five concentrations of $NaNO_2$ were used as indicated. Incubation temperature was 35°C.

### d. Data

See Table XX.

### e. Results and Conclusions

(1) C. botulinum 62A is known to be inhibited by low concentration of $NO_2^-$, 0.02% being quoted by Jensen (1954, p. 44) as a limiting value. Data in Table XX indicate that 0.01% $NaNO_2$ is inhibitory for C. botulinum in 4% trypticase.

(2) Jensen (1954, p. 44) reports that 0.02% $NaNO_2$ inhibits S. aureus; our data in Table XX indicate that S. albus was inhibited by 0.001% in this 4% trypticase medium.

TABLE XX

EFFECT OF NITRITE ON GROWTH OF BACTERIA IN 4% TRYPTICASE BROTH (EXPERIMENT 2)

| $NaNO_2$ (%) | Tube No. | *C. botulinum* | | | | *S. albus* | | | | *E. intermedium* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 18 | 36 | 120 | 168 | 18 | 36 | 120 | 168 | 18 | 36 | 120 | 168 |
| | | hour | | | | hour | | | | hour | | | |
| 0 | 1 | 3+ | 4+ | 4+ | 4+ | 2+ | 3+ | 4+ | 4+ | 2+ | 4+ | 4+ | 4+ |
| 0 | 2 | 3+ | 4+ | 4+ | 4+ | 2+ | 3+ | 4+ | 4+ | 2+ | 4+ | 4+ | 4+ |
| 1 | 1 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 2+ | 4+ | 4+ | 4+ |
| 1 | 2 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 2+ | 4+ | 4+ | 4+ |
| 0.1 | 1 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.1 | 2 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.01 | 1 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.01 | 2 | 0 | 0 | 0 | 0 | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.001 | 1 | 0 | 0 | 0 | 1+ | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.001 | 2 | 0 | 0 | 0 | 1+ | ? | ? | 0 | 0 | 3+ | 4+ | 4+ | 4+ |
| 0.0001 | 1 | 1+ | 4+ | 4+ | 4+ | ? | 3+ | 4+ | 4+ | 3+ | 4+ | 4+ | 4+ |
| 0.0001 | 2 | 1+ | 4+ | 4+ | 4+ | ? | 3+ | 4+ | 4+ | 3+ | 4+ | 4+ | 4+ |

(3) In Table XX this growth of *Escherichia intermedium* was shown to be more resistant to inhibition by $NaNO_2$ than either *C. botulinum* or *S. albus*. It was only slightly, if at all, affected by 1% $NaNO_2$, the maximum concentration used.

3. EXPERIMENT 3: Comparison of the Effect of NaCl on the Growth of Bacteria Isolated from PLM with Its Effect on *C. botulinum* 62A Spores

a. Object

Examine the effect of various NaCl concentrations on the development of *C. botulinum* spores, vegetative cells, and other bacteria.

b. Organisms

(1) C. botulinum 62A spores, grown in liver-extract broth, isolated by centrifugation, washed, heat-shocked at 85°C for 15 min, and suspended in distilled water.

(2) C. botulinum 62A vegetative cells, 0.1 ml of a young culture of C. botulinum 62A grown in liver-extract broth.

(3) Staphylococcus albus, isolated from PLM and grown in nutrient broth.

(4) Escherichia intermedium, isolated from PLM and grown in nutrient broth.

c. Medium

For these experiments a 4% trypticase broth containing the desired amount of NaCl was dispensed into 15-cm x 15-mm screw-cap tubes. The tubes of media were autoclaved, cooled, and inoculated, with 0.1 ml of a young culture from nutrient broth used for S. albus and E. intermidium. The C. botulinum inoculum was 0.1 ml of a heat-schocked spore suspension in distilled water. Incubation was carried out at 35°C

d. Data

See Table XXI.

e. Results and Conclusions

(1) C. botulinum was inhibited by 3 to 4% NaCl in 4% trypticase broth at 35°C.

(2) S. albus grew essentially as well at 6% salt concentration as in the absence of salt.

(3) Escherichia intermedium began to grow less well at 3% and barely grew at 6% salt concentration.

(4) Vegetative inocula of C. botulinum develop cultures more quickly than spore inocula but are inhibited by essentially the same concentration of NaCl.

