THE GROWTH AND TOXIN PRODUCTION OF CLOSTRIDIUM BOTULINUM IN COTTAGE CHEESE

BY

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY WELTON IVAN TAYLOR

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INTRODUCTION

Botulism is a constant threat to those who process foods. The low acid foods have caused many outbreaks. When outbreaks occur in acid foods which are generally believed to be immune, investigations are usually started to determine the cause. Among various procedures which may be used is the experimental preparation and inoculation of the food in question with detoxified spores of Clostridium botulinum under various conditions of preparation and storage to include the important variables. Cottage cheese is in this category because it has been incriminated in outbreaks of botulism on epidemiological evidence which, lacking conclusive bacteriological supporting data, has not been entirely convincing. The few attempts to determine whether or not one may obtain experimental evidence of botulism caused by cottage cheese have resulted in conflicting conclusions. Cottage cheese is generally an acid product and might be considered to be free of any hazard. The several outbreaks which have been attributed to it, especially one by Nevin, prompted the investigations, results of which are reported in this thesis.

HISTORICAL REVIEW

Botulism is an acute toxemia with a high mortality rate caused by ingestion of the exotoxin produced by the anaerobic micro-organism, Clostridium botulinum. The disease was first described and recorded in 1735, according to Dickson (1918). The name was taken from the Latin word botulus, meaning a sausage, since that was the foodstuff incriminated in most of the early outbreaks of the disease. Indeed, for many years it was known as "sausage poisoning" in Germany where it was first recognized and named.

The relatively frequent occurrence of the disease in one locality in Germany was explained by Ostertag; many cases of botulism were recorded in Würtemburg primarily because it was a sausage manufacturing center and leberwurst and blutwurst were prepared for eating at a later date with little thought of poor keeping qualities. As a result, much of the botulism reported in that period of the Middle Nineteenth Century was a result of eating habits of people in that particular locality.

The disease was known as allantiasis, and later, botulismus, but the assumption was that it was caused by eating of ordinary decomposed food and that normal products of decomposition were responsible. In an outbreak in Ellezelles, Belgium, van Ermengem (1897) isolated from the ham which caused botulism, and from the spleen of one of the patients who died, an anaerobic spore forming bacterium he named Bacillus botulinus, later to be known as Clostridium botulinum. This organism produced toxin with which he was able to reproduce the characteristic symptoms of botulism in animals.

Outbreaks of botulism had resulted in about 1,200 reported cases up to 1913, according to a survey made by Mayer (1913). In the period 1899 to 1932, in the United States and Canada mainly, Tanner (1933) lists 657 cases with 447 deaths, or a mortality of 68 per cent. The mortality for the American outbreaks reported has been twice as high as that of Europe. Rudel (1921) reported mortality of 18 - 20 per cent in Germany while Mayer's data show 25 per cent. A possible explanation of this difference is offered by Bronfenbrenner and Schlesinger (1920) who found ethyl alcohol to destroy the toxin of *Clostridium botulinum*, and therefore the habit of many Europeans of having alcoholic drinks with meals may have reduced the mortality in part. It is also possible that American strains of *Clostridium botulinum* are more toxic than European.

Botulism in Domestic Animals

Since van Ermengem (1896) described the symptoms of botulism in laboratory and domestic animals, the relationship between *Clostridium botulinum* and many diseases of animals has become increasingly apparent and important. Mitchell (1922) isolated *Clostridium botulinum* from ingesta from a horse dead of forage poisoning and demonstrated the ability of the organism to grow and produce toxin in fodder and, ultimately, clinical symptoms and death of a horse experimentally fed the fodder. Leighton (1923) reported occurrence of an outbreak of equine disease caused by *Clostridium botulinum*, type A. "Blind staggers" and "grass disease" are names given botulism in horses.

Forage poisoning of cattle was reported to be caused by Clostridium botulinum by Graham, et al, (1917). The literature incriminating Clostridium botulinum, type C, in outbreaks of "lamsiekte" disease in South Africa and "bush sickness" and "midland disease" of Tasmania was reviewed by Tanner (1933). Guinea pigs, rabbits, mice, monkeys and cats were reported to be susceptible to toxin by van Ermengem (1896). Chicken outbreaks were noticed after human outbreaks were more frequently recognized and Geiger, Dickson, and Meyer (1922) reported chicken deaths caused by feeding spoiled home-canned corn and spoiled sour milk to poultry flocks. The disease known as "limberneck", was reported by Thom, et al, (1919) to be caused by toxin of Clostridium botulinum, the Boise strain, type A. Bengtson (1924) compared type A, B, and C toxins and reported type A to be more toxic for chickens than B and C.

"Duck sickness" or "duck disease" is now known to be similar to limberneck in origin. Kalmbach (1934) reported a high incidence of duck sickness caused by a type C strain of Clostridium botulinum in alkaline soil areas of the western United States and Canada. He reported stagnant water, high temperatures, alkaline pH and dead organic matter as factors contributing to outbreaks. Quortrup and Schillinger (1941) reported 47.9 per cent of 3,000 birds autopsied in western lake areas died of botulism.

It is apparent from the above data that few animals are refractory to the toxin of the various types of Clostridium botulinum. Botulism may well become a conservation problem in addition to a public health problem.

Distribution of *Clostridium botulinum* in Nature

Although van Ermengem had isolated the causative organism of botulism from a toxic ham and the spleen of a victim, he did not discuss the natural habitat of the organism. It is now known to be widely disseminated. Burke (1919) isolated 7 toxic cultures of *Clostridium botulinum* from samples of fruit, leaves, twigs and dirt from central California and concluded that the organism was widely distributed in nature and that spiders and insects were able to disseminate it, and that the organism might be able to live in the intestinal tract of warm-blooded animals for months. The isolation of *Clostridium botulinum* from hog feces and sewage by Tanner and Dack (1922) give credence to that conclusion. Meyer and Dubovsky (1922a) (1922b)(1922c), Dubovsky and Meyer (1922), and Schoenholz and Meyer (1922) were able to isolate toxin-producing cultures of *Clostridium botulinum* with considerable frequency from California soils and from the whole of the United States excepting Virginia, and with less frequency from Belgium, Denmark, England, the Netherlands, and Switzerland. A high percentage of toxic cultures was found in soil samples collected in Canada, Alaska, China, and the Hawaiian Islands.

