AN ABSTRACT OF THE THESIS OF

Susan DeWitt Nester for the degree of Master of Science in

Foods and Nutrition presented on July 23, 1981

Title: Contamination and Growth of Bacillus cereus and Clostridium perfringens in Mexican-style Beans

Abstract approved: Dr. Margy Woodburn

Because Mexican foods have been implicated in a number of outbreaks of foodborne illness in the United States, production procedures used in Mexican restaurants were investigated by interviewing four managers. Two major problems identified through these interviews were failure to cool large quantities of beans rapidly and failure to reheat beans thoroughly before placement on the steam table. Experiments were designed to study the effects of varying temperatures, duration of incubation, and the location in the product as it might affect aeration on growth of B. cereus and C. perfringens, singly and combined, in cooked mashed pinto beans. Growth of both B. cereus and C. perfringens was rapid at 37°C, with numbers of cells associated with illness reached in 4 and 6 hours, respectively. B. cereus may present more of a health hazard, since obvious signs of spoilage did not occur in these beans until 12 hours, whereas C. perfringens caused obvious spoilage of beans within 6 to 8 hours. At 23°C with B. cereus, the numbers associated with illness were found at 12 hours. However, the beans appeared to be spoiled before this level was reached

with C. perfringens at 24 hours. Aeration, as indicated by location in the jar, appeared to have more of an effect on B. cereus growth than on C. perfringens, but good growth of the two species occurred in both top and bottom locations.

Restaurant samples of bean dip and mashed beans were analyzed for contamination with B. cereus and C. perfringens. Total aerobic and anaerobic counts were determined. Only two of the 42 samples were found to contain B. cereus or C. perfringens and these were present in low numbers. The total aerobic and anaerobic counts varied from less than 100 to 100,000,000 per gram: chiefly lactic acid bacteria which appeared to be related to the seasoning ingredients. However, one batch of bean dip and one of mashed beans were found to contain large numbers of coagulase-positive S. aureus (>100,000/g).

Contamination and Growth of *Bacillus cereus* and *Clostridium perfringens* in Mexican-style Beans

by

Susan DeWitt Nester

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed July 23, 1981

Commencement June 1982

APPROVED:

Head of Department, Foods and Nutrition

Dean of Graduate School

Date thesis is presented July 23, 1981

Typed by Michele Merfeld for Susan DeWitt Nester

ACKNOWLEDGEMENT

My sincere gratitude is expressed to Dr. Margy Woodburn, Head of Department of Foods and Nutrition, for her continued guidance and encouragement throughout my graduate studies.

Appreciation is expressed to Dr. Toshiko Morita for much helpful advice and support during my laboratory work.

Recognition is given to E. J. Schantz of the Food Research Institute, Madison, Wisconsin, who provided B. cereus and C. perfringens cultures for the study.

Thanks are extended to Kathy Morris, Benton County Sanitarian, for her help in obtaining restaurant samples.

Special appreciation is expressed to Gary Beem for his patient assistance during my laboratory work and encouragement during this study.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	5
Nutrient Analysis of Pinto Beans	5
Bacillus cereus Foodborne Illness	5
Diarrheal Syndrome	5
Emetic Syndrome	10
Growth Requirements	14
Sources	15
Clostridium perfringens Foodborne Illness	16
Etiology	16
Incidence	18
Growth Requirements	20
Sources	22
Microbial Competition	23
MATERIAL AND METHODS	25
Growth of *B. cereus* and *C. perfringens* in Mexican-style Beans	25
Bean Preparation	25
B. cereus Inoculum	25
C. perfringens Inoculum	27
Mixed Inoculum Study	28
Determination of Numbers of *B. cereus* and *C. perfringens*	28

Page

B. cereus and *C. perfringens* Contamination in Commercial Samples of Mexican-Style Beans and Bean Dip. . . 30

Samples. 30

Enumeration of the Samples. 32

Seasonings. 34

RESULTS AND DISCUSSION 37

Inoculated Growth Studies. 37

Sporulation. 45

Microbiology of Commercial Bean Samples. 48

Microbiology of Seasonings 57

Restaurant Practices. 59

Recommendations for Further Research and Application. . . . 62

SUMMARY. 65

REFERENCES 68

APPENDIX 74

LIST OF FIGURES

Figure		Page
1.	Procedure for preparation, inoculation, and incubation of beans in growth studies	26
2.	Procedure for enumeration of *B. cereus* and *C. perfringens* in growth studies	31
3.	Modifications of methods of AOAC as used in study	33
4.	Procedure for enumeration of microorganisms present in commercial bean samples	35
5.	Procedure used for enumeration of microorganisms present in seasonings	36
6.	Growth of *B. cereus* strain 4810 in cooked mashed pinto beans at 23°C and 37°C	38
7.	Growth of *B. cereus* strain 4810 in competition with *C. perfringens* strain 232 in cooked mashed pinto beans at 23, 30, 37 and 43°C	41
8.	Growth of *C. perfringens* strain 232 in cooked mashed pinto beans at 23°C and 37°C	42
9.	Growth of *C. perfringens* strain 232 in competition with *B. cereus* strain 4810 in cooked mashed pinto beans at 23, 30, 37, and 43°C	46

LIST OF TABLES

Table		Page
1	The amino acid composition of dry pinto beans (*Phaeseolus vulgaris* L. var. *Piratã*) (Sgarbieri et al., 1979)	6
2	Statistical analysis of the effect of location in the beans, time, and temperature on growth of *B. cereus* strain 4810 in cooked mashed pinto beans	40
3	Statistical analysis of the effect of location in the beans, time, and temperature on growth of *C. perfringens* strain 232 in cooked mashed pinto beans	44
4	Sporulation of *B. cereus* strain 4810 and *C. perfringens* strain 232 in cooked mashed pinto beans at 23°C and 37°C	47
5	Numbers of microorganisms (log viable cells per gram) in restaurant samples at time of purchase and after 5 hours incubation at 30°C, sample date, and temperature of sample upon arrival in laboratory	49
6	Microorganisms (log viable cells per gram) in cooked, spiced bean samples obtained by the Benton County Sanitarian at time of delivery to laboratory and after 5 hours incubation at 30°C, sample date, and temperature of sample when sample was taken, source of sample	52
7	Microorganisms (log viable cells per gram) in seasonings commonly used in Mexican foods	58

Contamination and Growth of *Bacillus cereus* and *Clostridium perfringens* in Mexican-style Beans

INTRODUCTION

Mexican food, such as tacos, burritos, enchiladas, and tostadas, is popular in the United States today, as indicated by a wide variety of restaurants and "fast food" places specializing in Mexican dishes. However, Mexican food has been identified as a vehicle for foodborne illness in a number of outbreaks since 1972. A total of 78 outbreaks associated with Mexican foods were reported to the National Centers for Disease Control (CDC) in the period 1972 through 1978 (Center for Disease Control, 1972b; Center for Disease Control, 1973; Center for Disease Control, 1974; Center for Disease Control, 1975b; Center for Disease Control, 1976; Center for Disease Control, 1977; Centers for Disease Control, 1978b). Of these 78 incidents, seven involved *Clostridium perfringens*, four involved *Staphylococcus aureus* and three involved *Salmonella*. *Bacillus cereus*, *Shigella*, and *Clostridum botulinum* were each responsible for one outbreak. As 61 outbreaks were of unknown etiology during this period, no clear pattern of causality is established. It would be helpful to identify the factors affecting contamination and growth of the microbial agents that may have been involved.

Recent outbreaks suggest bean dishes may be a vehicle for foodborne illness (Centers for Disease Control, 1978a). Since a majority of Mexican foods contain some form of beans, usually kidney or pinto, it would be of value to determine whether beans provide a suitable medium for growth of suspected pathogens. Both bean dip and refried beans seem likely sources of bacterial foodborne illness since they are often

cooked in large quantities and are held for long periods of time prior to consumption. Improper storage conditions could lead to growth if viable microorganisms are present in cooked foods. Since spores of Bacillus cereus (B. cereus) and Clostridium perfringens (C. perfringens) may survive a cooking process or be airborne contaminants later, the organisms are frequently associated with foodborne illness where improper holding practices have been followed.

The extent and importance of B. cereus as a causal agent of foodborne illness in the United States has yet to be determined. Consideration of B. cereus in the laboratory as well as from an epidemiological point of view is not common in investigations of foodborne illness in this country. Due to the similarity between the B. cereus food poisoning syndromes and the well known foodborne illnesses caused by C. perfringens and S. aureus, B. cereus may have been overlooked as a potential causative agent in some outbreaks (Portnoy et al., 1976).

B. cereus is involved in two distinct forms of foodborne illness, each caused by a different toxin. The first of these illnesses resembles C. perfringens foodborne illness and is characterized by severe abdominal pain and profuse diarrhea. Vomiting is seldom present. The incubation period and duration of this diarrheal syndrome also closely parallels those of C. perfringens foodborne illness. A wide variety of foods have been identified as vehicles for the B. cereus diarrheal syndrome, including meat, meat dishes, poultry, raw and cooked vegetables, puddings, fish, pasta, milk and ice cream (Gilbert, 1979). B. cereus was identified as the cause of one food poisoning outbreak in which Mexican food was incriminated (Centers for Disease Control, 1978).

The second type of illness involving B. cereus is the emetic syndrome which resembles S. aureus food intoxication. The symptoms include nausea and vomiting, with incubation periods ranging from one to seven hours. The duration of these symptoms is generally less than 24 hours. Reported emetic outbreaks have been associated almost exclusively with cooked rice and most frequently with fried rice obtained at Chinese restaurants and "take-out" shops (Raevovri et al., 1977; Gilbert and Parry, 1977; Melling et al., 1976).

Outbreaks of unknown etiology are classified by the Centers for Disease Control according to the incubation periods of the illnesses. In the past, incubation periods of between one and seven hours have been classified as "probable S. aureus" while those of eight to fourteen hours have been classified as "probable C. perfringens." It is possible that some of these outbreaks were indeed due to B. cereus, since it is seldom considered as a possible pathogen.

C. perfringens was the third most common bacterial pathogen responsible for foodborne illness in the United States between 1975 and 1979 (Centers for Disease Control, 1981). It was confirmed as the causative agent in 57 outbreaks involving 3223 persons. C. perfringens foodborne illness is characterized by acute, watery diarrhea and abdominal cramps. Fever, vomiting and nausea are seldom present. The incubation period generally ranges from 8 to 24 hours, with symptoms lasting less than 24 hours in most cases.

The foods involved in C. perfringens outbreaks are usually protein-type foods, including meat and poultry, alone or in casseroles and salads (Bryan, 1969). C. perfringens has been identified as the

causative agent in seven outbreaks of illness in which Mexican food was incriminated (Center for Disease Control, 1974; Center for Disease Control, 1975b, Center for Disease Control, 1977; Centers for Disease Control, 1978). In one outbreak, C. perfringens was isolated from bean burrito filling containing no meat or meat extracts, indicating that foods other than meat, poultry, and gravy do contain the essential amino acids to support growth (Centers for Disease Control, 1978a). Rockland and Gardiner (1969) found that dry beans contain an unidentified factor which stimulates rapid growth and gas production by C. perfringens type A. Labbe and Rey (1979) determined that raffinose, a fermentable carbohydrate present in dried beans, increases sporulation and enterotoxin production by C. perfringens type A.

The purpose of this study was to determine the conditions affecting growth of B. cereus and C. perfringens in cooked, mashed pinto beans. The incidence of B. cereus and C. perfringens in commercial samples of pinto beans and bean dip was also examined. The commercial samples were analyzed for total aerobic and anaerobic populations as well as flora capable of growth on Lactobacillus selection medium. Due to findings of large numbers of bacteria, an additional study involving the microbial flora of seasonings was done.