TABLE XXI

EFFECT OF NaCl ON GROWTH OF BACTERIA IN 4% TRYPTICASE BROTH
(EXPERIMENT 3)

| NaCl (%) | Tube No. | *C. botulinum* Spores 24 hours | *C. botulinum* Spores 72 hours | *C. botulinum* Veg. Cells 24 hours | *C. botulinum* Veg. Cells 72 hours | *S. albus* 24 hours | *S. albus* 72 hours | *S. albus* 120 hours | *E. intermedium* 24 hours | *E. intermedium* 72 hours | *E. intermedium* 120 hours |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 4+ | 4+ | 4+ | 4+ | 2+ | 4+ | 4+ | 3+ | 4+ | 4+ |
| | 2 | 4+ | 4+ | 4+ | 4+ | 2+ | 3+ | 4+ | 3+ | 4+ | 4+ |
| 1 | 1 | 0 | 4+ | 3+ | 4+ | 2+ | 3+ | 4+ | 3+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 3+ | 4+ | 2+ | 3+ | 4+ | 3+ | 4+ | 4+ |
| 2 | 1 | 0 | 4+ | 3+ | 4+ | 2+ | 3+ | 4+ | 3+ | 3+ | 4+ |
| | 2 | 0 | 4+ | 3+ | 4+ | 2+ | 3+ | 4+ | 3+ | 3+ | 4+ |
| 3 | 1 | 0 | 4+ | 2+ | 4+ | 2+ | 3+ | 4+ | 3+ | 3+ | 3+ |
| | 2 | 0 | 4+ | 2+ | 4+ | 2+ | 3+ | 4+ | 3+ | 3+ | 3+ |
| 4 | 1 | 0 | 0 | 0 | 1+ | 2+ | 3+ | 4+ | 2+ | 2+ | 3+ |
| | 2 | 0 | 0 | 0 | 1+ | 2+ | 3+ | 4+ | 2+ | 2+ | 3+ |
| 5 | 1 | 0 | 0 | 0 | 0 | 2+ | 3+ | 4+ | 2+ | 2+ | 2+ |
| | 2 | 0 | 0 | 0 | 0 | 2+ | 3+ | 4+ | 2+ | 2+ | 2+ |
| 6 | 1 | 0 | 0 | 0 | - | +2 | +3 | +4 | +1 | +1 | +1 |
| | 2 | 0 | 0 | 0 | - | +2 | +3 | +4 | +1 | +1 | +1 |

EXPERIMENT 4: Effect of NaCl Concentration in Trypticase Broth on the Growth of *C. botulinum*

a. Object

NaCl is known to affect the development of *C. botulinum*. The two Runs, 4a and 4b, designed to observe the effect of NaCl on the development of *C. botulinum* culture from spore and vegetative inocula.

b. Procedure

5% trypticase broth was despensed into 15-cm x 15-mm, screw-cap test tubes,

proper amounts of concentrated NaCl solution were added, and the tubes were autoclaved, cooled, and inoculated. Incubation was carried out at 35°C.

This experiment consisted of two runs. In Run 4a, small, screw-cap test tubes containing about 10 ml of media were inoculated with 0.1 ml of either a 48 hr culture of C. botulinum 62A in trypticase media or with 0.1 ml of a C. botulinum 62A spore suspension in distilled water. For Run 4b, 60-ml, screw-cap test tubes were inoculated with 0.5 ml of a spore suspension containing about $10^7$ spores per ml.

c. Data

See Tables XXII and XXIII.

TABLE XXII

EFFECT OF NaCl ON GROWTH OF C. BOTULINUM 62A IN 5% TRYPTICASE MEDIUM WITH BOTH VEGETATIVE AND SPORE INOCULA (EXPERIMENT 4, RUN 4a)

| NaCl (%) | Tube No. | Inoculum | | | |
|---|---|---|---|---|---|
| | | Veg. Cells | | Spores | |
| | | 24 hours | 96 hours | 24 hours | 96 hours |
| 0 | 1 | +4 | +4 | 0 | +4 |
| | 2 | +4 | +4 | 0 | +4 |
| 1 | 1 | +4 | +4 | 0 | +4 |
| | 2 | +4 | +4 | 0 | +4 |
| 2 | 1 | +4 | +4 | 0 | +4 |
| | 2 | +4 | +4 | 0 | +4 |
| 3 | 1 | 0 | +4 | 0 | 0 |
| | 2 | 0 | +4 | 0 | 0 |
| 4 | 1 | 0 | +4 | 0 | 0 |
| | 2 | 0 | +4 | 0 | 0 |
| 5 | 1 | 0 | +4 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 6 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |

TABLE XXIII

EFFECT OF NaCl ON GROWTH OF C. BOTULINUM 62A IN 5% TRYPTICASE BROTH (EXPERIMENT 4, RUN 4b)

| NaCl (%) | Tube No. | Spore Inoculum | | | |
|---|---|---|---|---|---|
| | | 24 | 48 | 72 | 96 |
| | | hours | | | |
| 0 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |
| 1.0 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |
| 2.0 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |
| 3.0 | 1 | 0 | 0 | 4+ | 4+ |
| | 2 | 0 | 0 | 4+ | 4+ |
| 4.0 | 1 | 0 | 0 | 4+ | 4+ |
| | 2 | 0 | 0 | 4+ | 4+ |
| 5.0 | 1 | 0 | 0 | 0 | 4+ |
| | 2 | 0 | 0 | 0 | 4+ |
| 6.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 7.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 8.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 9.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 10.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | | | | |

d. Results and Conclusions

(1) Comparing data in Tables XXII and XXIII, it will be observed that inhibition of C. botulinum growth occurs at a salt concentration of 5% in trypticase broth. This is slightly less than the value of 6.7% reported in the literature (Jensen, 1954, p. 17) for this organism in nutrient broth.