Bachmann and Haynes (1924) reported 146 soil samples negative in Wisconsin and isolated only 1 weakly toxic culture from canned mushrooms, but Damon and Payabal (1926) found that 76 per cent of the soil samples from Maryland produced toxic cultures of *Clostridium botulinum*. Leighton and Buxton (1928) isolated 4 toxic cultures from 106 samples of Scottish soils while 5 - 14 per cent of English soil specimens were reported to contain toxic Clostridium botulinum strains by Haines (1942). Pasricha and Panja (1940) reported Clostridium botulinum, type A, in soils near Calcutta, India. Jones and Tanner (1945) discovered soil of Illinois to produce 7.2 per cent toxic cultures, with southern Illinois having a larger percentage than northern Illinois. Parry (1946) isolated toxic cultures from 11.7 per cent of the soil samples from New York and reported all positive samples came from silt loam soils.

With these reports as a basis, it is logical to conclude that the organism is ubiquitous and may be isolated in the New World and the Old World, in virgin and cultivated soils, in and on damaged and undamaged fruits and vegetables, and in the droppings of vertebrates and invertebrates which disseminate it. Wide distribution of Clostridium botulinum as just shown from the literature, makes it possible for many human foods to be contaminated. Consequently foods in which it may develop must be held either at sufficiently low temperatures to repress development of this bacterium or treated in some matter to destroy the spores. Owing to marked heat resistance, this latter is a serious problem.

Pathogenicity of Clostridium botulinum

Spores of Clostridium botulinum were recovered from feces of surviving animals by Thom, Edmondson, and Giltner (1919). The same authors, Edmondson, et al, (1920), investigated further and reported deaths with clinical symptoms of botulism after feeding 50,000,000 or more detoxified spores of Clostridium botulinum, Boise strain, to guinea pigs. Irregular results were obtained with the Nevin strain. They concluded that there was a limited pathogenicity and that foods suspected of harboring Clostridium botulinum should be destroyed rather than heated and eaten. Orr (1922) produced death consistently with detoxified spores of a type B strain fed in 125,000,000 amounts and concluded that feeding massive doses of spores would cause death in animals. Disease produced by feeding 3,300,000,000 or more spores to animals caused Coleman and Meyer (1922) to draw similar conclusions. Intramuscular infection resulted after using formalin as a tissue debilitant with as few as 25 spores, according to Coleman (1929). Hall (1945) reported the occurrence of Clostridium botulinum in wounds in human beings, isolated and typed the organisms, but observed no symptoms of the disease in any of the 3 cases.

There seems to be little doubt that the primary source of intoxication is from ingestion of pre-formed toxin, but the organism is now thought to have a limited pathogenicity and power to invade tissue. It has been reported recoverable from spleen, liver, and brain as early as van Ermengem (1897).

Only recently has the toxin of Clostridium botulinum been purified. Abrams, Kegeles, and Hottle (1946) used Clostridium botulinum, type A, Hall strain, to produce toxin, and isolated a protein with toxic properties. They reported it to behave like a globulin, with an isoelectric point of pH 5.6 and a total nitrogen of 14.1 per cent. Twice crystallized toxin contained 220 x 10^6 mouse MLD per mg. of nitrogen. Kegeles (1946) reported it to have a molecular weight of 1,130,000, a shape according to the Simha theory of elongated ellipsoids with an axial ratio of 8.3, and a frictional ratio, from Perrin's theory, of 1.45. Lamanna, et al, (1946) obtained crystals of 125 x 7 microns when they purified the toxin of Clostridium botulinum, type A. They reported a tendency of these crystals to line up in parallel rows during crystallization.

Importance of Clostridium botulinum in Food Industry in the United States

The importance of Clostridium botulinum to the food industry cannot be overestimated. It existed for many years before it was recognized as the cause of the specific disease, botulism. Cases of so called "ptomaine poisoning" and "belladonna poisoning" were often actually unrecognized botulism. Geiger, Dickson, and Meyer (1922) reported that from 1899 to 1902 the first clinically characteristic cases of botulism were described in the United States. Wilbur and Ophüls (1914) recognized it in an outbreak in California which resulted in a death with clinical symptoms of botulism. Meyer (1931) stated that commercially canned foods caused botulism in 1919. An outbreak among persons who had eaten ripe olives at a banquet in Canton, Ohio caused 7 deaths out of 17 intoxicated. Outbreaks followed in quick succession, all from ripe olives grown and canned in California. This development caused an historical step to be taken which eventually stopped outbreaks from all commercially canned products.

The National Canners Association, Canner's League of California, and California Olive Association proposed an investigation of conditions which were making California preserved olives hazardous. A Botulinus Commission was formed, Geiger, et al, (1922), to make a survey of the industry and recommend what should be done. Such recommendations were made that botulism from olives was stopped. Processing and packing of the fruit was placed under the jurisdiction of the Bureau of Pure Food and Drugs of the California State Board of Health. Spinach and other vegetables grown and canned in California also caused many outbreaks of botulism, which resulted in regulations replacing agreements as to proper processing times and procedures, with confiscation of improperly processed foods as a penalty. Meyer (1931) listed as headings for investigation:

1. handling new products and plant sanitation
2. canning procedure and equipment
3. processing operations, temperatures and time to sterilize
4. examination of commercial pack

The result of standardization and enforcement of procedures proved adequate by investigation is that not one case of botulism has resulted from an American product that is normally commercially canned since 1925.

The outbreaks of botulism resulting from poor processing methods in home-canning still occur, although fewer in number than formerly. Tanner (1944) extensively reviewed the literature available to the homemaker from Farmer's Bulletin 359 in 1909 to recent cook books, and condemned careless home canning methods. Of the "cold-pack" method, still greatly used by homemakers, he states:

> It has been responsible for much spoilage and many outbreaks of botulism since its introduction about 1917. When used for preserving vegetables and meats, it is a dangerous method.

A cook book printed as late as 1930 recommended times and temperatures insufficient for safe home canning, according to Tanner (1933). Meyer (1931) stated that eventual prevention of outbreaks from home-canned foods will depend largely on education of the masses as to the necessity for proper canning procedures and boiling home-canned foods before eating.