REVIEW OF LITERATURE

Nutrient Analysis of Pinto Beans

Pinto beans and other legumes are an excellent source of protein, generally containing about 23% protein (Watt and Merrill, 1975). The amino acid composition of pinto bean protein is presented in Table 1. In addition to protein, pinto beans contain approximately 35 to 40% starch (% dry weight). The oligosaccharides, sucrose, raffinose, stachyose, and verbascose, together are approximately 10% of the dry weight of the beans (Hodge, 1976). Lipids comprise about 0.5% of the dry weight of pinto beans.

On a cooked basis, the beans contain approximately 8% protein and 21% starch (Watt and Merrill, 1975).

Bacillus cereus Foodborne Illness

Diarrheal Syndrome

There are two main syndromes, diarrheal and emetic, associated with Bacillus cereus; the most well known of these is the diarrheal syndrome. The symptoms include diarrhea, abdominal cramps, and rectal tenesmus. Vomiting and fever are seldom present and incubation is generally between nine and fifteen hours in length. Symptoms do not last more than 24 hours in most cases. Children, however, appear to be more severely affected than most adults in B. cereus foodborne illness (Bodnar, 1962).

The diarrheal toxin involved in this syndrome has been named according to various physiological reactions it induces in both humans

Table 1
The amino acid composition of dry pinto beans
(*Phaeseolus vulgaris* L. var. *Piratã*) (Sgarbieri et al., 1979)

Amino Acid	g/16gN
Lysine	6.54
Histidine	2.94
Arginine	5.14
Tryptophan	1.32
Aspartic Acid	14.12
Threonine	4.53
Serine	5.92
Glutamic Acid	19.41
Proline	3.67
Glycine	3.99
Alanine	4.23
1/2 Cystine	1.31
Valine	4.35
Methionine	1.22
Isoleucine	3.50
Leucine	7.75
Tyrosine	2.60
Phenylalanine	5.62

and test animals. According to Turnbull et al. (1979), the diarrheagenic toxin, diarrheal agent, fluid accumulation factor, vascular permeability factor, dermonecrotic toxin, and intestino-necrotic toxin all refer to a single toxin. This toxin is a relatively heat labile protein with a molecular weight of approximately 50,000 and an isoelectric point of about pH 4.9. It is a metabolite produced by almost all B. cereus strains during vegetative growth. It is unstable at low ionic strength, at pH outside the range 5.0-10.0 and is sensitive to trypsin (Spira and Goepfert, 1975, and Turnbull et al., 1979). This sensitivity may be the reason it is not involved in food poisoning more frequently.

Production by B. cereus of the diarrheal toxin is generally determined using the rabbit ligated ileal loop test and monkey feeding trials. The Y-1 adrenal cell assays and suckling mouse assays have also been used to detect enterotoxin in samples from B. cereus foodborne outbreaks (Melling et al., 1976).

B. cereus has been considered a major cause of foodborne illness in Northern and Eastern Europe for many years. All of the reported B. cereus outbreaks between 1950 and 1973 involved similar symptoms, incubation periods and durations of illness characteristic of the diarrheal syndrome. Between 1960 and 1968, B. cereus was the third most common cause of bacterial food poisoning identified in Hungary. It was responsible for 8.2% of the reported outbreaks and a total of 15% of reported cases (Ormay and Novotny, 1969). Meat and meat dishes were the most frequently implicated foods in these outbreaks. Ormay and Novotny attribute this to the highly spiced dishes of Hungary,

since spices have frequently been shown to contain large numbers of aerobic sporeformers including B. cereus (Ormay and Novotny, 1969; Speck, 1976; Powers et al., 1976; Kim and Goepfert, 1971a).

The number of reported outbreaks caused by B. cereus is rather low in the United States. Most laboratories still do not test for this organism in food samples or fecal specimens obtained from outbreaks of foodborne illness. One reason may be that B. cereus is still considered to be a harmless saphrophyte for the most part (Portnoy et al., 1972; Goepfert et al., 1972). The symptoms of B. cereus food poisoning are usually not severe enough to require medical care, so many incidents are never reported. If a group of individuals becomes ill at the same time with similar symptoms, foodborne illness may be suspected and reported to the authorities (Goepfert et al., 1972; Terranova and Blake, 1978).

Recognition of B. cereus as a health hazard may be improving according to Terranova and Blake (1978). Six out of ten B. cereus outbreaks reported to the Center for Disease Control between 1966 and 1975 were reported during 1974 and 1975. Kim and Goepfert (1971b) also mention that the low number of B. cereus outbreaks reported prior to that time may have been due to the lack of a selective isolation medium and lack of examination of suspected foods for B. cereus rather than no actual outbreaks occurring.

Not much was written concerning B. cereus associated foodborne illness in the United States prior to 1970. However, a 1969 outbreak involving contaminated meatloaf fed to a University of California fraternity was described by Midura et al. (1970). This diarrheal

outbreak had characteristics of the diarrheal syndrome. Turkey loaf was the incriminated food in an outbreak of the diarrheal-type in a Kentucky Veterans Administration Hospital in 1977. In this outbreak B. cereus was isolated from all 14 fecal samples taken from the affected patients. Both food and fecal isolates were shown to have enterotoxigenic activity (Giannella and Brasile, 1975a).

An outbreak in 1975 traced to mashed potatoes from a "take-out" restaurant was investigated (Center for Disease Control, 1975a). The mashed potatoes contained 1.8×10^7 organisms per gram of B. cereus with no other bacterial pathogens isolated. All three isolates from the mashed potatoes were positive for enterotoxigenic activity as measured by the rabbit ligated ileal loop test, as well as the skin permeability test. These tests are indicators of enterotoxigenic activity. Another outbreak involving mashed potatoes occurred in a South Dakota high school in 1978 (Beebe et al., 1978). In both of these outbreaks, symptoms, incubation periods and duration of illness followed the general pattern of the diarrheal syndrome (Beebe et al., 1978).

A wide variety of foods have been identified as vehicles for the B. cereus diarrheal syndrome. These foods include meat, meat dishes, poultry, raw and cooked vegetables, puddings, fish, pasta, milk and ice cream (Gilbert, 1979). Sprouts were identified as a vehicle for B. cereus food poisoning in one outbreak (Portnoy et al., 1976). A seed sprouting kit containing soy, mustard and cress sprouts, was found to contain B. cereus organisms in varying amounts. The soy seeds contained only B. cereus, but the mustard and cress seeds contained

B. cereus as a minor part of the bacterial flora. Cross-contamination during sprouting resulted in large numbers of B. cereus on all sprouts consumed. The individual consuming only a small quantity of sprouts suffered from nausea and vomiting, while the other two individuals who ate sprouts twice that day experienced nausea and vomiting followed by abdominal cramps and diarrhea. The two strains of B. cereus isolated from the contaminated sprouts were positive for enterotoxigenic activity using the rabbit ligated ileal loop technique, the dermal reaction in guinea pigs and the rabbit skin permeability test.

Emetic Syndrome

Bacillus cereus has been identified only recently as also causing an emetic type of illness (Gilbert, 1979). This syndrome, characterized by an incubation period ranging from one to five hours, symptoms of nausea and vomiting, and a duration of less than 24 hours, is most frequently associated with boiled and fried rice. Diarrhea is not a common feature of the illness caused by the emetic toxin.

The emetic toxin is a highly stable molecule, resistant to heating at 126°C for 90 minutes. This toxin is also resistant to pepsin, trypsin and various pH levels. This toxin has a low molecular weight (<5,000) and is not a protein. It is not yet known whether or not the production of this toxin is related to sporulation (Turnbull el al., 1979).

This emetic toxin has been differentiated from the diarrheal toxin with the use of monkey feeding trials and the rabbit ligated ileal loop test (Melling et al., 1976). Turnbull (1976) found the rabbit ligated ileal loop test to be of value only in the diarrheal

toxin test and stresses the need for a more convenient and reliable system than monkey feeding for routine detection of the emetic toxin in food and fecal specimens. In a recent study strains isolated from emetic outbreaks grew and produced the emetic toxin on rice cultures (Turnbull et al., 1979). These strains also produced the diarrheal toxin in culture media. Low levels of the emetic toxin have been obtained by growth in tryptone soy broth and on tryptone soy agar. In addition, the association of two vomiting-type outbreaks with pasteurized cream and a pastry, respectively, suggests there may be substrates other than rice on which the emetic toxin can be produced (Turnbull et al., 1979).

A serotyping system for B. cereus has been investigated in attempts to differentiate isolates from the two syndromes of B. cereus food-borne illness. Eighteen serotypes isolated from cooked rice and fecal specimens were studied by Gilbert and Parry (1977). Serotype 1 is the most common serotype involved in the vomiting type outbreaks, associated with 69% of those studied. Turnbull et al. (1979) suggest this may indicate that only certain strains are capable of producing the emetic toxin. According to Gilbert and Parry (1977), serotypes 3, 4, 5, and 8 were most frequently associated with the emetic outbreaks though serotypes 1 and 8 were also involved in diarrheal episodes. Serotypes 2, 6, 9, 10, and 12 were implicated in different diarrheal outbreaks involving numerous foods.

Since 91% of the uncooked rice samples contained B. cereus, it can be considered part of the normal flora. It is interesting to note, however, that serotypes 1, 3, and 5 made up only 7.5% of the total

serotypes identified on 66 isolates from the 31 samples of rice. Gilbert and Parry concluded that serotyping alone is not sufficient to distinguish between the cultures from the two syndromes.

Between 1971 and 1976 there were more than 70 outbreaks of food poisoning attributed to B. cereus reported in Great Britain (Gilbert, 1976). These episodes followed the general pattern of the emetic syndrome concerning symptoms, incubation periods, and duration of illness. All of these incidents were associated with cooked rice. The rice was usually fried and most frequently obtained at Chinese restaurants and "take-out" shops. Gilbert et al. (1974) found that Chinese restaurants allow the boiled rice to "dry off" at room. temperature for varying periods of time. This rice is left out for a few hours up to three days, usually overnight. The rice to be fried is not stored in refrigerators since this causes the rice grains to stick together and makes "tossing" the rice in beaten egg more difficult. The eggs used in the fried rice were found to be a possible source of contamination since they are not always fresh. In addition, the fried rice is sometimes stored at room temperature and then "flash fried" before serving (Gilbert et al., 1974).

Beckers (1976) investigated a Chinese restaurant involved in two different cases of B. cereus foodborne illness within one week. Fried rice was incriminated as the vehicle of illness. Beckers found that the rice was boiled sometime in the morning between ten and twelve o'clock. The rice was cooked in approximately 6 kg batches and then stored at room temperature until the fried rice was prepared. Samples taken at 2:00 PM revealed low counts of B. cereus in the boiled rice

and freshly prepared fried rice, 2×10^5 and $.5 \times 10^2$ organisms per gram, respectively. Samples taken later in the afternoon at 4:30 PM had counts of 8×10^6 and 8×10^5 organisms per gram in the boiled and freshly prepared fried rice from the same batch of rice. The rice was also examined for Staphylococcus aureus, but it was not isolated from any samples (Beckers, 1976).

Beckers (1976) observed that some fried rice is fried twice. Data indicated that the handling of rice between the two heat treatments permitted substantial growth of bacteria. The brief frying of the rice does not always substantially reduce the number of viable cells. Since the emetic toxin is extremely heat stable, it may not be affected by this short heat treatment.

In another vomiting outbreak, rice had been cooked and refrigerated overnight (Raevouri et al., 1976). On the following morning, it was divided into small portions and put into containers holding hot meat and gravy. The lunch boxes were stored at room temperature for approximately six hours. The incubation period and symptoms followed those of the B. cereus emetic syndrome. Food samples were found to contain large numbers of B. cereus; the rice, 1.0×10^8 organisms per gram, and the meat 1.0×10^6 organisms per gram. The implicated foods were examined and found negative for Staphylococcus aureus, Clostridium perfringens, and Salmonella.