(2) Data in Table XXII show that vegetative inocula develop cultures more quickly; but the results finally produced by spore and vegetative inocula are similar, if sufficient time is allowed for growth.

5. EXPERIMENT 5: Effect of $NaNO_3$ on the Growth of C. botulinum 62A in Trypticase Broth

a. Object

Sodium nitrate is one of the chemicals used in curing PLM. It is not particularly antiseptic for C. botulinum; Jensen (1954, p. 32) states that a concentration of 2 to 4% has been reported to irregularly inhibit C. botulinum growth. Jensen (1954, p. 34) also points out that pH may affect the antiseptic action of $NO_3^-$ against C. botulinum.

The two Runs (5a and 5b) made for testing the antiseptic effect of $NaNO_3$ in 5% trypticase broth are included here.

b. Procedure

(1) Run 5a—Screw-cap test tubes measuring 15 cm x 15 mm were used. A total of 15 ml of media was present at inoculation; 13.5 ml of 5% trypticase media were sterilized in the tube and they 1.5 ml of a suitable $naNO_3$ solution were aseptically added to provide the desired concentration.

Two kinds of inocula, spores and vegetative cells of C. botulinum 62A, were used. One half ml of a spore inoculum containing $10^7$ spores per ml in distilled water were used in the spore series; three drops of a trypticase culture were added per tube for the vegetative inocula.

(2) Run 5b—The procedure in this run was similar to that in Run 5a except that the pH of the trypticase broth was lowered from its usual value of 6.82 to 5.72 with acid for part of the studies; 1 ml of the spore suspension was used for the inoculum; and the nitrate solution was added before autoclaving.

c. Data

See Tables XXIV and XXV.

d. Results and Conclusions

(1) At pH 6.82, the usual pH value for trypticase broth, concentrations of $NaNO_3$ as high as 1% did not affect the development of C. botulinum spores or vegetative cells in 5% trypticase broth. This is shown in Table XXIV.

(2) As suggested by Jensen (1954, p. 32) nitrate is more effective in controlling development of bacteria in acid than in neutral solutions this is demonstrated by the data in Table XXV, where an effect of nitrate began to be evident at 0.5% and inhibition of C. botulinum growth occurred at 1%, when the pH of the broth was 5.72. At a pH of 6.72 in the broth, the only effect noted was a slight delay in development of the culture, even with 1% $NaNO_3$.

6. EXPERIMENT 6: Inhibition of Bacterial Growth by Nitrite in Trypticase Broth

a. Object

Nitrite is one of the principal preservatives incorporated into PLM. It is known to inhibit bacterial growth (Jensen, 1954, p. 42). Evaluation of the effect of nitrite on our strain of C. botulinum 62A is therefore important for interpretation of results of the inoculated pack studies described in Part A of this report.

b. Procedure

Screw-cap test tubes measuring 15 cm x 15 mm and containing 15 ml at the time of inoculation were used. First, 13.5 ml of trypticase broth was added to each tube and these were autoclaved. Then 1.5 ml of a suitable $NaNO_2$ solution was added aseptically; this was followed with 0.5 ml of an inoculum containing approximately $10^6$ C. botulinum 62A spores per ml. The tubes were incubated at 35°C. In Run 6a concentrations of 0.0005 to 0.5% $NaNO_2$ were used; in Run 6b the effect of added Fe metal was tested; and in Run 6c the pH of the medium was adjusted from its usual value of approximately 6.8 to three arbitrary levels of pH 6.0, 7.0, and 8.0. In Run 6a, a vegetative inoculum of 3 drops per tube of a culture of C. botulinum 62 grown in trypticase broth was used.

TABLE XXIV

EFFECT OF $NaNO_3$ ON THE GROWTH OF C. BOTULINUM 62A IN 5% TRYPTICASE BROTH WITH BOTH VEGETATIVE AND SPORE INOCULA (EXPERIMENT 5, RUN 5a)

| $NaNO_3$ (%) | Tube No. | Vegetative Inoculum | | | Spore Inoculum | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 48 hours | 144 | 24 | 48 hours | 144 |
| 0 | 1 | +4 | +4 | +4 | +1 | +1 | +4 |
| | 2 | +4 | +4 | +4 | +1 | +1 | +4 |
| 1 | 1 | +4 | +4 | +4 | 0 | +4 | +4 |
| | 2 | +4 | +4 | +4 | 0 | +4 | +4 |
| 0.5 | 1 | +4 | +4 | +4 | 0 | +4 | +4 |
| | 2 | +4 | +4 | +4 | 0 | +4 | +4 |
| 0.1 | 1 | +4 | +4 | +4 | 0 | +4 | +4 |
| | 2 | +4 | +4 | +4 | 0 | +4 | +4 |