Relation of Acidity of Food to Botulism

The relative infrequency with which certain foodstuffs are incriminated in outbreaks of botulism is probably largely a matter of reaction. Acid foods have been thought to be safe when canned by methods that would surely be insufficient if neutral or alkaline reactions were encountered. Dozier (1924) reported the optimum and limiting pH's for some strains of *Clostridium botulinum*. The optimum pH for vegetative cells was said to be from pH 6.0 to 8.2, and the optimum for the inoculation of spores, pH 6.0 to 7.2. The limiting range for a three day growth was from pH 5.0 to 9.0. Since this publication there have been many exceptions to these general rules reported. Liefson (1931) found pH 6.2 to 6.3 to be the optimum for sporulation and reported that no growth occurred below pH 6.0. Cutter (1922), upon investigation of an outbreak of botulism resulting in the deaths of 2 men, found tomato-onion-chili sauce to be incriminated, and stated, "The sauce provided an acid medium, the first instance recorded in which *Bacillus botulinus* had developed under these conditions." He neglected to mention the pH of the sauce, however. It is now generally accepted that the critical pH is 4.5 and that foods below that pH are classified as acid foods, with relation to botulism.

Acidity of Foods and Toxin Formation

Thompson and Tanner (1925) reported results of observations in inoculated canned foods in which no toxicity developed where the pH was 4.4 or less, but toxicity did appear at pH 4.6 or above. They concluded that acid fruits were more easily sterilized and that very acid vegetables were not suitable for toxin production by Clostridium botulinum if cells survived the processes. Despite the belief pH 4.5 is the critical acidity and that foods which are more acid will be safe, a few outbreaks have occurred in such acid foods as pears and tomatoes. Meyer and Gunnison (1929) reported toxin formation in Bartlett pears which caused 2 deaths although the pH of the juice was 3.86. Wallace and Park (1933) were able to demonstrate toxic cultures resulting from inoculation of dextrose of pH 4.0 and cherry juice of pH 3.5, and Tanner, Beamer, and Rickher (1940) found fruits toxic at pH's of 3.25, 3.75, and 3.8, however these latter three papers will be discussed in more detail later as mixed cultures were the rule and pH is evidently not the only factor involved. The critical pH value of 4.5 previously mentioned was determined in pure culture experiments and not in experiments where mixed cultures were present to introduce other variables.

An outbreak of botulism from home-canned tomatoes was reported by Slocum, Welch, and Hunter (1941) which resulted in 2 deaths and isolation of Clostridium botulinum, type B, producing toxin at a pH of 4.21. A tomato residue was filtered and the filtrate proved non-toxic, but when the pH was adjusted to 6.8 it proved toxic and produced symptoms of botulism in experimental animals receiving it. This organism was added to commercially canned tomatoes and incubated for 32 days but produced no toxic samples although the organism was recoverable in 10 cans.

Acidification of toxin to a pH of 4.0 showed an increase in toxicity, according to Bronfenbrenner and Schlesinger (1922). Stehle (1922) was opposed to the statement made by Bronfenbrenner on the basis that a dilution of 3×10^{-21} would contain no toxin because of the assumed size of the toxin molecule itself. Bronfenbrenner (1922) agreed with Stehle's criticism and stated that his results at that dilution were irregular, producing only 5 per cent mortality and thereby indicating that some such dilutions contained toxin while others didn't. Snipe and Sommer (1928) did work which originally started to bear out Bronfenbrenner's contention that acidification of the toxin of Clostridium botulinum increased its toxicity until they discovered the pipette to be a source of experimental error. When a clean pipette was used for each transfer no toxin potency increase was noted. They did find, however, that acidifying toxin to a pH of 1.2 or less did not destroy it, which verified the findings of Bronfenbrenner and Schlesinger (1924) that an acidity equivalent to that of the stomach was resisted by the toxin for 24 hours at 37°C. The latter authors also noted that peptic and tryptic digestion did not alter the toxin.

Antibiotic and Symbiotic Effects of Other Organisms on the Growth and Toxin Production of *Clostridium botulinum*

It has long been known that variance between results obtained with pure cultures in an otherwise sterile medium and with mixed cultures may be caused by symbiosis and antibiosis between organisms. Hall and Peterson (1925) reported aerobic bacteria to have the ability to inhibit, in varying degrees, toxin production of *Clostridium botulinum* in glucose broth. Some organisms toxin production in glucose broth did not do so in plain broth. Organisms which caused non-toxic or weakly toxic filtrates to be produced by *Clostridium botulinum* while growing with them usually produced the greatest acidities in glucose broth. They concluded that, "....certain fermentative aerobes destroy botulinus toxin through the operation of some mechanism involving a factor other than acid but dependent on it."

Dack (1926) showed that some anaerobes are also capable of causing measurable changes in toxin production by *Clostridium botulinum*, and concluded that filtered toxin of the organism was destroyed by growth of *Clostridium sporogenes* in beef heart medium, the organisms with greatest proteolytic abilities produced most rapid destruction of toxin, and that this destruction of toxin was not caused by changes in hydrogen ion concentration. The ability of *Clostridium sporogenes* to destroy the toxin of *Clostridium botulinum* has been reported by Jordan and Dack (1924) and Sommer and Glunz (1927). Francillon (1924) reported *Pseudomonas pyocyaneus* to inhibit toxin production by Clostridium botulinum and Staphylococcus to be necessary for the production of toxin by another strain of Clostridium botulinum, thus illustrating antibiosis and possibly symbiosis. Ruyle and Tanner (1935) reported a higher percentage of toxic samples resulting from inoculation of processed meats than from unprocessed, and concluded that since processed meat has a lower bacterial load the presence of other organisms may inhibit the formation of toxin by Clostridium botulinum. Streptococcus lactis and Lactobacillus casei were reported by Sherman, Stark, and Stark (1928) to have the ability to destroy the toxin of Clostridium botulinum.

Production of toxin by Clostridium botulinum is often enhanced by organisms growing concomitantly. Meyer and Gunnison (1929) found that in an outbreak caused by home-canned pears, the organism isolated had grown and formed toxin in the syrup with a pH of 3.33 to 4.44 with a yeast and a lactobacillus. Without the yeast Clostridium botulinum did not produce toxin and the highest toxicity was obtained when both yeast and lactobacillus were present. The authors concluded:

> It is probable that certain fruits and acid vegetables owe their immunity from botulinum spoilage less to their acidity than to the absence of food substances essential to the elaboration of toxin.

Wallace and Park (1933) produced toxic cultures by utilizing Saccharomyces ellipsoideus in mixed culture with Clostridium botulinum, types A and B, at pH's of 3.5 and 4.0. Tanner, Beamer, and Rickher (1940) have also noted the effect of fungi on toxin production. Black raspberries at pH 3.25 proved toxic after Penicillium spp. had grown and the pH was 5.4, cherries at pH 3.75 had a pH of 5.1

to 5.3 after Mycoderma spp. had grown and were toxic, but peaches at pH 3.8 were toxic with no noticeable changes.