Between 1971 and 1978 five cases of food poisoning involving Chinese food, in which B. cereus was identified as the causal agent, were reported to the Center for Disease Control (Center for Disease Control, 1971; Center for Disease Control, 1972b; Center for Disease

Control, 1973; Center for Disease Control, 1974; Center for Disease Control, 1975a; Center for Disease Control, 1976; Center for Disease Control, 1977; Centers for Disease Control, 1978b). During this period 84 outbreaks of unknown etiology involving Chinese food were also reported. It would be interesting to know how many of these cases could have been traced to B. cereus, had the investigating laboratories tested for its presence in food and fecal specimens.

Growth Requirements

B. cereus is a large, gram positive, aerobic rod though it is capable of growing under anaerobic conditions. It is a sporeformer whose spores are ellipsoidal to cylindrical, central to terminal (Buchanan and Gibbons, 1974). This organism is usually motile.

The optimum temperature for growth of B. cereus is 30-35°C. The minimum temperatures ranges from 10-20°C while the maximum is between 35-45°C. Growth has, however, been reported at 49-50°C (Goepfert et al., 1972). The pH range favorable to growth is approximately 4.9-9.3. The minimum a_w is 0.95 and it is capable of growth in 7% NaCl (Buchanan and Gibbons, 1974).

Citrates can be used as the sole source of carbon by B. cereus. It produces acid from glucose but does not ferment arabinose, xylose or mannitol (Gordon et al., 1973). It is able to hydrolyze starch. The heat resistance of B. cereus spores is generally similar to that of other mesophelic bacteria which are usually destroyed within 30 minutes at 100°C (Banwart, 1979). Some spores, however, are extremely heat resistant, surviving four hours at 135°C (Goepfert et al., 1972). Gilbert and Parry (1977) note that the spores of serotype 1 are more

heat resistant than the spores of other serotypes. Serotype 1 is often associated with the emetic syndrome of B. cereus but has also been isolated from diarrheal outbreak samples.

It has been shown by Gilbert et al. (1974) that B. cereus spores can survive cooking. Rice was inoculated with two strains of B. cereus BC 2 and BC 9. After cooking, less than 20 organisms per gram were found in samples of the rice. Viable spores were present, however, since vegetative growth did occur during subsequent storage. After 24 hours storage at room temperature large numbers of B. cereus were detected in the rice. In addition spores were observed during microscopic examination. Rapid frying and subsequent storage yielded the same result with a small number of viable spores surviving the heat process and growth occurring during storage (Gilbert et al., 1974).

The heat resistance of B. cereus spores is of concern to those involved in food safety since they are present in many foods including spices and dried foods (Speck, 1976; Powers et al., 1976; Blakey and Priest 1979; Ayres et al., 1980).

Sources

B. cereus is widely distributed throughout nature, the environment, and many foods. In a recent study, Blakey and Priest (1979) found that a variety of pulses and cereals, including red lentils and kidney beans contained B. cereus. During normal soaking procedures and storage following cooking, the numbers of B. cereus could increase to a level where toxin production was of concern as a possible health hazard.

Kim and Goepfert (1971a) found 25.3% of the dried foods they examined contained B. cereus, 40% of the spices, 55% of the seasoning

packages, 40% of the dried potatoes, and 37.5% of both milk powder and dried spaghetti sauces. The level of contamination in any of these positive samples did not exceed 4×10^3 organisms per gram and most samples had less than 1×10^3 organisms per gram.

Black and white pepper are known to contain very high microbial counts. Organisms of the genus Bacillus generally dominate the flora of whole spices, herbs, and spice blends, as well as white and black pepper. These bacilli are generally present in spore rather than vegetative cell form. Paprika, celery seed, and ginger also often have high microbial populations (Speck, 1976). Powers et al. (1976) tested 110 spices, including bay leaves, cayenne pepper, chili powder, garlic powder, and cinammon. They found that 53% of the spices tested contained B. cereus, with counts ranging from 5×10^2 to 8.5×10^3 organisms per gram. Eighty-nine of 99 isolates examined were positive for enterotoxigenic activity, using the rabbit ligated ileal loop assay and vascular permeability tests. Each kind of spice tested contained enterotoxigenic strains of B. cereus.

The B. cereus counts of spices and foods are important, since B. cereus spores may survive cooking or be introduced to the food after cooking through dust or contact with unclean equipment, kitchen surfaces, and food handlers, as well as B. cereus-containing spices.

Clostridium perfringens Foodborne Illness

Etiology

Clostridium perfringens type A is recognized as one of the most common causes of foodborne illness in the United States. This illness

is characterized by acute, watery diarrhea and abdominal cramps. Fever, vomiting and nausea are seldom present. The incubation period generally ranges from 8 to 24 hours, with symptoms usually lasting between 12 and 24 hours. The illness most frequently follows consumption of a meat or poultry dish which has been cooked and then held at improper temperatures for a period of time. Other foods which have been implicated in outbreaks of C. perfringens food poisoning include casseroles, salad dressings, and bean burrito filling. Most of these outbreaks were traced to foods cooked in large quantities in restaurants, schools, and other food services.

C. perfringens foodborne illness results from the ingestion of large numbers of viable cells, often between 10^8 and 10^9 cells per gram of food. The ability of these cells to survive the acid environment of the stomach may be due to the buffering action of the food. The cells then multiply and sporulate in the alkaline intestine. The enterotoxin which has been identified as the source of illness has been directly linked to sporulation of C. perfringens in the intestine. This relationship between enterotoxin formation and sporulation was demonstrated by Duncan et al. (1972) using mutants with an altered ability to speculate. Only when these mutants reverted to sporulation was enterotoxin produced. The enterotoxin is formed during the sporulation process and released when sporulation is complete and the vegetative cell lyses to release the free spore. Duncan (1973) detected C. perfringens enterotoxin intracellularly approximately three hours after the inoculation of vegetative cells into the sporulation medium. The increase in intracellular toxin concentration roughly paralleled but

followed by 2.5 to 5 hours, the increase in heat resistant spores (Duncan, 1973).

C. perfringens enterotoxin causes accumulation of fluid in the human intestine. McDonel and Duncan (1977) found that the enterotoxin reversed the net transport from absorption to secretion of water, sodium, and chloride, as well as inhibiting the absorption of glucose when injected into rat and rabbit ileum. Epithelial desquamation was also observed in response to the enterotoxin. McDonel (1979) reported that C. perfringens enterotoxin is also capable of inhibiting energy metabolism and macromolecular synthesis in addition to causing membrane damage to the microvillus brush borders of individual cells.

Though C. perfringens sporulates readily in the intestine, it does not sporulate easily in foods (Genigeorgis, 1975). There have been no reports of C. perfringens food poisoning resulting from ingestion of performed toxin. Stressing that the potential for sporulation is poor in cooked foods, Hobbs (1979) stated that even if toxin is produced in the food it will probably not be enough to cause illness. Nack and Duncan (1976) demonstrated enterotoxin production in cooked chicken, turkey, chuck roast and ground beef. They conclude that preformed enterotoxin may account for the short incubation times observed in some C. perfringens outbreaks. However, in another study Hauschild (1971) found that by the time enterotoxin is detectable in food, the food is not longer considered palatable.

Incidence

In the United States, the number of reported outbreaks of C. perfringens type A foodborne illness has increased since the early

1960's. During the period 1975-1979, the number of reported outbreaks confirmed as C. perfringens food poisonings was 57 (Centers for Disease Control, 1981). These 57 outbreaks involved 3223 persons. This makes C. perfringens the third most common bacterial pathogen (after Salmonella and Staphylococcus). However, these figures do not include the large number of outbreaks in which C. perfringens was suspected but not confirmed as the etiological agent. Outbreaks of C. perfringens food poisoning may be difficult to confirm, due to the problems involved in transporting and culturing the anaerobic specimens (Center for Disease Control, 1977).

An incident in 1972 in which beef strogonoff was implicated as the vehicle for C. perfringens foodborne illness represents a typical outbreak. Approximately 130 of 140 persons who consumed the beef strogonoff suffered from acute gastroenteritis. Of the 38 interviewed over the telephone, 35 had some symptoms and of these, 100% had diarrhea and 83% had abdominal pain. The incubation period ranged from 8 to 15 hours with a mean of 10 hours. A majority of those ill recovered within 24 hours. C. perfringens was isolated from 10 stool specimens. The food had been prepared by a caterer who also became ill from consumption of the strogonoff. Inadequate refrigeration and slight reheating before serving was reported to have led to growth of C. perfringens and the resulting outbreak of food poisoning (Center for Disease Control, 1972).

Though meat and poultry dishes are by far the most common vehicles for C. perfringens food poisoning, an outbreak involving no meat extracts was reported in 1978 (Centers for Disease Control, 1978a).

This outbreak involved contaminated bean burrito filling served at an outdoor fund-raising event in California. The Ventura County Environmental Health Division identified 181 people who became ill, with most suffering from diarrhea (96%) and cramps (79%). Vomiting was a much less common symptom (1.7%). The mean incubation time was 11 hours and symptoms were gone within 24 hours. The bean burrito filling and the unrefrigerated whole bean burrito had counts of 4.0×10^6 and 7.1×10^6 C. perfringens per gram, respectively.

Growth Requirements

C. perfringens is a short, thick, gram positive, sporeforming rod with square ends which is nonmotile and encapsulated (Genigeorgis, 1975). Though C. perfringens is an anaerobe, it is aerotolerant, with strict anaerobic conditions not required for growth. Initiation of growth does depend on the oxidation-reduction potential, the E_h of the medium. The upper E_h limit ranges from +31 mV at pH 7.7 to +230 mV at pH 6.0. The optimum E_h, approximately -200 mV, also varies with the pH as well as the size of inoculum, the strain of C. perfringens, and the metabolic status of the cells. Actively metabolizing cultures are not inhibited by atmospheric oxygen and C. perfringens can grow well in foods that are not extremely anaerobic (Genigeorgis, 1975).

The optimum temperature for growth ranges from 37°C to 47°C with a minimum and maximum of 15°C and 52°C, respectively. Growth occurs between pH 5.5 and 8.0 with a pH of 5.5 resulting in vegetative growth but no sporulation or enterotoxin production (Labbe and Duncan, 1974). A pH range of 6.5 to 7.3 and a temperature of 37°C were reported as

optimum for sporulation and enterotoxin production of C. perfringens.

The minimum a_w for growth is approximately 0.95 to 0.97 but depends on the pH, the strain of C. perfringens, size of inoculum, temperature, and type of solute used for the adjustment of a_w (Kang et al., 1969). It appears that a higher level of a_w is required for spore formation than for vegetative growth. C. perfringens can grow in concentrations of curing salts (sodium nitrate, sodium nitrite, sodium chloride) considerably higher than those used in normal curing procedures (Gough and Alford, 1965). C. perfringens can ferment fructose, galactose, glucose, inositol, lactose, maltose, mannose, sucrose, and starch (Buchanan and Gibbons, 1974). This organism requires 13 to 14 amino acids (see Table 1 for amino acid composition of beans) for growth, in addition to five to six growth factors including calcium, magnesium, iron, sodium, and potassium (Boyd et al., 1948; Fuchs and Bonde, 1957).

Vegetative cells of C. perfringens are rapidly destroyed by heat though the spores vary in their thermal resistance. Ray et al. (1975) found spores of five strains of C. perfringens survived 10-35 minutes at 95°C and one strain survived for three hours at 95°C. In another study, Roberts (1969) found the D_{90} for spores of C. perfringens type A ranged from 3 to 145 minutes. It was assumed for a time that only heat resistant strains were involved in food poisoning outbreaks. Hall et al. (1963), however, found that the C. perfringens foodborne illness outbreaks were not limited to heat resistant strains and stressed the importance of the number of C. perfringens present in the implicated food rather than emphasizing the serology or heat resistance

of the strains. Tong et al. (1962) stated that heat resistant spores may be more often involved in C. perfringens food poisoning, since these are the spores that are more likely to survive the cooking or reheating processes.