TABLE XXV

EFFECT OF $NaNO_3$ AT TWO pH LEVELS ON THE GROWTH OF C. BOTULINUM (EXPERIMENT 5, RUN 5b)

| $NaNO_3$ (%) | Tube No. | Spore Inoculum | | | | |
|---|---|---|---|---|---|---|
| | | 24 | 48 | 72 hours | 96 | 144 |
| pH 6.82 | | | | | | |
| 0 | 1 | 0 | 4+ | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ | 4+ |
| 1.0 | 1 | 0 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 0 | 4+ | 4+ | 4+ |
| 0.5 | 1 | 0 | 1+ | 4+ | 4+ | 4+ |
| | 2 | 0 | 1+ | 4+ | 4+ | 4+ |
| 0.25 | 1 | 0 | 3+ | 4+ | 4+ | 4+ |
| | 2 | 0 | 3+ | 4+ | 4+ | 4+ |
| pH 5.72 | | | | | | |
| 0 | 1 | 0 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 0 | 4+ | 4+ | 4+ |
| 1.0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 1 | 0 | 0 | 0 | 0 | 2+ |
| | 2 | 0 | 0 | 0 | 0 | 0 |
| 0.25 | 1 | 0 | 0 | 2+ | 2+ | 4+ |
| | 2 | 0 | 0 | 0 | 0 | 4+ |

c. Data

See Tables XXVI, XXVII, and XXVIII.

TABLE XXVI

EFFECT OF $NaNO_2$ ON THE GROWTH OF C. BOTULINUM 62A IN 5% TRYPTICASE BROTH (EXPERIMENT 6, RUN 6a)

| $NaNO_2$ (%) | Tube No. | Vegetative Inoculum | | | Spore Inoculum | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 144 | 24 | 48 | 144 |
| | | | hours | | | hours | |
| 0.0 | 1 | 4+ | 4+ | 4+ | 1+ | 1+ | 4+ |
| | 2 | 4+ | 4+ | 4+ | 1+ | 1+ | 4+ |
| 0.5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.05 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.005 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0025 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0005 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 4+ | 4+ | 4+ | 0 | 0 | 0 |

d. Results and Conclusions

Tarr is quoted by Jensen (1954, p. 43) as observing that 0.02% nitrite inhibits C. botulinum but that above pH7, nitrite is much less effective. These statements are supported by data in Tables XXVI, XXVII, and XXVIII in which it is whown that 0.01% nitrite was effective in preventing growth of C. botulinum 62A in trypticase media. Even at pH 8.0, 0.01% nitrite showed some inhibitory effect. Actually the data in these three runs indicate that in trypticase broth concentrations of $NaNO_2$ as low as 0.001% have some inhibitory effect on the growth of C. botulinum.

TABLE XXVII

EFFECT OF $NaNO_2$ ON THE GROWTH OF C. BOTULINUM 62A IN 5% TRYPTICASE BROTH IN THE PRESENCE OF METALLIC Fe (EXPERIMENT 6, RUN 6b)

| $NaNO_2$ (%) | Tube No. | Without Iron | | | With Iron | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 120 | 24 | 48 | 120 |
| | | hours | | | hours | | |
| 0 | 1 | 0 | 3+ | 4+ | 0 | 4+ | 4+ |
| | 2 | 0 | 2+ | 4+ | 0 | 4+ | 4+ |
| 1.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.001 | 1 | 0 | 0 | 4+ | 0 | 4+ | 4+ |
| | 2 | 0 | 2+ | 4+ | 0 | 4+ | 4+ |
| 0.0001 | 1 | 0 | 2+ | 4+ | 0 | 4+ | 4+ |
| | 2 | 0 | 3+ | 4+ | 0 | 4+ | 4+ |

TABLE XXVIII

EFFECT OF $NaNO_2$ ON THE GROWTH OF C. BOTULINUM 62A IN 4% TRYPTICASE BROTH AT VARIOUS pH LEVELS (EXPERIMENT 6, RUN 6c)

| $NaNO_2$ (%) | pH 6.0 | | pH 7.0 | | pH 8.0 | |
|---|---|---|---|---|---|---|
| | 48 | 168 | 48 | 168 | 48 | 168 |
| | hours | | hours | | hours | |
| 0 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| 1 | 0 | 0 | 0 | 0 | 0 | 1+ |
| 0.1 | 0 | 0 | 0 | 0 | 0 | 1+ |
| 0.01 | 0 | 0 | 0 | 0 | 0 | 1+ |
| 0.001 | 0 | 0 | 0 | 0 | 0 | 4+ |
| 0.0001 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |

7. EXPERIMENT 7: Inhibition of C. botulinum 62A in Trypticase Broth by Mixture of $NaNO_3$, $NaNO_2$, and NaCl

a. Object

Sodium nitrate, sodium nitrite, and sodium chloride are each known to have preservative action in meat and to inhibit bacterial growth. It is also recognized (Jensen, 1954, p. 37) that mixtures of these salts are perhaps more effective in curing meats than are these salts when used individually. This experiment was designed to observe the inhibitive effects of various combinations of these salts on the growth of C. botulinum 62A in 4% trypticase broth.