Botulism in Dairy Products

Dairy products have not often been incriminated in outbreaks of botulism for a number of reasons some of which have been discussed. Acid souring of milk with its resultant low pH is probably most important because it is certainly not conducive to growth and toxin production by an organism which so manifestly prefers a neutral to slightly alkaline environment. Edmondson, Thom, and Giltner (1923) inoculated sterile, laboratory pasteurized, and commercial raw milks with toxin-free spores and incubated them at various temperatures and times. The raw milk did not become toxic but pasteurized and sterile samples did. Evidence of abnormality accompanied toxicity and thereby provided an indication that an organo-leptic test may be all that is required for "safe" milk as far as botulinum toxin is involved.

Possible contamination of milk by Clostridium botulinum before it reaches the dairy has been investigated by Graham, Schwarze, and Boughton (1922) who fed unfiltered toxin to cows and sows to observe possible effects on nursing young. They could not demonstrate toxin in the milk, but recovered the organism in the feces. In one case where an experimental infection of the udder with toxin-free spores was accomplished, they were able to demonstrate free toxin in the milk. Sherman, Stark, and Stark (1928) have perhaps provided another clue as to why dairy products have been as free of botulism as they have been in their work on toxin destruction by Streptococcus lactis and Lactobacillus casei, both of which are important in normal souring of milk, forming an acid curd for cottage cheese and curing of cheese. Glotova and Chebotareva (1938) reported that when inoculating acid soybean milk with toxin-free spores of Clostridium botulinum, factors favoring development of lactic acid organisms had the opposite effect on toxin production by Clostridium botulinum, and that the abrupt change in the degree of acidity had the most inhibitory effect.

Shippen (1919) inoculated sterile milk with the Nevin strain of Clostridium botulinum and a yeast and got a toxic culture. Nevin strain, yeast, and Escherichia coli produced toxic cultures, as did Nevin strain and M. aureus. Canned milk caused an outbreak of botulism among 4 persons using it in their coffee, reported by Geiger, et al, (1922), and also a chicken outbreak resulting in the death of 20 chickens caused by spoiled sour milk. The authors stated:

> Spoiled sour milk is unquestionably an excellent medium for B. botulinus and it is surprizing that only one single chicken outbreak epidemiologically attributed to it has thus far been recorded.

Evaporated milk was inoculated with 50,000,000 or more spores of Clostridium botulinum by Schoenholz, Esty, and Meyer (1923) with irregular results in degree of spoilage and toxin production. The pH was not given. Inoculation of sweetened condensed milk produced no growth of Clostridium botulinum. Lewis and Hill (1947) recommend powdered milk diluted to a final concentration similar to that of whole milk as a substrate for the production of highly toxic cultures. Stark, Sherman, and Stark (1928) reported milk to increase the potency of pre-formed toxin. They filtered cells from an eight day growth at 37°C in 4 per cent peptone beef infusion broth and found it to have a titre of 700 MLD while fresh. After 4 days at 37°C the titre was 1 MLD. Similar portions, however, when incubated with sterile skimmed milk gave the following titres:

Filtrate : milk	Titre in MLD
1 : 9	2,000
1 : 19	2,000
1 : 49	5,000

The authors believed this phenomenon to be the result of the action of enzymes of the organism on casein.

Botulism from Cottage Cheese

Cottage cheese has been incriminated in 2 outbreaks of botulism resulting in 5 deaths. Nevin (1921) had the opportunity to observe a sample of cottage cheese sent to the New York State Department of Health Laboratory. Three people who had partaken of this cheese, died with clinical symptoms of botulism. Nevin isolated an organism identified as Clostridium botulinum, type B. It has been widely used since then as the "Nevin strain". Nevin used a loopful of 48 hour broth culture of this organism to inoculate an emulsion of fresh cottage cheese, sterilized and unsterilized. When administered orally, 1 ml. produced death in the guinea pigs used for the 2 cheeses, and the control fed unsterilized market cheese was not affected. Geiger, et al, (1922) reported an outbreak in which 2 deaths resulted from 7 people who ate home-made cottage cheese. No laboratory data were available.

EXPERIMENTAL METHODS

Preparation of Inocula

Several strains of Clostridium botulinum, types A, B, and C, which have been used for a long time in studies on botulism, were available in the laboratory and were used in this investigation. Sheep brain medium, long known to be a satisfactory medium for toxin production by Clostridium botulinum, was made by boiling fresh ground sheep brains in an equal volume of water for two hours and tubing in 20 x 200 mm. tubes to about one-half full with solids and liquid. To test this medium, it was inoculated with spores of Clostridium botulinum and incubated 6 days at 37°C. Guinea pigs were fed 1 ml. of each of such cultures, and strains of Clostridium botulinum which did not cause death of 250 gr. guinea pigs in less than 24 hours were discarded as non-toxic or weakly toxic.

Deep tube cultures of the most toxicogenic strains of Clostridium botulinum were made in pork tryptose infusion agar. This medium was made by adding dried ground pork, 2 per cent Difco tryptose, and 2 per cent Difco agar to fresh pork infusion and adjusting pH to 7.0. Isolated colonies were transferred to pork tryptose infusion medium to secure satisfactory sporulation, which was accomplished in 12 days at 37°C. Spore suspensions were made by filtering the culture through sterile glass wool, centrifuging, and decanting the liquid from the spores; they were then resuspended in

100 ml. sterile distilled water in 8 oz. screw-capped bottles. Spore suspensions were detoxified by heating for 30 minutes at 80°C in an oil bath. This treatment insures the destruction of both toxin and vegetative cells capable of elaborating toxin.

The spores of <u>Clostridium botulinum</u> were counted by means of the following technique. Modified Agarslant tubes were three-fourths filled with pork tryptose agar. Spore suspensions were diluted from 1:1 to 1:10,000,000 by the use of 99 ml. sterile water blanks. Duplicate dilutions were added to the cooled melted agar, shaken throughout it and the agar allowed to solidify. Stratifying agar was then added to fill the tubes and complete the anaerobic conditions.