Sources

C. perfringens is widely distributed throughout nature. It is frequently found in soil, sewage, and water. This bacterium is also recognized as a common inhabitant of the human gastro-intestinal tract. C. perfringens type A can often be isolated from the feces of healthy individuals in moderately large numbers (Collee, 1974). Counts of between 10^2 and 10^4 organisms per gram of wet weight are not uncommon although there are wide fluctuations in the numbers excreted by any given individual (Genigeorgis, 1975; Bryan, 1969). However, Hobbs (1979) doubts that the role of food handlers as fecal excreters of C. perfringens has any importance in transmission of this illness.

C. perfringens type A is commonly found in raw, cooked, and processed foods. It is often isolated from vegetables, raw meats and poultry and dehydrated soups and sauces. C. perfringens spores are also present on many spices at relatively low levels, generally ranging from ten to several hundred spores per gram (Speck, 1976; Nakamura and Kelly, 1968).

Since the spores of some strains of C. perfringens are very heat resistant surviving 100°C for more than 1 hour, their presence in cooked foods may be unavoidable (Harmon and Duncan, 1980). Spores that do survive cooking or are introduced to the food after preparation, may present a public health hazard if improper holding or storage

conditions exist. These spores can germinate and grow rapidly in the low redox environment of the cooked foods. Consumption of these foods will result in illness, if they have not been thoroughly reheated before serving.

Microbial Competition

The effect of competition between two or more microorganisms depends on the nutrient needs and environmental conditions required for growth as well as any specific antimicrobial compound which may be produced. Some organisms grow in the same environment with little or no effect on their own growth or growth of the other organisms (Pelczar et al., 1977). However, if the organisms are competing for the same nutrients or oxygen, the organism which is capable of more rapid growth under the given conditions, may deplete nutrients needed for growth of the other organism. Growth of one may also alter the pH in a direction (usually more acid) unfavorable for the other, such as the growth of Streptococcus diacetilactis inhibiting Clostridium perfringens and Staphylococcus aureus (Daly et al., 1972).

In contrast to a detrimental effect, an organism may be able to enhance the growth of another organism by providing it with essential nutrients or improved environmental conditions. B. cereus and C. perfringens have been shown to be capable of growth in the same food since an outbreak involving "Meals-On-Wheels" in Great Britain was traced to a chicken dinner. Large numbers of both B. cereus and C. perfringens were isolated from patients' stools (Jephcott et al., 1977). However, since B. cereus is favored by aerobic conditions

and C. perfringens by anaerobic conditions, this may affect how well they compete in cooked foods. B. cereus may consume available oxygen during growth resulting in anaerobic conditions more rapidly than C. perfringens can create growing by itself. To date, no research has been reported to determine if this effect does occur.

MATERIALS AND METHODS

Growth of B. cereus and C. perfringens in Mexican-style Beans

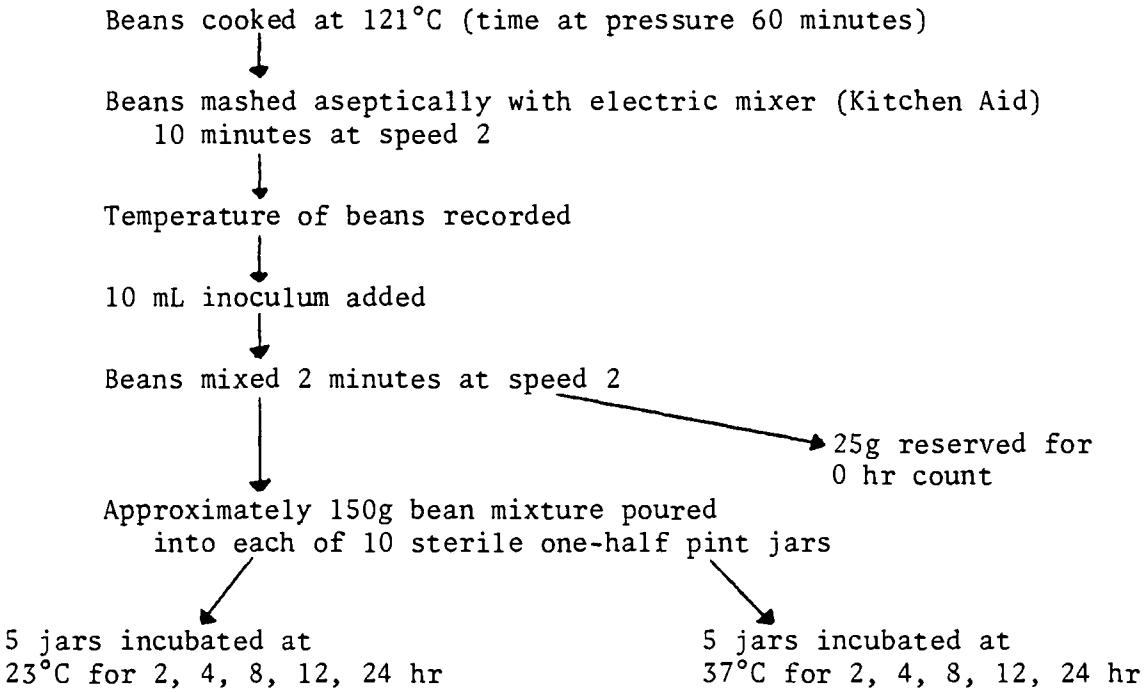
Bean Preparation

The beans were prepared by placing 323g dry pinto beans, 13g NaCl, and 1183 mL distilled water into a Kitchen Aid mixing bowl (Kitchen Aid, Hobart Mfg. Co., Troy, Ohio). The bowl was covered with aluminum foil and placed in an autoclave. The beans were cooked for 60 minutes after the autoclave temperature reached 121°C. The total cooking time was approximately 1 hour 50 minutes which included the time until 121°C was reached and the cooling time. Upon removal from the autoclave, the beans were immediately beaten aseptically for 10 minutes using a mixer (Kitchen Aid) on speed 2. After 10 minutes the temperature of the beans was recorded and 10 mL of spore inoculum was added to the beans. To distribute the inoculum uniformly throughout the bean mixture an additional two minutes of mixing at Speed 2 was done. Following this, approximately 150g of the beans were poured into each of ten sterile one-half pint jars. At the same time, 25g of the bean mixture were reserved for 0 hour count. Five of the jars were incubated at 23°C, while the remaining five jars were incubated at 37°C for the varying lengths of time.

The outline for the experimental procedure is presented in Figure 1.

B. cereus Inoculum

The strain of B. cereus selected for the growth studies was Bc 4810/73. This stock culture was obtained from the Food Research



For mixed inoculum: Same as above until:

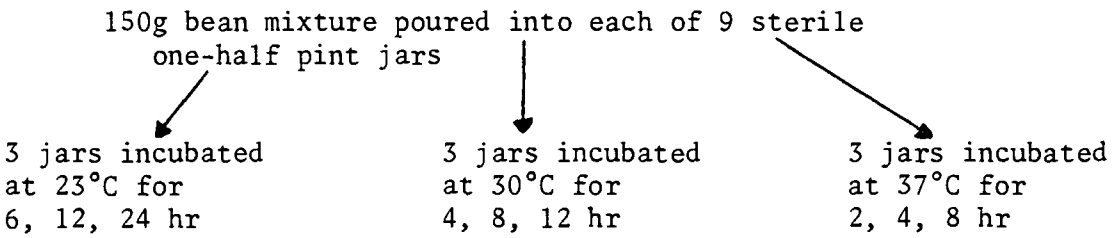


Figure 1. Procedure for preparation, inoculation, and incubation of beans in growth studies

Institute (FRI, University of Wisconsin, Madison, Wisconsin). Bc 4810/73 was originally isolated from vomitus and had been implicated in a typical rice-associated B. cereus food poisoning outbreak (Turnbull et al., 1976).

The lyophilized spores from FRI were suspended in B. cereus spore preparation liquid (Yousten, 1975) and shaken at 200 rpm in a 30°C waterbath for 20 hours. This suspension was held at room temperature and aliquots taken as needed for replications. Enumeration of spores was done using Plate Count Agar (Difco Laboratories, Detroit, Michigan).

C. perfringens Inoculum

The strain of C. perfringens type A used in the growth studies was Cw 232 obtained from E. J. Schantz (Food Research Institute, University of Wisconsin, Madison, Wisconsin). The strain was isolated from a stool sample of a patient who became ill following a dinner party in 1972. Cw 232 was selected on the basis of preliminary tests comparing sporulation of strains Cw 232, Cw 256, Cw 270, Cw 300, and Cw 335. These five strains had been isolated from patients suffering from C. perfringens gastroenteritis and all were obtained from E. J. Schantz at FRI. Cw 232 exhibited greater sporulation than did the four other strains tested.

The lyophilized C. perfringens spores were suspended in C. perfringens sporulation medium of Kim et al. (1967). This preparation was then incubated at 37°C for 24 hours, held at room temperature and used as the stock culture for all growth experiments. The number of spores per mL in the broth was determined with Plate Count Agar (Difco)

incubated anaerobically using a GasPak Anaerobic System (BBL Microbiology Systems, Becton Dickinson and Company, Cockeysville, Maryland).

Mixed Inocolum Study

In order to study the effects of competition between these two organisms, the beans and inocula of B. cereus and C. perfringens were prepared with the same method used for the individual growth studies. For cooking, 1173 mL, rather than 1183 mL of water, were added to the raw dry beans to allow for the additional 10 mL of inoculum. Beans were incubated at three temperatures; 23°C, 30°C, and 37°C, for three replications. An additional replication was done using two temperatures, 37°C and 43°C. Three jars were incubated at each temperature.

Determination of Numbers of B. cereus and C. perfringens

Before removal of samples, changes were noted in visual appearance, including texture and color, and odor. For each jar of beans, 25g from the top portion of the bean mixture were weighed into 225 mL of phosphate-buffered (Lancette and Harmon, 1980) 0.1% peptone (Difco) water. Following this, the center portion of the beans was aseptically removed and discarded. Finally 25g from the bottom portion of the bean mixture were weighed into 225 mL of the phosphate buffered peptone water.

The diluted sample was blended on low speed for 30 seconds in the B. cereus growth study and 10 seconds in the C. perfringens and mixed inoculum growth studies. The blade and gasket of the blender (Osterizer, Model 432A, John Oster Manufacturing Company, Milwaukee, Wisconsin) were sterilized in 70% ethanol for 20 minutes, rinsed in sterile distilled water and then placed on the jar.

Appropriate dilutions of the samples were made using phosphate buffered 0.1% peptone water. All diluent used for the C. perfringens and mixed inocolum growth studies was freshly steamed and cooled.

For the individual growth studies, both B. cereus and C. perfringens enumeration was done using Plate Count Agar (PCA, Difco). For some incubation periods Mannitol-egg yolk-polymyxin (MYP) agar (Lancette and Harmon, 1980) and the Polymyxin Pyruvate Egg Yolk Mannitol Bromthymol Blue Agar (PEMBA) medium of Holbrook and Anderson (1980) were used to observe differences in the recovery of B. cereus due to the use of selective media. The same procedure was done with the C. perfringens growth study using SFP Agar (Difco). All C. perfringens incubation was carried out under anaerobic conditions using a GasPak Anaerobic System (BBL). No antibiotics were added to these media during the individual growth studies.

Enumeration of B. cereus during the mixed inoculum growth study involved use of MYP agar, PEMBA medium and the Plate Count Agar. SFP agar with Kanamycin (Kantrex Bristol Laboratories, Syracuse, New York) and Polymyxin B (Aerosporin Laboratories Division, Pfizer, Inc., New York, New York) added was used for enumeration of C. perfringens along with Plate Count Agar, both incubated under anaerobic conditions.

Sporulation of B. cereus was determined during the individual growth studies by heating the 10^{-1} dilutions at 65°C for 60 minutes before plating. Sporulation determination of C. perfringens involved heating the 10^{-1} dilution at 80°C for 20 minutes before samples were plated.

The outline of the procedure used to enumerate B. cereus and C.

perfringens in the growth studies is shown in Figure 2.