b. Procedure

The general procedure was similar to that outlined for Experiment 6.

c. Data

See Table XXIX

TABLE XXIX

INHIBITORY EFFECTS ON C. BOTULINUM OF VARIOUS COMBINATIONS OF $NaNO_3$, $NaNO_2$, AND NaCl IN 4% TRYPTICASE BROTH (EXPERIMENT 7)

| $NaNO_2$ (%) | Tube No. | 1% $NaNO_3$ 72 hours | 1% $NaNO_3$ 168 hours | 3.5% NaCl 72 hours | 3.5% NaCl 168 hours |
|---|---|---|---|---|---|
| 0 | 1 | 4+ | 4+ | 0 | 3+ |
| | 2 | 0 | 4+ | 0 | 3+ |
| 0.01 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.001 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.0001 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.00001 | 1 | 4+ | 4+ | 0 | 4+ |
| | 2 | 0 | 4+ | 0 | 2+ |

d. Results and Conclusions

Neither 3.5% NaCl nor 1% $NaNO_3$ alters the inhibitory effect of $NaNO_2$ for C. botulinum 62A in 4% trypticase broth.

EXPERIMENT 8: Effect of Ascorbic Acid on the Growth of C. botulinum in 4% Trypticase Broth

a. Object

This experiment was carried out to learn whether ascorbic acid, one of the chemicals used in curing PLM, affects growth of C. botulinum.

b. Procedure

The general procedure was similar to that described for Experiment 6 except that the ascorbic acid was added to the 4% trypticase broth before autoclaving.

c. Data

See Tables XXX and XXXI.

Ascorbic acid, being an acid could lower the pH of the broth. The broth was not adjusted to a common pH value. The pH of 4% trypticase broth containing ascorbic acid is given in Table XXX.

d. Results and Conclusions

(1) The broth in Test 1 appears to have not been properly exhausted of oxygen before inoculation.

(2) The reducing action of ascorbic acid appears to have helped initiate growth at an earlier time where it occurred.

(3) C. botulinum 62A growth was inhibited by 1% ascorbic acid. The inhibition in the instance could have been caused by lowered pH, although C. botulinum can grow at pH 5.25.

9. EXPERIMENT 9: Effect of Metals on Growth of C. botulinum 62A in Liquid Media

a. Object

Hydroxylamine ($NH_2OH$) is one of the reduction products of $NO_2$ (Jensen, 1954, p. 47). It is known to be an inhibitor of bacterial growth. Jensen quotes Tarr (Jensen, 1954, p. 47) as finding 0.005% $NH_2OH$ inhibitory for C. botulinum in fish digest cultures at pH 6.3 or above, whereas at pH 5.9, 0.0025% prevented multiplication.

TABLE XXX

EFFECT OF ADDED ASCORBIC ACID ON THE pH OF 4% TRYPTICASE BROTH (EXPERIMENT 8)

| Ascorbic Acid (%) | pH |
|---|---|
| 0 | 6.91 |
| 0.125 | 6.65 |
| 0.25 | 6.35 |
| 0.50 | 5.89 |
| 1.00 | 5.25 |

TABLE XXXI

EFFECT OF ASCORBIC ACID ON THE GROWTH OF C. BOTULINUM 62A IN 4% TRYPTICASE BROTH (EXPERIMENT 8)

| Ascorbic Acid (%) | Tube No. | Hours | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 72 | 96 | 120 | 144 | 192 |
| Test No. 1 | | | | | | | | |
| 0 | 1 | 0 | 0 | 0 | 0 | +4 | +4 | +4 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1 | 0 | 0 | +4 | +4 | +4 | +4 | +4 |
| | 2 | 0 | 0 | +4 | +4 | +4 | +4 | +4 |
| Test No. 2 | | | | | | | | |
| 0 | 1 | 0 | +4 | +4 | +4 | | | |
| | 2 | 0 | +4 | +4 | +4 | | | |
| 1 | 1 | 0 | 0 | 0 | 0 | | | |
| | 2 | 0 | 0 | 0 | 0 | | | |
| 0.5 | 1 | 0 | +4 | +4 | +4 | | | |
| | 2 | 0 | +4 | +4 | +4 | | | |
| 0.1 | 1 | +4 | +4 | +4 | +4 | | | |
| | 2 | +4 | +4 | +4 | +4 | | | |

This experiment is intended to evaluate the concentration of $NH_2OH$ that would inhibit growth of our strain of C. botulinum 62A in 4% trypticase broth.

b. Procedure

The general procedure in the initial stages was similar to that described for Experiment 6. $NH_2OH$ was used in the hydrochloride salt form. Its addition to the medium had a minor effect on pH at concentrations stronger 0.01%. The pH of solutions weaker than 0.01% was 6.95, whereas at 0.1% the pH was 6.34 and at 1% it was 5.5.

c. Data

See Table XXXII.