After 24 to 48 hours incubation at 37°C., macroscopic colonies in the agar were counted. With some strains used, and after long storage in the refrigerator, an incubation period of 5 days was sometimes necessary to insure the development of all colonies. This method was chosen because it is a count of only the viable spores which will germinate after the heating procedure. The spore suspensions were adjusted to 1,000,000 spores per ml. concentrations and stored at 4°C. when not actually in use.

In the later experiments, spore suspensions of <u>Clostridium botulinum</u> were made differently. Isolated colonies in deep agar tubes were transferred to pork tryptose infusion and incubated 3 - 5 days at 37°C. When a noticeable characteristic odor had developed in the medium, Gram stains were made to verify morphology, and typical <u>Clostridium botulinum</u> plated out on milk tryptose agar in Kolle flasks and 16 oz. screw-capped bottles. Anaerobic conditions were secured by use of 100 gr. of pyrogallic acid and 500 ml. of 20 per cent aqueous NaOH in a 10 liter glass museum jar. Incubation at 37°C. for 12 days produced excellent multiplication and sporulation. Milk tryptose agar was made by adding 2 per cent Difco tryptose and 2 per cent Difco agar to milk, adjusting the pH to 7.0, and sterilizing. Spores were washed of the agar with sterile distilled water into sterile 6 oz. screw-capped bottles, detoxified, counted, and stored as previously described.

Preparation of Cottage Cheese

Several batches of cottage cheese were made to gain proficiency and standardize techniques before inoculation with spore suspensions of _Clostridium botulinum_. The method of cheese-making was one described by Tuckey (1942).

Cheese batches were made from both raw and pasteurized skimmed milk. When milk was inoculated with toxin-free spore suspensions of _Clostridium botulinum_ before curdling occurred, it was referred to as "Early". When milk was made into cheese and bottled and was inoculated only when ready for incubation, it was known as "Late".

Raw skimmed milk was poured into large rectangular shallow sterile porcelain pans in one-half gallon amounts, covered with sterile laboratory towels, and allowed to sour naturally at room temperature in a room other than the one in which cultures were kept and inoculating done. Souring occurred and a firm curd obtained in 36 to 72 hours, and when whey was noticeable on the surface of the curd, it was ready to be cut. The curd was cut with a sterile spatula into three-fourth inch cubes, cooked in a water bath for 2 hours at temperatures gradually increased from 32°C. to 55°C., and drained. Whey was bottled in 100 ml. amounts. Curd was washed in cool tap water twice for 5 minute periods and salted with 2 teaspoons NaCl per gallon of original milk. Cheese was then bottled in 100 gm amounts in sterile 4 or 8 oz. wide-mouthed screw-capped jars. Pasteurized skimmed milk was handled similarly except that a starter was added to assist curdling. Starter consisted of 150 ml. sterile milk curdled by Streptococcus lactis in pure culture at 37°C. for 24 hours, and used for a gallon of milk. Later 300 ml. of starter was used per gallon of milk.

Milk samples designated "Early" received 1,000,000 toxin-free spores of Clostridium botulinum per 100 ml. of milk prior to curdling, and those designated "Late", 1,000,000 spores per 100 gr. cottage cheese. Bottled cheese was incubated at 4°C. and at room temperature (22 - 26°C.), as those temperatures would be the ones most likely to be involved in the spoilage of the product in the home.

The pH of control samples of cheese and whey was recorded as read on a Beckman pH meter in the early experiments, and on a MacBeth pH meter later, at the time of bottling and at intervals during the incubation period of the samples.

Feeding of Experimental Animals

Guinea pigs of as near 250 gr. in weight as possible were fed 1 ml. amounts of the cheese or whey by pipette at 5 day intervals. Experimental animals were individually caged and received no food or water after being fed incubated samples of cheese. Animals were closely observed for clinical symptoms of botulism, and those dying in 5 days or less were carefully inspected at autopsy to determine cause of death. In later experiments, all animals not observed to have exhibited clinical symptoms of botulism before death were autopsied immediately, the brain removed aseptically and added to heated tubes of sheep brain medium under vaseline seal. These tubes were incubated for 6 days at 37°C. and observed for the development of gas and the characteristic odor of Clostridium botulinum. One milliliter of this medium was fed to 350 to 500 gr. guinea pigs which were observed for clinical symptoms of botulism. This method was chosen as nearest to fulfilling Koch's (Henle's) postulates as was practicable. Coleman and Meyer (1922) reported the brain as second only to the liver in the recovery of Clostridium botulinum in fatal cases of botulism, and stated:

> It is possible that this organ [brain] is the last to be invaded....we have frequently failed to recover the organism from the brains of animals dying within 48 hours.

From this statement it was seen as likely that when Clostridium botulinum was recovered from the brain of a guinea pig, botulism was actually the cause of death, and that when symptoms of botulism were observed before death this method could be used as confirmation.

EXPERIMENTAL DATA

Experiment 1

The first work was a duplication of experiments performed by Nevin (1921) because of the fact that she attributed an outbreak of botulism to cottage cheese.

One milliliter of a 72 hour culture of Clostridium botulinum of the same type as the Nevin strain, type B, strain 791, in sheep brain medium, was fed to a 211 gr. guinea pig. The survival of the animal indicates there was little toxin production in that length of time. One loopful of this culture was then used to inoculate 100 gr. of cottage cheese newly made from pasteurized skimmed milk. A loopful of the same organism in a 48 hour culture of pork tryptose infusion was also used to inoculate 100 gr. samples of cheese and whey. Controls were uninoculated cheese samples.

These samples were incubated at 37°C. for 72 hours and 1 ml. amounts fed to guinea pigs of 199 to 259 gr. in weight. All animals lived. The cultures in pork tryptose medium and sheep brain medium were fed at the same time and produced death of 157 and 191 gr. guinea pigs in less than 20 hours. This indicates that the strains of Clostridium botulinum used were toxicogenic under the conditions afforded by pork tryptose or sheep brain media but were not capable of producing sufficient toxin in cottage cheese to cause deaths of guinea pigs.