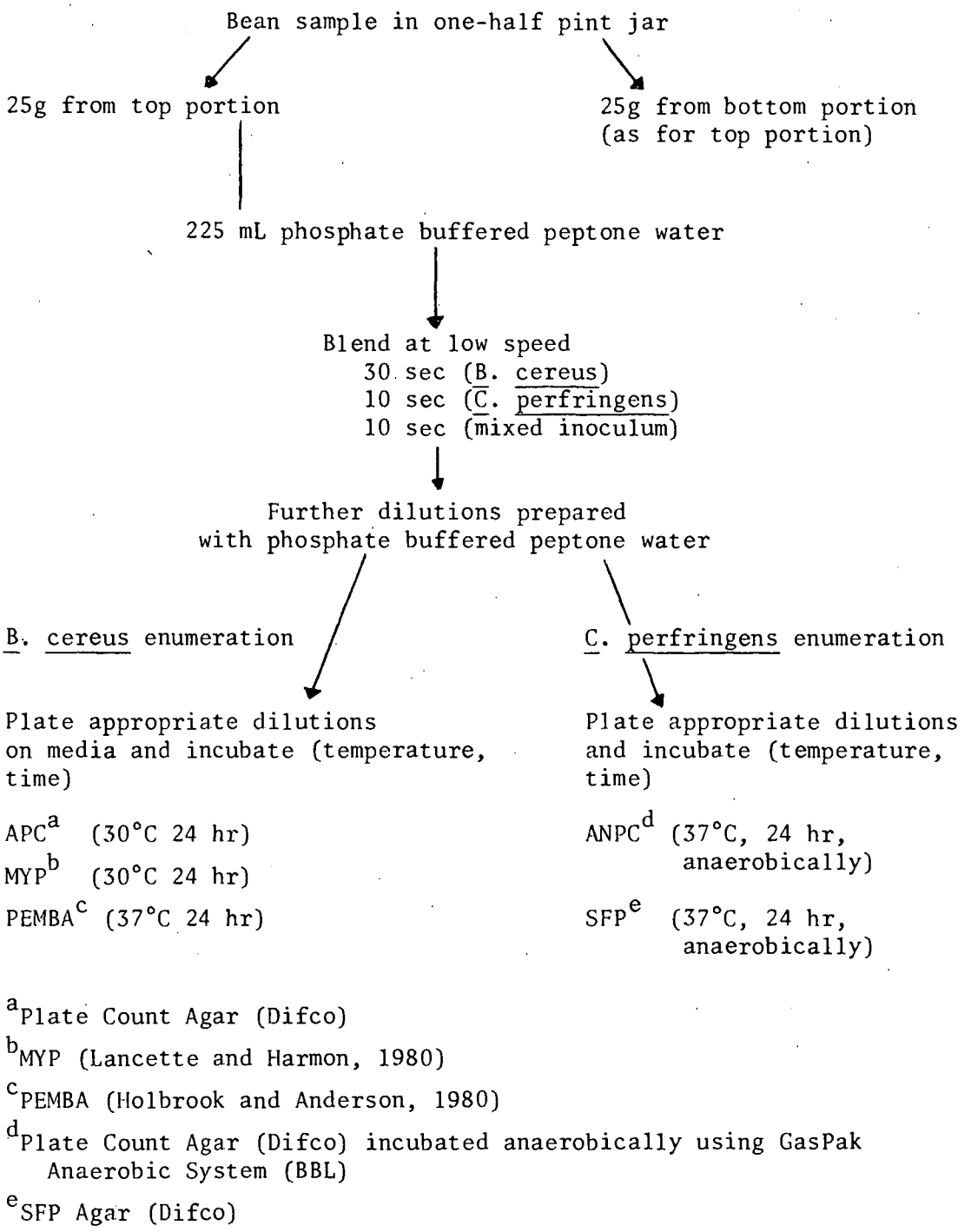
Three replications were completed for the individual growth studies at 23°C and 37°C. A single replication at 30°C of the growth of C. perfringens was also done. Three replications were completed for the mixed inocolum study at 23°C and 30°C while four replications were done at 37°C and one additional replication was done at 43°C.

An analysis of variance for the three factorial experiment was done for numbers of B. cereus and C. perfringens in cooked, mashed pinto beans at selected times. Missing datum was estimated by the method given for randomized blocks by Snedecor and Cochran (1967).

B. cereus and C. perfringens Contamination In Commercial Samples of Mexican-style Beans and Bean Dip

Samples

Samples were obtained from four local restaurants (A, B, C, D) specializing in Mexican food. It was found that two of these restaurants (C and D) microwaved their products immediately before serving. In order to examine samples which had not been exposed to the microwaves, the Benton County Sanitarian, Kathy Morris, obtained cooked, seasoned bean samples from steam tables, refrigerators, and beans set out on the counter to cool. The Sanitarian obtained a total of 15 samples on two different days early in the afternoon from restaurants C and D, as well as two other local restaurants (E and F) serving large quantities of beans. A total of 27 retail samples were obtained on six different days from each Mexican restaurant (A and B) not using the microwave with three samplings in the afternoon (4:30 to



[a]Plate Count Agar (Difco)

[b]MYP (Lancette and Harmon, 1980)

[c]PEMBA (Holbrook and Anderson, 1980)

[d]Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)

[e]SFP Agar (Difco)

Figure 2. Procedure for enumeration of B. cereus and C. perfringens in growth studies

5:00 PM) and three samplings in the evening (8:45 to 9:15 PM).

Two portions of each product sampled were purchased each sampling day. Upon arrival in the laboratory, one portion of each product was placed in a 30°C incubator for 5 hours. The temperature of the other portion was taken immediately and analysis of the product begun. All retail samples were analyzed within 30 minutes of purchase. The samples obtained by the Sanitarian were analyzed within 30 minutes of arrival at the laboratory.

Enumeration of the Samples

The Official First Action of Association of Official Analytical Chemists (AOAC) (Lancette and Harmon, 1980) was followed for *B. cereus* enumeration. An additional medium, PEMBA (Holbrook and Anderson, 1980) was used to determine presence of *B. cereus* in the samples. Alterations from the AOAC Official First Action for *B. cereus* are outlined in Figure 3.

The Official Final Action for *C. perfringens* (Association of Official Analytical Chemists, 1980) was followed for enumeration of *C. perfringens*; spread plates were used throughout the study. Figure 3 outlines alterations made from the AOAC method for determining numbers of *C. perfringens*.

In addition to the selective media used to determine *B. cereus* and *C. perfringens* counts, Plate Count Agar (Difco) was used to determine total aerobic plate counts and total anaerobic plate counts.

Lactobacillus Selective Medium (BBL) was used to determine the presence of lactic-acid bacteria in the samples.

Step	AOAC[a]	Modification
Quantity of sample	50g	25g
Diluent used	phosphate buffer for *B. cereus* .1% peptone water for *C. perfringens*	phosphate buffered .1% peptone water
Blending	2 minutes high speed *B. cereus* 2 minutes low speed *C. perfringens*	10 seconds low speed
Plating	Duplicate plates used	*B. cereus*: one plate of MYP and one plate of PEMBA[b] used *C. perfringens*: no duplicate
Dilutions plated	10^{-2} ——> 10^{-6} for *B. cereus* and *C. perfringens*	0 hr 10^{-2}, 10^{-3} 5 hr 10^{-2} ——> 10^{-5}

[a]Association of Official Analytical Chemists, 1980 and Lancette and Harmon, 1980

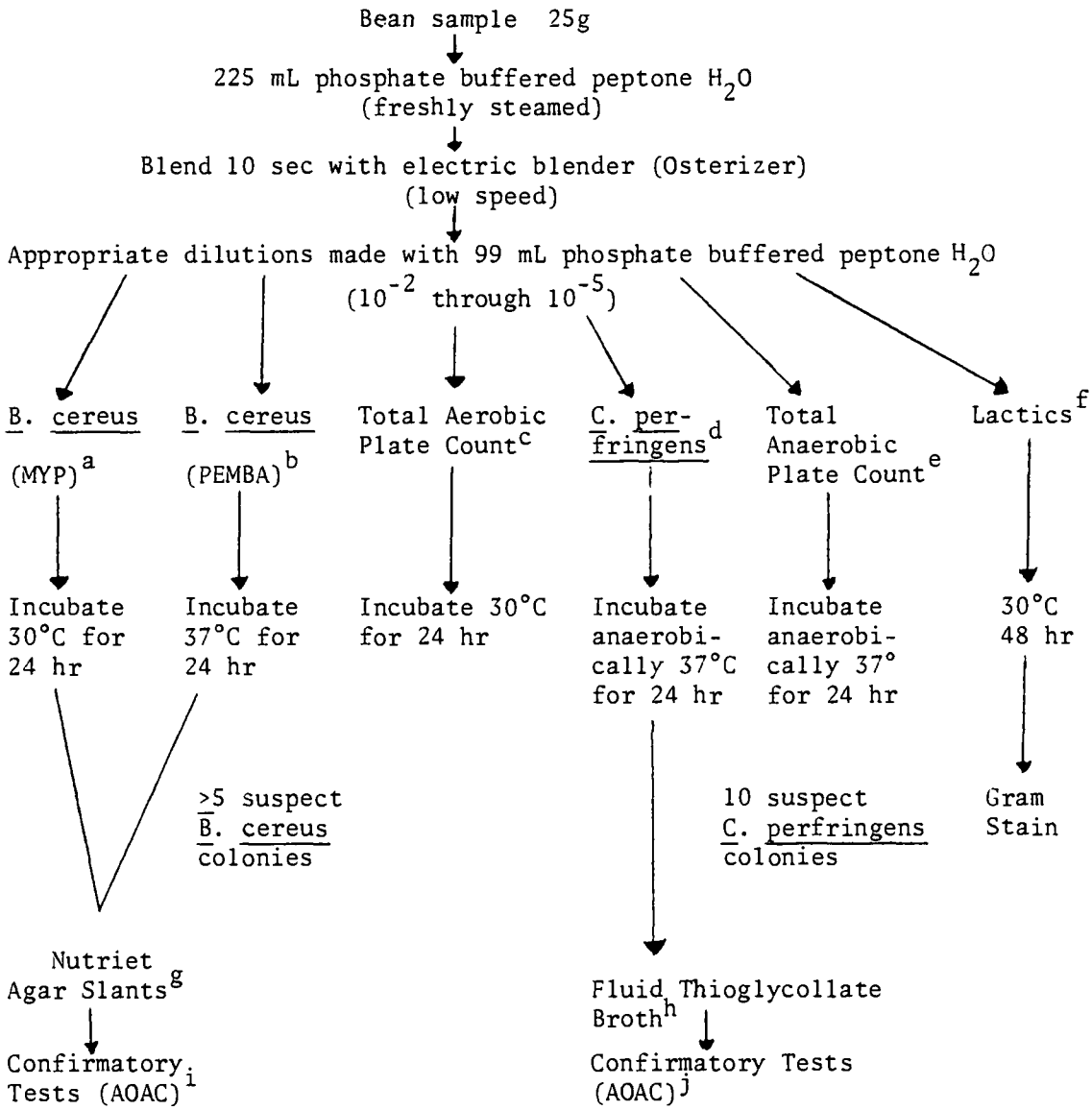
[b]PEMBA (Holbrook and Anderson, 1980)

Figure 3. Modifications of methods of AOAC as used in study

The outline of the procedure used for enumeration of bacteria of the restaurant samples is shown in Figure 4.