TABLE XXXII

EFFECT OF HYDROXYLAMINE ON THE GROWTH OF C. BOTULINUM 62A IN 4% TRYPTICASE BROTH (EXPERIMENT 9)

| $NH_2OH \cdot HCl$ (%) | Tube No. | hours | | | |
|---|---|---|---|---|---|
| | | 24 | 48 | 72 | 144 |
| 0 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |
| 1.0 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.1 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.01 | 1 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 |
| 0.001 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |
| 0.0001 | 1 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 4+ | 4+ | 4+ |

d. Results and Conclusions

C. botulinum 62A was inhibited by 0.001% $NH_2OH \cdot HCl$ in 4% trypticase broth. This value is not significantly different from the level of 0.005% previously mentioned.

10. EXPERIMENT 10: Identification of Bacteria Isolated from Spoiled PLM

Early in these studies two bacteria that developed cultures in spoiled PLM were isolated. These spoiled cans of meat had merely been repacked into smaller cans in our laboratory and incubated at 30°C. One of these organisms was a bacterium and the other a coccus. A number of tests were conducted with these bacteria to classify them; standard techniques were used. These tests are recorded in Table XXXIII.

TABLE XXXIII

CLASSIFICATION REACTIONS OF A BACTERIUM AND A COCCUS ISOLATED FROM SPOILED PLM (EXPERIMENT 10)

| Test | Bacterium | Coccus |
|---|---|---|
| Gram stain | small; negative rod | small, positive coccus |
| Dextrose broth | acid and gas | acid but no gas |
| Lactose broth | acid and gas | no acid or gas |
| Sucrose broth | gas, slowly turning acid | no gas, slowly turning acid |
| Maltose broth | gas, slowly turning acid | no gas, slowly turning acid |
| $NO_3^-$ broth | acid; $NO_3^- \rightarrow NO_2^-$ | acid; $NO_3^- \rightarrow NO_2^-$ |
| Citrate broth | positive | negative |
| MB milk | acid; white | acid; white |
| Blood agar | no hemolysis | no hemolysis (perhaps slight β at 48 hr) |
| Litmus milk | lavender (24 hr) | no change |
| Gelatin | no liquefaction | liquefied in 6 days |
| Mannitol broth | acid and gas | no gas or color change |
| Methyl red broth | positive | negative |
| Voges Proskauer | negative | positive |
| Nutrient broth | pellicle; cloudy throughout | no pellicle; cloudy throughout |
| Catalase | positive | positive |
| Motility | positive | negative |
| Coagulose | | negative |
| EMB | pink colonies | |
| Kliglers iron agar | dextrose, acid and gas; lactose neg; $H_2S$, neg | |
| | Escherichia intermedium | Staphylococcus albus (maybe Pediococcus cerevisiae which does not ferment lactose, but which according to Bergey does not reduce $NO_3^-$ to $NO_2^-$ either) |

## IV. EFFECT OF METALS ON GROWTH OF C. BOTULINUM

### a. Object of the Experiment

To test the effects of various metals on development of cultures from C. botulinum 62A spores in liquid media. This is desirable in order to learn whether the metals themselves inhibit, stimulate, or do not affect the growth of the bacterium.

### b. Organism

C. botulinum 62A spores grown in liver extract medium; 0.1 ml of a heat-shocked suspension were used as inocula.

### c. Medium

Four runs in trypticase broth are reported; for the fifth run, other ingredients besides trypticase which are present in PLM were included, viz.:

| | |
|---|---|
| Distilled $H_2O$ | 917.2 ml |
| Trypticase | 36.7 gms |
| NaCl | 31.8 gms |
| Dextrose | 28.6 gms |
| Karo syrup | 18.2 gms |
| Ascorbic acid | 0.18 gms |
| $NaNO_3$ | 0.14 gms |
| $NaNO_2$ | 0.10 gms |

In all cases the media were "exhausted" before inoculation. The exhaustion process involved removal of oxygen, and for these systems was accomplished by one of two methods. Either the medium was used as it cooled from having just been autoclaved, or—if it had stood in the laboratory—it was reheated in a water bath and used as it cooled.

The media were dispensed into 20-ml screw-cap culture tubes or into 250-ml flasks that were closed with a cotton plug.

When nitrite was used, a sufficient amount of sterile concentrated solution was added aseptically after sterilization to produce the desired concentrations.

d. Data

See Tables XXXIV - XXXVIII.

TABLE XXXIV

GROWTH OF C. BOTULINUM 62A IN THE PRESENCE OF VARIOUS METALS IN 4% TRYPTICASE MEDIA WITHIN 48 HOURS*

| Metal | Flasks | Culture Tubes |
|---|---|---|
| No Metal | poor | good |
| Fe | good | good |
| Co | good | good |
| Ni | good | good |
| Al | good | fair |
| Mn | good | good |
| Mg | good | poor |
| Zn | good | good |
| Sn | good | good |

*Development of culture was slow, requiring nearly 48 hours in all cases. Much gas was produced with Mg.

e. Results and Conclusions

(1) As shown in Tables XXXIV, XXXVI, and XXXVII the metals Fe, Co, Ni, Al, Mn, Mg, Zn, and Sn do not inhibit the growth of C. botulinum 62A in trypticase broth.