The quantity of cheese which Nevin (1921) inoculated was not reported and it was thought that 100 gr. amounts might be too large a sample for the small inoculum, so in the next attempt to duplicate the work of Nevin a smaller sample was used. Pasteurized and raw cheese samples in 30 gr. amounts were put into 20 x 200 mm. tubes. Cultures of Clostridium botulinum, type A, strains 701 and 702, and type C, strain 827, were incubated 48 hours at 37°C. in sheep brain and a loopful of each used to inoculate duplicate tubes of raw and pasteurized cheese. In addition, 1 ml. amounts of each culture, from the sheep brain and the cheese, were fed to guinea pigs in order to determine whether there was sufficient preformed toxin in the loopful of viable vegetative cells to cause death of the animals without further growth and toxin production in the cheese. The samples were incubated for 70 hours at 37°C. and fed to guinea pigs. Guinea pigs fed 48 hour sheep brain cultures weighed 484, 449, and 474 gr. and died with symptoms of botulism in less than 20 hours, 70 hours, and 6 days respectively. The 12 guinea pigs fed the cheese samples at 0 and 70 hours weighed from 218 to 425 gr.; all survived. The indication is that the strains of Clostridium botulinum used were capable of producing toxin in 48 hours in a substrate of sheep brain under the conditions reported, but were not able to produce detectable amounts of toxin in cottage cheese under the conditions imposed.

The attempts to duplicate the experiments of Nevin (1921) gave negative results since no deaths occurred in guinea pigs fed cheeses inoculated with vegetative cells of Clostridium botulinum, types A, B, and C, strains 701, 702, 791, and 827. Nevin neglected to report the quantity of cheese which she used, and since she used a commercially prepared product, the method of preparation is unknown and the pH was not given. Her inoculum was not heat treated and consisted of vegetative cells of Clostridium botulinum, consequently she may have been adding some preformed toxin to her cheese samples, and in a small sample it may have been in sufficient quantity to intoxicate the guinea pig without further growth and toxin production by Clostridium botulinum in the cheese. It is possible, however, that the Nevin strain of Clostridium botulinum was capable of growth and toxin production under conditions unsuitable for growth by the strains used in this experiment, but because of the lack of certain information, as previously mentioned, the exact conditions of Nevin's work can not be ascertained. The results reported here would seem to duplicate the negative findings of Edmondson, et al, (1923), and both low pH and toxin destruction by other organisms are responsible, possibly.

Experiment II

Since it was obvious that pH of a product as acid as cottage cheese would affect growth and toxin production of Clostridium botulinum, it was thought advisable to acidify sterile milk with dl lactic acid and determine the effect of pH on Clostridium botulinum in the absence of other organisms and possible antibiotic and symbiotic relationships.

Skimmed milk was placed in 100 ml. amounts in 8 oz. screw-capped bottles and autoclaved 30 minutes at 15 pounds pressure. It was stored for 2 months at room temperature with n o signs of spoilage. A 1:10 dilution of dl lactic acid and sterile distilled water was made to facilitate measuring, and proper amounts of acid necessary to produce known acidities were determined by using a MacBeth pH meter. The pH of the sterile milk was 6.15. Samples of pH 6.15, 6.0, 5.5, 5.0, 4.5, 4.0, and 3.5 were made and inoculated with freshly heated and detoxified, newly counted spore suspension of Clostridium botulinum, type B, strain 783. Samples received an inoculum of 1,000,000 spores per 100 ml. milk. Lewis and Hill (1947) reported satisfactory growth of Clostridium botulinum in powdered milk of the concentration of whole milk in 100 to 150 ml. amounts in 6 or 8 oz. screw-capped bottles. They stated:

> Under these conditions no special procedures were required to insure adequate anaerobiosis, provided agitation was minimized to avoid undue aeration of the medium.

The results shown in Table 1 bear out this statement.

TABLE 1

TOXIN PRODUCTION OF CL. BOTULINUM
IN ACIDIFIED STERILE MILK

Guinea pig wt. in gr.	Incubation Time in Days	pH	Strain of Cl. botulinum	Results: Death in Hours
237	5	6.15	--	--
223	10	6.15	--	--
309	5	6.15	783	23
291	10	6.15	783	25, symptoms
303	5	6.0	783	96, symptoms
280	10	6.0	783	47, symptoms
299	5	5.5	783	--
276	10	5.5	783	94
296	5	5.0	783	46
275	10	5.0	783	25, symptoms
285	5	4.5	783	--
262	10	4.5	783	94, symptoms
275	5	4.0	783	--
256	10	4.0	783	--
270	5	3.5	783	--
255	10	3.5	783	--
250	5	3.5	--	--
245	10	3.5	--	--

Data shown in Table 1 indicate that sterile milk is a suitable substrate for the germination of spores, growth, and toxin production by Clostridium botulinum, type B, strain 783, at room temperature in 10 days, provided the pH of the milk is 4.5 or more alkaline. Under the conditions imposed, pH's of less than 4.5 are not conducive for toxin production by Clostridium botulinum.

Experiment III

Detoxified spore suspensions of Clostridium botulinum were fed in 1 ml. amounts to guinea pigs to assure absence of toxin. All animals so fed survived proving absence of toxin.

Cottage cheese was made from pasteurized milk and curd and whey were bottled then inoculated with Clostridium botulinum, type B, strains 783 and 791. The inoculum consisted of 260,000 spores of strain 783 and 920,000 spores of strain 791 per 100 gr. of cheese. The pH of cheese and whey at time of inoculation was 4.05. Samples of cheese and whey, inoculated and uninoculated, were incubated at room temperature, about 24°C., and 4°C. and fed to guinea pigs in 1 ml. amounts after 5 and 10 days incubation. No deaths caused by botulism occurred among the 16 guinea pigs fed, showing absence of toxin.

Experiment IV

Raw and pasteurized skimmed milk received inoculations of spores of Clostridium botulinum, type B, strains 783 and 791 in the amount of 1,000,000 spores per 100 ml. milk (Early), or 100 gr. bottled cheese (Late). Curd formation required 3 days at room temperature. Early samples showed lenticular gas holes in the curd while uninoculated samples did not. One sample received two inocula, Early and Late. Uninoculated controls of each type of cheese and whey were fed. Incubation periods were 0, 5, and 10 days at room temperature and 5, 10, 15, and 20 days at 4°C. A total of 74 guinea pigs were fed with no occurrence of botulism. One death occurred among control animals fed uninoculated pasteurized cheese which had been incubated 20 days at 4°C., but the animal showed no symptoms of botulism before death or at autopsy.

Experiment V

Cheese was made from pasteurized and raw skimmed milk, inoculated Early or Late with <u>Clostridium botulinum</u>, type B, strains 783 and 791, incubated at room temperature and fed at 1, 5, and 11 days, or at 4°C and fed at 5, 11, 16, and 20 days. A firm curd was obtained in 3 days and pH's were taken on a MacBeth pH meter 3 days after bottling. Control raw cheese had a pH of 4.25; raw whey, pH 4.0; pasteurized cheese, pH 4.1; and pasteurized whey, pH 4.2.