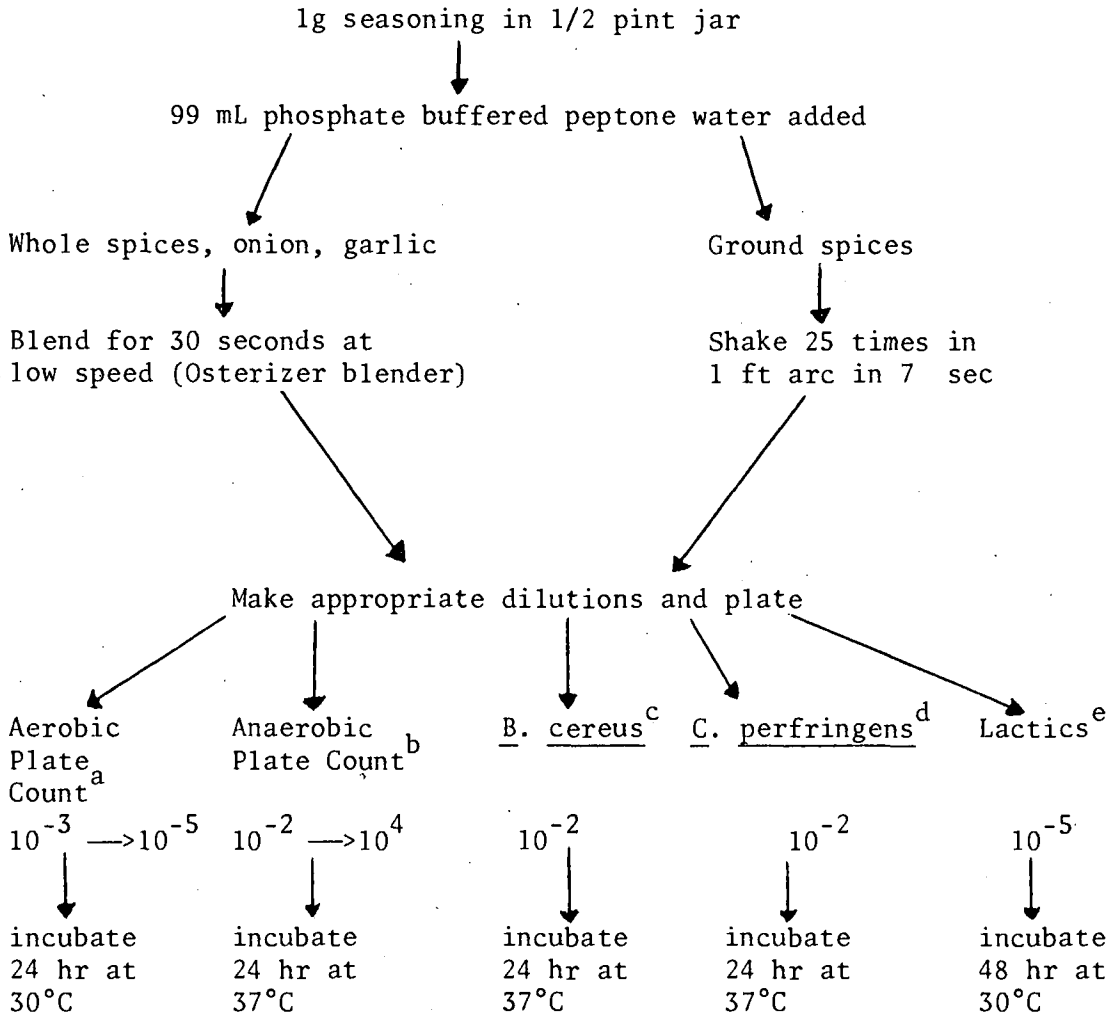
Seasonings

Seasonings commonly used in seasoning Mexican-style foods, as determined in the preliminary interviews with managers, were analyzed for the presence of *B. cereus*, *C. perfringens* and lactobacilli in addition to determining total aerobic and anaerobic counts. The procedure used for this study is presented in Figure 5.



[a]MYP (Lancette and Harmon, 1980)
[b]PEMBA (Holbrook and Anderson, 1980)
[c]Plate Count Agar (Difco)
[d]TSC (AOAC, 1980)
[e]Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)
[f]Lactobacillus selection medium (BBL)
[g]Nutrient Agar (Difco)
[h]Fluid Thioglycollate Broth (Difco)
[i]Lancette and Harmon, 1980
[j]AOAC, 1980

Figure 4. Procedure for enumeration of microorganisms present in commercial bean samples



[a]Plate Count Agar (Difco)

[b]Plate Count Agar (Difco) incubated anaerobically with GasPak Anaerobic System (BBL)

[c]PEMBA (Holbrook and Anderson, 1980)

[d]TSC (AOAC, 1980)

[e]Lactobacillus selection medium (BBL)

Figure 5. Procedure used for enumeration of microorganisms present in seasonings

RESULTS AND DISCUSSION

Prior to planning the studies, practices related to the production and safety of beans and bean products were determined through personal interviews with managers of local Mexican restaurants. Based upon methods commonly used, laboratory experiments were designed to examine the growth of B. cereus and C. perfringens in cooked, mashed pinto beans. The effects of varying temperatures and duration of incubation as well as location in the product, as it might affect aeration, were studied. Restaurant samples were analyzed for contamination with B. cereus and C. perfringens as well as total aerobic and anaerobic populations.

Inoculated Growth Studies

Sterile mashed pinto beans were inoculated while hot (mean inoculation temperature of 68.2°C) with spores of B. cereus and/or C. perfringens. The beans were then incubated at different temperatures for varying lengths of time. After incubation, samples from both the top and bottom portions of the beans were enumerated for the organism(s) being studied. Since counts obtained on the PEMBA selective medium were very close to those on PCA, these plates were treated as duplicates in the enumeration of B. cereus. Since SFP with Kanamycin and Polymyxin B gave counts close to those from the APC incubated anaerobically, these plates were treated as duplicates for enumerating C. perfringens.

Beans inoculated with B. cereus were incubated at 23°C and 37°C. Growth was very rapid at 37°C, following a lag phase of approximately 2 hours (see Figure 6). The lag phase at 23°C lasted about four hours and growth remained slower at this lower temperature.

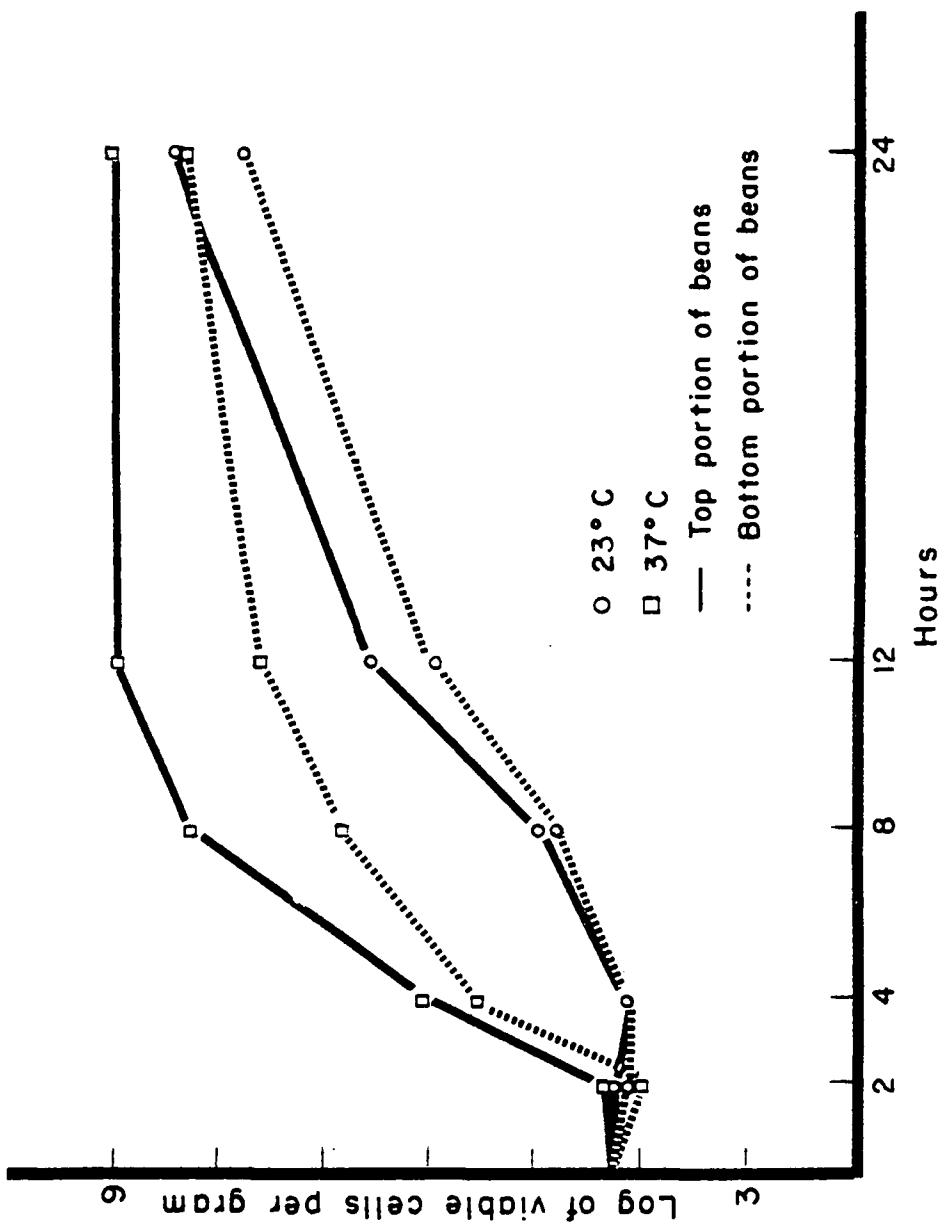


Figure 6. Growth of B. cereus strain 4810 in cooked mashed pinto beans at 23°C and 37°C

In examining differences in growth between the top aerobic portion of the beans and the bottom portion of the beans, it appears that B. cereus grew more rapidly in the top portion of the beans at both 23°C and 37°C. Significantly larger numbers of B. cereus were found; the size of the difference varying with the temperature and time of incubation (significant interaction of location with time and with temperature ≤ 0.01) (Table 2).

A level of 10,000 to 100,000 B. cereus per gram is reported as the minimum for causing B. cereus foodborne illness (Goepfert et al., 1972). This level was reached in the beans incubated at 37°C after 4 hours while it took between 8 and 12 hours to reach this level in the beans at 23°C, in both top and bottom locations. The beans incubated at 37°C had a distinct off-odor after 12 hours of incubation when B. cereus reached a level of approximately 930,000,000 per gram. This indicates an 8 hour interval in which the beans appeared organoleptically acceptable but were a health hazard. After 24 hours the odor was much stronger and, in addition, the top surface of the beans was covered with a white film. The color of the beans had shifted from brown to dark pink. Although the beans incubated at 23°C reached the minimum level for causing illness in between 8 and 12 hours, the beans did not appear to be spoiled after 24 hours incubation.