(2) C. botulinum spores did not develop cultures in 4% trypticase broth containing concentrations of 0.001% $NaNO_2$ and higher when Fe and Sn were present. In the presence of Zn, however, a concentration of 0.01% $NaNO_2$ did not inhibit C. botulinum, as is shown in Table XXXV.

(3) The data in Table XXXVII show that Al powder did not affect the inhibitory quality of $NaNO_2$ for C. botulinum 62A spores in 4% trypticase broth. Similarly, Table XXXVI shows that Mg powder did not affect the inhibitory quality of NaCl for this microorganism in 8% trypticase broth.

(4) Data in Table XXXVIII show that the chemicals used for curing PLM inhibit the growth of C. botulinum spores when these chemicals are present in 4% trypticase broth in the concentration used in PLM. These data also show, however, that metallic Mn slightly reduces the antiseptic effects of the curing ingredients and that Fe powder is even more effective in this regard.

TABLE XXXV

GROWTH OF C. BOTULINUM 62A IN THE PRESENCE OF VARIOUS METALS AND ADDED NITRITE IN 4% TRYPTICASE BROTH*

| Metal | Conc. of $KNO_2$ | Hours | | | |
|---|---|---|---|---|---|
| | | 24 | 48 | 96 | 120 |
| No metal | 0 | 0 | 4+ | 4+ | 4+ |
| | 1.0 | 0 | 0 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 |
| | 0.01 | 0 | 0 | 0 | 0 |
| | 0.001 | 0 | 0 | 0 | 0 |
| | 0.0001 | 0 | 4+ | 4+ | 4+ |
| Mg | 0 | 0 | 0 | 0 | 0 |
| | 1.0 | 0 | 0 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 |
| | 0.01 | 0 | 0 | 0 | 0 |
| | 0.001 | 0 | 0 | 0 | 0 |
| | 0.0001 | 0 | 4+ | 4+ | 4+ |
| Zn | 0 | 0 | 4+ | 4+ | 4+ |
| | 1.0 | 0 | 0 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 |
| | 0.01 | 0 | 4+ | 4+ | 4+ |
| | 0.001 | 0 | 4+ | 4+ | 4+ |
| | 0.0001 | 0 | 4+ | 4+ | 4+ |
| Fe | 0 | 0 | 4+ | 4+ | 4+ |
| | 1.0 | 0 | 0 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 |
| | 0.01 | 0 | 0 | 0 | 0 |
| | 0.001 | 0 | 0 | 0 | 0 |
| | 0.0001 | 0 | 4+ | 4+ | 4+ |
| Sn | 0 | 0 | 1+ | 4+ | 4+ |
| | 1.0 | 0 | 0 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 |
| | 0.01 | 0 | 0 | 0 | 0 |
| | 0.001 | 0 | 0 | 0 | 0 |
| | 0.0001 | 0 | 4+ | 4+ | 4+ |

*A qualitative nitrite test was made on the media in the "No metal" and "Zn" series using the Greiss test from the Association of Official Agricultural Chemists, Methods of Analyses. The following results were obtained:

| | Nitrite Test | | | | | |
|---|---|---|---|---|---|---|
| % of $NO_2^-$ | 0 | 1.0 | 0.1 | 0.01 | 0.001 | 0.0001 |
| No metal | - | + | + | + | + | - |
| Zn | - | + | + | ± | - | - |

Comparison of these results with those in Table XXXV shows that when growth occurs, $NO_2^-$ is not detected by the A.O.A.C. test.

TABLE XXXVI

GROWTH OF C. BOTULINUM 62A IN THE PRESENCE OF Mg AND VARIOUS CONCENTRATIONS OF NaCl IN 8% TRYPTICASE BROTH

| NaCl (%) | Tube No. | No Mg Powder | | | | | With Mg Powder | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 72 | 96 | 120 | 24 | 48 | 72 | 96 | 120 | 144 |
| | | hours | | | | | hours | | | | | |
| 0 | 1 | 0 | 0 | 0 | 1+ | 4+ | 0 | 0 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 0 | 0 | 0 | 4+ | 0 | 0 | 0 | 4+ | 4+ | 4+ |
| 1.0 | 1 | 0 | 0 | 0 | 3+ | 4+ | 0 | 0 | 0 | 4+ | 4+ | 4+ |
| | 2 | 0 | 0 | 0 | 3+ | 4+ | 0 | 0 | 0 | 4+ | 4+ | 4+ |
| 2.0 | 1 | 0 | 0 | 0 | 3+ | 4+ | 0 | 0 | 0 | 0 | 4+ | 4+ |
| | 2 | 0 | 0 | 0 | 3+ | 4+ | 0 | 0 | 0 | 0 | 4+ | 4+ |
| 3.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE XXXVII