Spore suspensions of <u>Clostridium botulinum</u>, type B, strains 783 and 791 were used to inoculate sheep brain medium. After 20 days incubation at 37°C. both strains caused deaths of 427 and 422 gr. guinea pigs with symptoms of botulism in less than 22 hours. Spore suspensions were fed to guinea pigs of 391 and 491 gr. in 1 ml. amounts and produced no deaths, thus proving the suspension to be detoxified.

A total of 92 feedings resulted in 1 death with clinical symptoms of botulism. The sample was raw Late cheese inoculated with strain 791, incubated 20 days at 4°C., fed to a 266 gr. guinea pig. Death with symptoms of botulism occurred in 52 hours. The brain of this animal was removed aseptically and added to sterile sheep brain medium under vaseline seal and incubated at 37°C. for 7 days, at which time it exhibited the typical odor and gas production characteristic of Clostridium botulinum. When 1 ml. of this medium was fed it produced death with symptoms of botulism of a 412 gr. guinea pig in less than 23 hours. The original cheese sample was fed at 23 days, when symptoms were apparent in the 266 gr. guinea pig, to a 334 gr. animal, and it lived.

Several explanations for the fact that this cheese proved toxic to 1 guinea pig when fed at 20 days, and non-toxic to another at 23 days, are possible. One is that alkaline areas may have existed in the cheese sample where conditions for growth and toxin production by Clostridium botulinum were favorable because of the action of either bacteria or fungi, and that the contents of such an area were included in the 1 ml. cheese fed to the 266 gr. guinea pig that died with symptoms of botulism, and none was present in the 1 ml. sample fed to the 334 gr. guinea pig which survived. Another possibility is that a greater resistance of the latter guinea pig may have been the deciding factor. Partial aeration of the cheese resulting from the removal of the first milliliter sample by pipette may have changed growth conditions, or the sample may have contained close to 1 MLD of toxin per milliliter, and 1 death and 1 survival may be within the range of such a toxicity. Still another possibility is that toxin destruction may have been caused by the presence of bacteria antagonistic to it, as reported by Sherman, Stark, and Stark (1928).

Experiment VI

Raw and pasteurized skimmed milk of pH 6.6 was curdled in 30 hours. Cheese at the time of bottling had pH's of 4.6, raw, and 4.25, pasteurized. Early samples showed gas bubbles in the curd while uninoculated curd did not.

The inocula consisted of Clostridium botulinum, type A, strain 702, and type C, strain 827. The detoxified spores of these strains, when grown in sheep brain medium, produced toxin which caused death of guinea pigs in 19 and 66 hours. Cheese samples inoculated with these strains were incubated for 10 days at room temperature and 20 days at 4°C. and fed to guinea pigs at 5 day intervals.

A total of 78 feedings resulted in the survival of all guinea pigs fed inoculated samples. One control animal fed uninoculated pasteurized cheese which was incubated 15 days at 4°C., died. This animal showed no symptoms of botulism but was markedly unthrifty in appearance at the time of feeding. The 245 gr. animal died in 96.5 hours.

Experiment VII

Pasteurized skimmed milk produced a firm curd in 41 hours, and raw, in 46 hours. Clostridium botulinum, type A, strain 701, and type C, strain 827 were used as inocula. Strain 701 was toxic to a 317 gr. guinea pig in 22 hours in sheep brain culture. Incubation times and temperatures, and experimental procedures for the cheeses were similar to those of Experiment VI.

All of the 76 guinea pigs fed survived.

Experiment VIII

Curd formation was aided by the addition of a rennet extract as described by Tuckey (1942), to make a "sweet curd" cottage cheese. Rennet extract in a solution of such strength that 2 drops were sufficient for 10 gal. of milk was diluted 1:20 with sterile distilled water so that 4 drops were sufficient for a gallon of milk. A firm curd was produced in 71 hours at room temperature in raw and pasteurized milk samples to which the rennet was added. Detoxified spores of Clostridium botulinum, type B, strains 783 and 791 were the inocula. Techniques described in Experiment VI were followed otherwise.

The pH's of the control cheese and whey taken with a MacBeth pH meter at various times in the experiment are given in Table 2.

There were 92 guinea pigs fed and 3 deaths resulting from botulism. Specific details of these deaths follow.

Raw Early whey inoculated with Clostridium botulinum, type B, strain 783 was fed after 5 days incubation at room temperature and produced death of a 281 gr. guinea pig in 48 hours. The brain of this animal was removed aseptically and incubated in sheep brain medium under vaseline seal for 6 days at 37°C. A typical odor of putrefaction caused by Clostridium botulinum, and considerable gas

TABLE 2

ACIDITIES OF RENNET CURD CHEESE

Sample	Incubation		pH
	Time in Days	Temp. C.	
Raw whey	3	RT	4.5
Raw whey	10	RT	4.15
Past. whey	3	RT	4.75
Past. whey	10	RT	4.15
Raw cheese	3	RT	4.35
Raw cheese	10	RT	3.95
Past. cheese	3	RT	4.3
Past. cheese	10	RT	4.1
Raw cheese	3	4^{o}	4.5
Raw cheese	10	4^{o}	4.3
Raw cheese	20	4^{o}	4.7
Past. cheese	3	4^{o}	4.65
Past. cheese	10	4^{o}	4.45
Past. cheese	20	4^{o}	4.8

were evolved. A 397 gr. guinea pig was fed 1 ml. of this medium and died in less than 22.5 hours.

Raw Early cheese inoculated with strain 791 and incubated 5 days at 4^{o}C. produced death with symptoms of botulism of a 219 gr. guinea pig in 48 hours. The cultured brain of this animal produced the characteristic odor of Clostridium botulinum spoilage and caused the death of a 433 gr. guinea pig in less than 22.5 hours.

Strain 791 in pasteurized Late cheese at 4^{o}C. for 5 days caused the death of a 235 gr. guinea pig in less than 52 hours with symptoms of botulism. The brain of this animal produced a culture with typical odor and gas production which killed a 430 gr. guinea pig in 7.5 hours with symptoms of botulism.