The growth of B. cereus in the mixed inoculum study was similar to that in the single cultures (Figure 7). Beans inoculated with C. perfringens were incubated at 23°C and 37°C: In addition, one replication at 30°C was completed. The lag phase at 37°C lasted approximately 2 hours whereas at 23°C it appeared to last 4 hours (Figure 8). As

Table 2
Statistical analysis of the effect of location in the beans, time, and temperature on growth of B. cereus strain 4810 in cooked mashed pinto beans

Source of Variation	Degrees of Freedom[a]	Mean Square[b]	F value[c]
Replications	2	0.15	2.9
Time	2	15.43	308.6**
Temperature	1	41.11	822.2**
Location	1	3.86	77.2**
Time x Temperature	2	0.64	12.7**
Time x Location	2	0.46	9.2**
Temperature x Location	1	1.40	28.0**
Time x Temperature x Location	2	.14	2.72
Error	21	1.15	.05

[a] corrected for estimation of missing datum

[b] logarithmic transformations of viable cells per gram

[c] indicates significance at .05 level; ** at .01 level

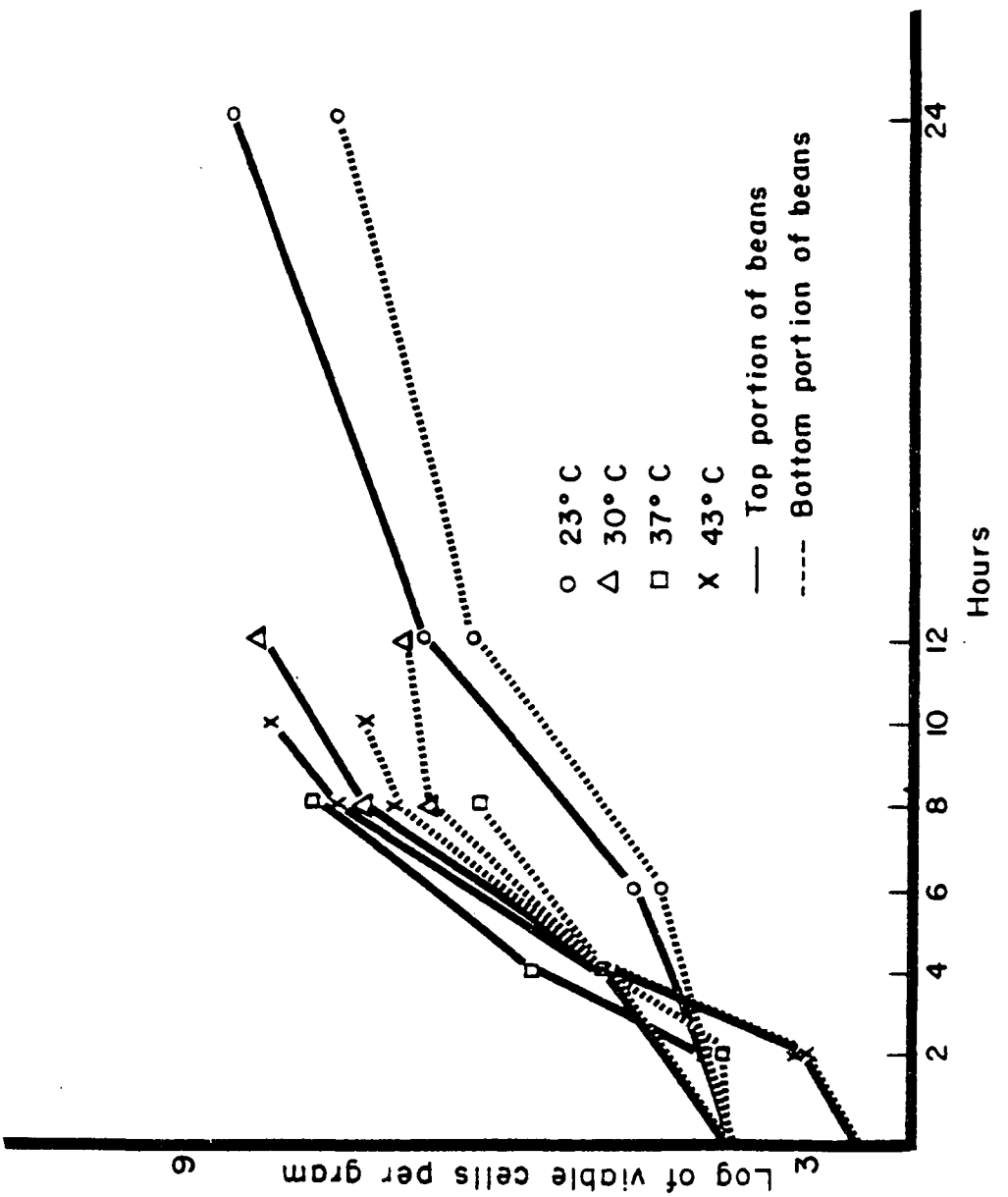


Figure 7. Growth of B. cereus strain 4810 in competition with C. perfringens strain 232 in cooked mashed pinto beans at 23, 30, 37 and 43°C

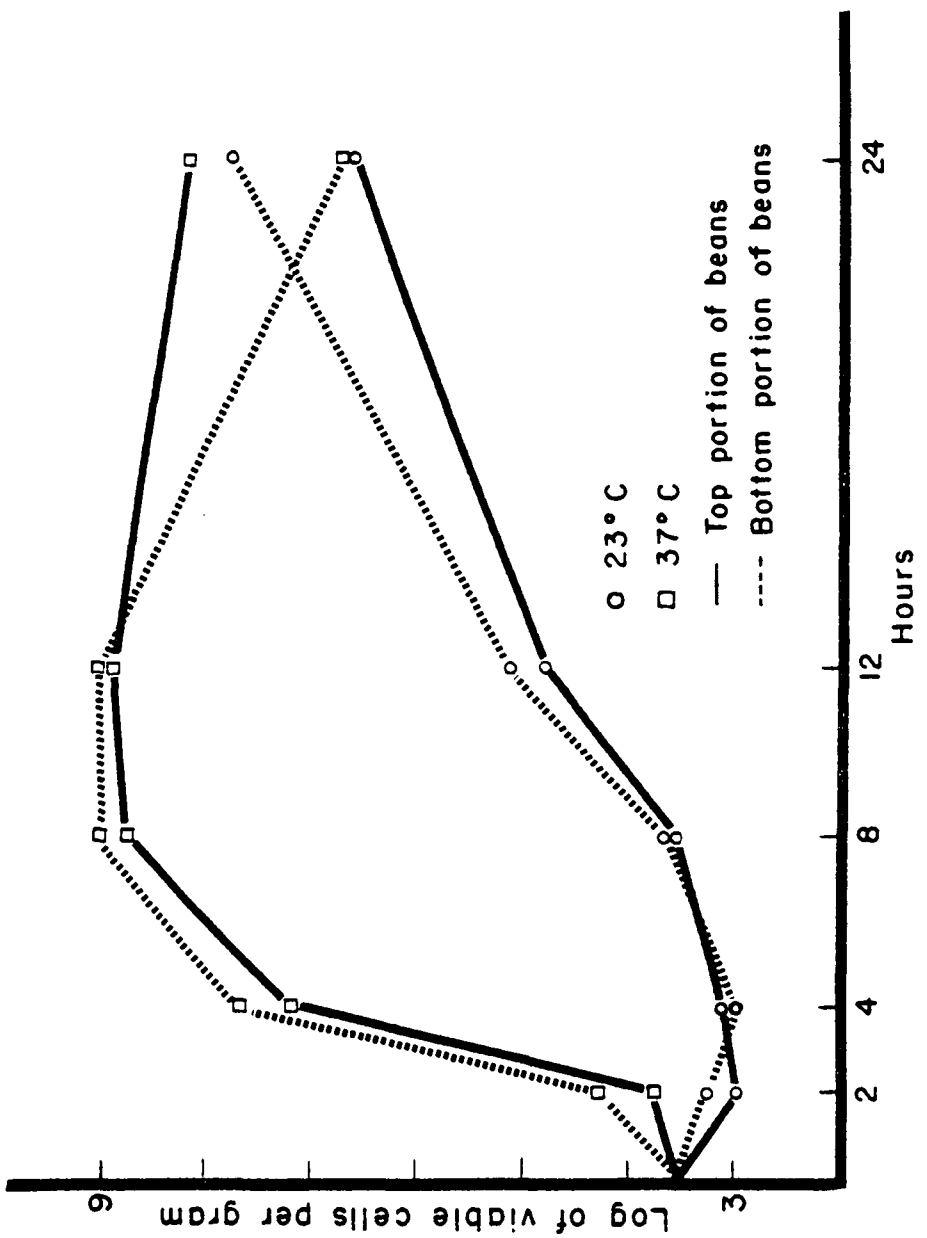


Figure 8. Growth of C. perfringens strain 232 in cooked mashed pinto beans at 23°C and 37°C

with B. cereus, growth of C. perfringens was more rapid at 37°C than at 23°C. At 37°C, C. perfringens reached a maximum level of approximately 800,000,000 per gram by 8 hours and a decrease of about 2 logs was evident in the bottom portion of the beans by 24 hours. At 24 hours, C. perfringens incubated at 23°C was still in its log phase of growth. Location had no significant effect (>0.05, Table 3).

The variability in replications was significant (<.05, Table 3) and may be due to the lower amount of inoculum used in the fourth replication. This inoculum was approximately 10 times less than that used previously. This replication was completed after a four-week interval, due to necessary equipment repair. The variability does not appear to be related to the temperature of the beans at the time of inoculation.

C. perfringens gastroenteritis usually results from the ingestion of between 100,000,000 and 1,000,000,000 organisms per gram of food. This level was reached at 37°C after 6 to 8 hours of incubation. At 23°C, the beans had not yet reached this level after 24 hours incubation. However, in both cases, the beans were obviously spoiled by the time the minimum level for causing illness was reached. The beans inoculated with C. perfringens and incubated at 37°C showed signs of spoilage after 6 hours incubation. The beans had become dry and lumpy, separating into several uneven layers. Gas bubbles were observed on the sides of the jar and the beans had gone from a brown to a pink color. The putrid off-odor noticeable at 6 hours was much more pronounced after 8 hours' incubation. The beans incubated at 30°C and 23°C showed similar signs of spoilage after 12 and 24 hours of incubation, respectively.

Table 3
Statistical analysis of the effect of location in the beans, time, and temperature on growth of C. perfringens strain 232 in cooked mashed pinto beans

Source of Variation	Degrees of Freedom	Mean Square[a]	F value[b]
Replications	1	0.73	6.06*
Time	1	33.15	276.25**
Temperature	1	42.87	357.25**
Location	1	0.09	0.75
Time x Temperature	1	16.46	137.17**
Time x Location	1	0.00	0.00
Temperature x Location	1	0.23	1.92
Time x Temperature x Location	1	0.09	0.75
Error	7	0.87	.12

[a] logarithmic transformations of viable cells per gram
[b] indicates significance at .05 level; ** at .01 level

During the mixed inoculum studies, the beans spoiled in the same manner and time period as in the individual C. perfringens growth study, with one exception. Improved growth of C. perfringens did occur in the beans incubated at 23°C, with a similar growth response in both the top and bottom portion of the beans (Figure 9).

Since spoilage was rapid in beans inoculated with C. perfringens, this organism may not present as great a health problem in beans as B. cereus. However, variability among strains does exist and other strains of C. perfringens may not cause equally obvious signs of spoilage before the danger level is reached. No mention of off-odors or off-flavors was reported in the outbreak of C. perfringens gastroenteritis in California that was traced to bean burrito filling (Center for Disease Control, 1978a).

Sporulation

Sporulation of B. cereus appears to be dependent on aerobic conditions. Spores were present in all bean samples after 2, 12, and 24 hours (Table 4). At 2 hours there were approximately 3000 spores per gram of beans. This probably represented the portion of inoculated spores which had not germinated. The top, aerobic portion of the beans favored sporulation at 37°C. In two of the three replications, there were greater than 100,000 spores per gram after 24 hours. After 24 hours of 23°C, sporulation ranged from 2,700 to 220,000 in the top portion of the beans. The bottom portion of the beans did not show an increase in spore counts above 7,000 after 24 hours at either 23°C or 37°C.