GROWTH OF C. BOTULINUM 62A IN THE PRESENCE OF Al AND VARIOUS CONCENTRATIONS OF $NaNO_2$ IN 4% TRYPTICASE BROTH

| $NaNO_2$ (%) | Tube No. | No Al Powder hours | | | With Al Powder hours | | |
|---|---|---|---|---|---|---|---|
| | | 24 | 48 | 72 | 24 | 48 | 72 |
| 0 | 1 | 0 | +4 | +4 | 0 | +4 | +4 |
| | 2 | 0 | +4 | +4 | 0 | +4 | +4 |
| 1.0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.001 | 1 | 0 | +2 | +4 | 0 | +4 | +4 |
| | 2 | 0 | +2 | +4 | 0 | +4 | +4 |
| 0.0001 | 1 | 0 | +4 | +4 | 0 | +4 | +4 |
| | 2 | 0 | +4 | +4 | 0 | +4 | +4 |

TABLE XXXVIII

GROWTH OF C. BOTULINUM 62A IN A BROTH CONTAINING TRYPTICASE PLUS POWDERED METALS AND THE CHEMICAL PRESERVATIVE INGREDIENTS IN PLM

| Sample | Tube | 48 hr | 96 hr | Remarks |
|---|---|---|---|---|
| Trypticase | 1 | +4 | +4 | Control on trypticase broth alone |
| | 2 | 0 | 0 | |
| No Metal | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |
| Fe | 1 | ++ | ++ | Poor growth |
| | 2 | ++ | ++ | |
| Mn | 1 | 0 | + | Gas at 48 hr |
| | 2 | 0 | + | Gas at 48 hr |
| Mg | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |
| Sn | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |
| Zn | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |
| Ni | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |
| Co | 1 | 0 | 0 | |
| | 2 | 0 | 0 | |

## V. GROWTH OF C. BOTULINUM SPORES IN IRRADIATED PLM

### a. Object of the Experiment

It is known that $NO_2^-$ and $NO_3^-$ are affected by irradiation. Hougham and Watts (1954) state that irradiation may cause oxidation of nitrite to nitrate and simultaneous reduction of nitrate to nitrite. Furthermore, nitrite may be reduced to nitric oxide in meats.

Since nitrite is probably the principal curing agent preventing the growth of bacteria, any change in its concentration could affect the stability of PLM on storage. Indeed, pasteurization with irradiation could be dangerous because it could reduce the antiseptic effect of the cure and also prevent the warning effected by bacteria that produce gas but no toxin. Hence radiation-pasteurized PLM might be more dangerous than non-radiation-pasteurized PLM from the standpoint of possible toxicity upon spoilage.

### b. Organism

C. botulinum 62A spores grown in liver extract broth. One ml of a heat-shocked spore suspension in distilled water was used for an inoculum.

### c. Procedure

PLM was removed from the refrigerated 6-lb tins, ground, packed in 204 x 204 tin cans, irradiated with the can covers loosely in place, inoculated with 1 ml of the C. boutlinum 62A spore suspension, sealed with a commercial-tube vacuum-closing machine, and then incubated at 30°C.

Irradiation was carried out in the center well of the large cobalt-60 source in the Fission Products Laboratory. All cans were irradiated for 23 hr, 45 min. Different dosages were the result of different positions in the center well. The irradiation temperature was approximately 5°C.

This is an expanded repeat of Run LA2 conducted in 1959 as part of other studies.

### d. Data

See Table XXXIX.

TABLE XXXIX

SPOILAGE AND GROWTH OF C. BOTULINUM 62A SPORES INOCULATED INTO IRRADIATED PLM

| Radiation Level, Mg | Can No. | No. of Days Until Spoilage | Toxicity |
|---|---|---|---|
| 0.000 | 1 | > 460 | |
| | 2 | > 460 | |
| | 3 | > 460 | 0/4 |
| | 4 | > 460 | |
| 1.470 | 4 | > 147 | |
| | 8 | > 147 | |
| | 6 | > 147 | |
| 2.150 | 7 | 32 | 4/4 |
| | 8 | > 147 | |
| | 9 | > 147 | |
| 2.440 | 10 | 25 | 4/4 |
| | 11 | 26 | |
| | 12 | 32 | |
| 2.680 | 1 | 32 | |
| | 2 | 25 | |
| | 3 | 25 | |

d. Results and Conclusions

(1) With the minimum amount of radiation, 1.470 megarad, the PLM did not spoil within 147 days.

(2) As the amount of irradiation was increased beyond 1.470 megarad, spoilage of the PLM occurred progressively sooner.

(3) The radiation-pasteurized PLM developed toxin upon spoilage.

(4) Irradiation pasteurization of PLM is useless and dangerous because the preservative capacity of the cure is reduced and the removal of vegetative bacteria permits C. botulinum to develop in the meat. It should be noted that the C. botulinum 62A spores were not irradiated in this experiment; they were added after irradiation. It should also be noted that these remarks apply to radiation pasteurization as distinguished from radiation sterilization.

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