The higher incidence of deaths with clinical symptoms of botulism which resulted from this rennet curd cheese and the more alkaline pH's of these samples may well be related. The pH's were often more alkaline than the critical pH of 4.5 at the time the samples produced deaths of guinea pigs (Table 2). These pH's were more acid at 10 days and more alkaline at 20 days than at 3 days. It may be assumed that the addition of rennet extract aided the formation of a curd firm enough for cutting, cooking, and bottling before the low acid pH which inhibits the growth and toxin production of Clostridium botulinum has developed, and it is conceivable that such a cheese might develop high toxicity. In 20 days the samples are so overgrown with molds that the more alkaline pH noted is no longer in the inhibitory range for Clostridium botulinum, but so many changes have taken place that predictability of toxicity is poor since the flora of the cheese has altered greatly in character.

Experiment IX

Pasteurized and raw skimmed milk coagulated with rennet extract were bottled after 71 hours at room temperature. Detoxified spores of Clostridium botulinum, type A, strain 701, and type C, strain 827 were used as inocula. Times and temperatures were as in Experiment VIII but feedings were at 0, 5, 12, and 16 days. The pH's of the samples are given in Table 3.

There were 72 experimental feedings of the cheese and whey samples to guinea pigs, resulting in 1 death from botulism. A 332 gr. guinea pig fed raw Late cheese inoculated with Clostridium botulinum, type C, strain 827 and incubated for 12 days at room temperature died in less than 42 hours. The cheese sample causing death

TABLE 3

ACIDITIES OF RENNET CURD CHEESE

Sample	Incubation		pH
	Time in Days	Temp. C.	
Raw whey	0	RT	4.3
Raw whey	5	RT	4.53
Past. whey	0	RT	4.5
Past. whey	5	RT	4.25
Raw cheese	0	RT	4.3
Raw cheese	5	RT	4.05
Raw cheese	5	4^{o}	4.35
Past. cheese	0	RT	4.55
Past. cheese	5	RT	4.05
Past. cheese	5	4^{o}	4.7

was most odoriferous. The brain culture from this animal caused the death of a 392 gr. guinea pig in less than 23 hours with typical symptoms of botulism.

DISCUSSION OF RESULTS

The results of Experiment III through IX are given in Table 4.

TABLE 4

EXPERIMENTAL MORTALITY

Experiment Number	Total Animals Fed	Uninoculated Control Animals	Deaths with Symptoms Botulism	Deaths w/o Symptoms Botulism	% Mort. Bot.
III	16	6	0	2	0
IV	74	16	0	1	0
V	92	14	1	0	1.28
VI	78	14	0	1	0
VII	76	16	0	0	0
VIII	92	20	3	0	4.16
IX	72	16	1	0	1.78
Total	500	102	5	4	1.26

Of a total of 500 guinea pigs fed cheese or whey, of which 398 received samples which had been inoculated with detoxified spores of <u>Clostridium botulinum</u>, only 5 deaths were proven to be from botulism, or a mortality of 1.26 per cent, and that largely from the deaths of guinea pigs less than 250 gr. in weight. This would seem to indicate that it is possible for cottage cheese made by the techniques described and under the conditions imposed, to become toxic as a result of the germination, growth, and toxin production of spores of <u>Clostridium botulinum</u>, however the probability seems slight that toxicity sufficient to be measurable by methods used here will result.

Of the 5 deaths attributable to botulism, Clostridium botulinum, type B, strain 783 caused 1; type B, strain 791 caused 3; and type C, strain 827 caused 1. Type A, strains 701 and 702, did not cause any samples of cheese or whey to become toxic. Four of the 5 deaths resulted from cheese made by the addition of rennet as a coagulating agent. These 4 deaths from 128 animals fed inoculated samples (Experiments VIII and IX) constitute a mortality of 3.125 per cent, as compared to 1 death of botulism from 270 inoculated samples of acid curd cheese, or a mortality of 0.37 per cent. The less acid pH's occurring in the rennet curd cheese is probably partly if not wholly responsible for the greater number of deaths resulting from botulism.

The toxicity of sterile milk inoculated with Clostridium botulinum spores (Experiment II) at pH's of 3.5 to 6.15 indicates that sterile milk is a suitable substrate for growth and toxin production of Clostridium botulinum at pH 4.5 through pH 6.15, with the particular strains used, and unsatisfactory for toxin production at pH's of 3.5 and 4.0. These results, when compared with those obtained with unsterilized cheese, may indicate that pH is not the only factor determining whether a cheese sample will or will not become toxic because of the presence of Clostridium botulinum, but that a combination of factors, such as pH, symbiotic relationships, acid utilization by other organisms, and toxin destruction by other organisms determines toxicity.

The strains of Clostridium botulinum and techniques utilized in attempting to duplicate the work reported by Nevin (1921) did not reproduce her results (Experiment I). Cottage cheese inoculated with a loopful of 48 hour broth culture of Clostridium botulinum was not rendered toxic to 250 gr. guinea pigs in 1 ml. amounts.

The fact that the sheep brain cultures from which the loopful of vegetative cells of Clostridium botulinum used as an inoculum was taken, proved toxic when fed to guinea pigs, proves that the inoculum itself contains preformed toxin. This work of Nevin's was the pioneer work on botulism from cheese in America, and a lot that is now known was not then known, which probably accounts for her technique not employing detoxified spores. In spite of the addition of preformed toxin to the cheese, sufficient toxin was not present to kill guinea pigs fed the cheese after incubation. This may indicate the unsuitableness of the acid curd cheese for further growth and toxin production by the vegetative cell inoculum of Clostridium botulinum, or the destruction of even the preformed toxin by organisms or environmental conditions in the cheese.

CONCLUSIONS

Cottage cheese inoculated with vegetative cells of *Clostridium botulinum*, using techniques reported by Nevin (1921), did not become toxic.

Sterile milk acidified with lactic acid to pH's of 6.15 to 3.5 became toxic after inoculation with detoxified spores of *Clostridium botulinum*, type B, strain 783 at pH's of 4.5 to 6.15 but remained non-toxic at pH's 3.5 and 4.0.

In seven experiments involving the making of cottage cheese and its inoculation with various strains of *Clostridium botulinum*, 500 experimental feedings were made to guinea pigs. Of the 398 animals fed inoculated cheese, 5 deaths were traceable to botulism for a mortality of 1.26 per cent, indicating that under the conditions imposed by the experimental techniques described and with the strains used, it is possible for *Clostridium botulinum* to produce toxin in cottage cheese, but not probable.

More deaths resulted from inoculated rennet curd cheese than from acid curd cheese, and from *Clostridium botulinum*, type B, than from types A and C.

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