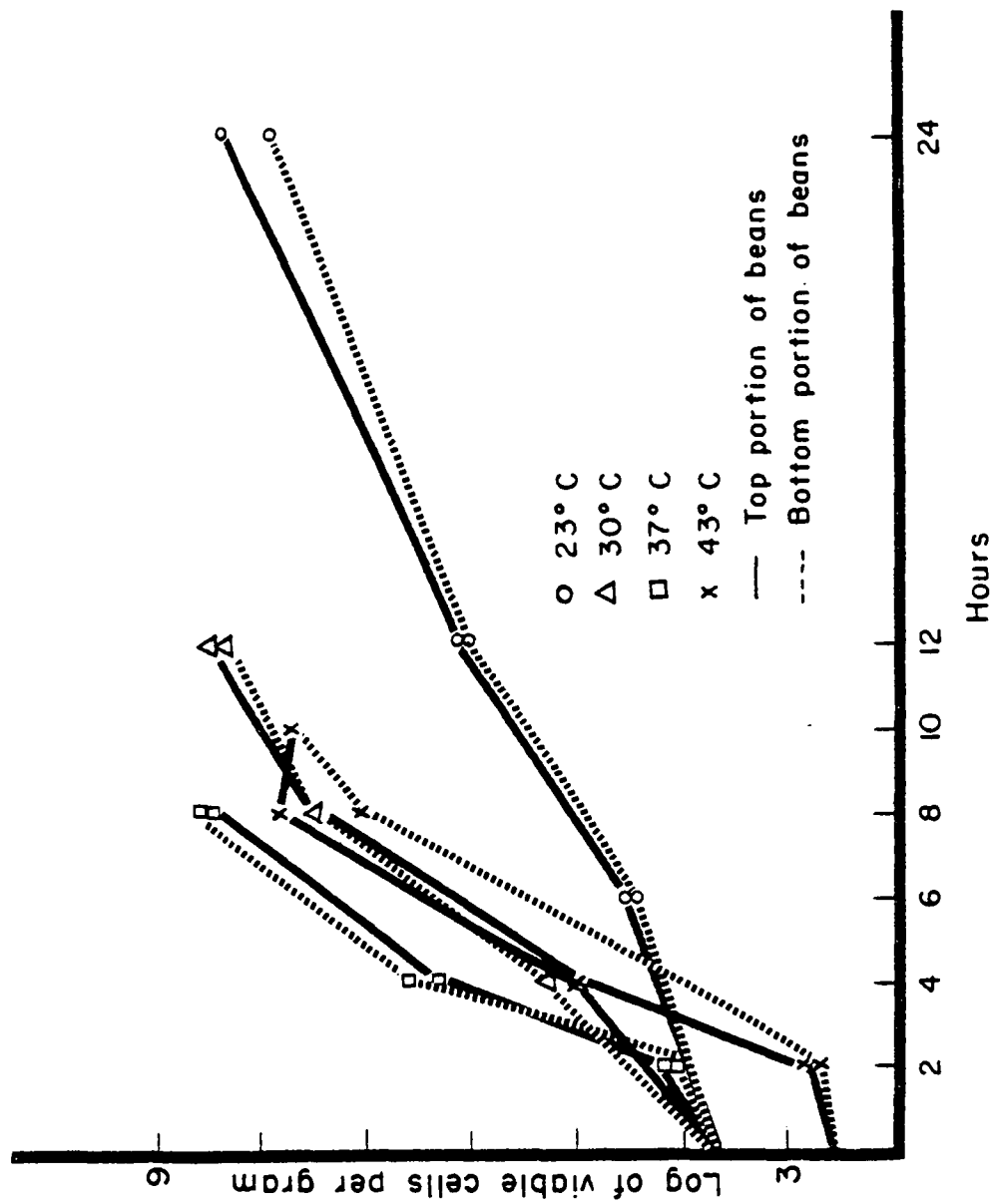


Figure 9. Growth of C. perfringens strain 232 in competition with B. cereus strain 4810 in cooked mashed pinto beans at 23, 30, 37, and 43°C

Table 4
Sporulation of B. cereus strain 4810 and C. perfringens strain 232 in cooked mashed pinto beans at 23°C and 37°C

B. cereus[a]

Temperature (°C)	Location in beans	Time (hr)	Replications 1	2	3
23	Top	2	ND[c]	ND	3.6×10^3
		12	ND	ND	4.5×10^3
		24	2.2×10^5	6.7×10^3	2.7×10^3
	Bottom	2	ND	ND	1.2×10^4
		12	ND	ND	4.0×10^2
		24	7.3×10^3	4.1×10^3	3.1×10^3
37	Top	2	ND	ND	3.1×10^3
		12	ND	ND	est. 9.0×10^5
		24	6.0×10^2	$>3.0 \times 10^4$	3.2×10^7
	Bottom	2	ND	ND	2.8×10^3
		12	ND	ND	5.0×10^2
		24	6.2×10^2	8.0×10^2	4.0×10^3

C. perfringens[b]

23	Top	24	$<1 \times 10^2$	$<1 \times 10^2$	$<1 \times 10^2$
	Bottom	24	$<1 \times 10^2$	8.3×10^3	$<1 \times 10^2$
30	Top	24	ND	ND	$<1 \times 10^2$
	Bottom	24	ND	ND	$<1 \times 10^2$
37	Top	24	$<1 \times 10^2$	ND	$<1 \times 10^2$
	Bottom	24	$<1 \times 10^2$	ND	$<1 \times 10^2$

[a]Plate Count Agar (Difco)

[b]Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)

[c]Not done

Sporulation of C. perfringens was not detected in any of the bean samples with the exception of one in which a count of 8,300 was obtained. Genigeorgis (1975) stated that C. perfringens does not sporulate readily in foods and Hobbs (1979) agreed.

Microbiology of Commercial Bean Samples

Samples of cooked pinto beans and bean dip were obtained from four local restaurants specializing in Mexican food and two other restaurants serving large quantities of pinto beans. These samples were analyzed for the presence of B. cereus and C. perfringens as well as total aerobic and anaerobic plate counts.

B. cereus was found in low numbers on two samples (see Tables 5 and 6). These isolates were restreaked on both MYP agar and the PEMBA medium to observe the presence of typical B. cereus colonies. Both samples yielded typical colonies of B. cereus (Lancette and Harmon, 1980 and Holbrook and Anderson, 1980). The presence of B. cereus in these samples did not constitute an immediate hazard, since the numbers present were not greater than 100 per gram in either sample. Holding the samples at favorable temperatures for a longer period of time may, however, have resulted in a health hazard.

C. perfringens was not present in the restaurant samples examined. It is possible, however, that growth of the lactic acid bacteria present in the samples may have inhibited C. perfringens. Daly et al. (1972) observed 99.90% inhibition of C. perfringens by Streptococcus diacetilactis after 24 hours in broth. Streptococcus diacetilactis was also shown to inhibit Staphylococcus aureus in that study.

Table 5
Numbers of microorganisms (log viable cells per gram) in restaurant samples at time of purchase and after 5 hours incubation at 30°C, sample date, and temperature of sample upon arrival in laboratory

Date	Temp °C	*B. cereus*[a]		*C. perfringens*[b]		Aerobic Plate Count[c]		Anaerobic Plate Count[d]		Lactics[e]	
		0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr
Restaurant A											
Bean Dip											
4-23[f]	54	<1.70	<2.0	<1.70	<2.0	1.0	<2.0	<1.70	<2.0	ND[j]	ND
4-25[f]	42	<1.70	<2.0	<1.70	<2.0	2.68	7.73est	3.62	7.73est	ND	+[k]
4-29[g]	42	<1.70	<2.0	<1.70	<2.0	2.91	4.75	2.96	4.47	ND	ND
4-30[g]	44	<1.70	<2.0	<1.70	<2.0	<1.0	2.48	5.72	8.04	ND	ND
5-6[g]	48	<1.70	<2.0	<1.70	<2.0	4.54	4.64	4.51	4.20	ND	+
5-22[f]	42	<1.70	<2.0	<1.70	<2.0	5.25	7.46[h]	5.08	7.48[h]	5.23	5.97
Refried Beans											
4-23[f]	63	<1.70	<2.0	<1.70	<2.0	1.48	2.48	<1.0	<2.0	ND	+
4-25[f]	55	<1.70	<2.0	<1.70	<2.0	<1.0	2.85	<1.0	2.30	ND	ND
4-29[g]	50	<1.70	<2.0	<1.70	<2.0	<1.0	5.99	<1.0	2.0	ND	ND
4-30[g]	53	<1.70	<2.0	<1.70	<2.0	2.11	<2.0	1.95	2.0	ND	ND
5-6[g]	51	<1.70	<2.0	<1.70	<2.0	3.67	5.85	4.75	4.05	ND	+
5-22[f]	67	<1.70	<2.0	<1.70	<2.0	4.71	4.74	4.90	6.62	5.09	6.67

Table 5 Continued

Date	Temp °C	*B. cereus*[a]		*C. perfringens*[b]		Aerobic Plate Count[c]		Anaerobic Plate Count[d]		Lactics[e]	
		0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr
Restaurant B											
Bean Burrito Filling											
4-25[f]	42	<1.70	<2.0	<1.70	<2.0	4.59	2.30	3.27	2.30	ND	ND
4-29[g]	54	<1.70	<2.0	<1.70	<2.0	2.20	2.30	1.0	2.0	ND	ND
4-30[g]	53	<1.70	<2.0	<1.70	<2.0	1.0	<2.0	1.70	2.30	ND	ND
Refried Beans											
4-25[f]	49	<1.70	<2.0	<1.70	<2.0	1.84	2.30	1.90	<2.0	ND	ND
4-29[g]	52	<1.70	<2.0	<1.70	<2.0	2.26	<2.0	<1.0	<2.0	ND	ND
4-30[g]	53	<1.70	<2.0	<1.70	<2.0	1.85	2.0	1.48	2.90	ND	ND
5-4[f]	57	<1.70	<2.0	<1.70	<2.0	4.69	MD[i]	4.76	LA	ND	+
5-6[g]	56.5	<1.70	<2.0	<1.70	<2.0	5.01	4.65	5.03	4.61	ND	+
5-22	45	<1.70	<2.0	<1.70	<2.0	5.45	5.38	5.51	5.36	5.48	5.34
Restaurant C											
Bean Dip											
4-23[f]	53	<1.70	<2.0	<1.70	<2.0	<1.7	<2.0	<1.0	<2.0		
Refried Beans											
4-23	62.5	<1.70	<2.0	<1.70	<2.0	<1.7	<2.0	<1.0	<2.0		

Table 5 Continued

Date	Temp. °C	*B. cereus*[a]		*C. perfringens*[b]		Aerobic Plate Count[c]		Anaerobic Plate Count[d]		Lactics[e]	
		0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr
Restaurant D											
Bean Burrito Filling											
4-11[f]	53	<2.0	<2.0	<2.0	<2.0	2.61	<4.0	3.10	<4.0		
4-11[g]	63	<2.0	<2.0	<2.0	<2.0	2.60	<4.0	2.93	<4.0		
Refried Beans											
4-11[f]	55	<2.0	<2.0	<2.0	<2.0	3.98	<24.0	3.52	<4.0		
4-11[g]	63	<2.0	<2.0	<2.0	<2.0	3.13	< 4.0	3.92	6.59		

[a] PEMBA (Holbrook and Anderson, 1980) and MYP agar (Lancette and Harmon, 1980) treated as duplicate plates

[b] TSC agar (AOAC, 1980)

[c] Plate Count Agar (Difco)

[d] Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)

[e] Lactobacillus Selection medium (BBL)

[f] Time of sampling 4:30 - 5:00 PM

[g] Time of sampling 8:45 - 9:15 PM

[h] Confirmed as coagulase-positive *S. aureus*

[i] Missing data

[j] Not done

[k] + indicates growth on Lactobacillus selection medium; -, no growth on this medium

[l] samples heated in microwave range immediately before purchase

Table 6
Microorganisms (log viable cells per gram) in cooked, spiced bean samples obtained by the Benton County Sanitarian at time of delivery to laboratory and after 5 hours incubation at 30°C, sample date, and temperature of sample when sample was taken, source of sample

Restaurant	Location and Date (1981)	Temp. (°C)	*B. cereus*[a]		*C. perfringens*[b]		Aerobic Plate Count		Anaerobic Plate Count		Lactics[e]	
			0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr
C	Steam Table											
	4-7	60	<1.70	ND	<1.70	ND	5.05	ND	4.51est	ND	ND[i]	ND
	4-21	49	<1.70	<2.0	<1.70	<2.0	3.84	4.83	4.10	4.61	4.91	5.61
	4-21	43	<1.70	<2.0	<1.70	<2.0	4.54	4.72	4.77	4.66	4.70	4.78
	Walk-In Refrigerator											
	4-7	3.5	<1.70	ND	<1.70	ND	5.05	ND	5.06	ND	ND	ND
D	Steam Table											
	4-7	MD[f]	<1.70	<2.0	<1.70	<2.0	5.49est	6.99	4.92	5.01	ND	ND
	4-7	65.5	ND	<2.0	ND	<2.0	ND	5.26	ND	5.03	ND	ND
	Walk-In Refrigerator											
	4-7	MD	<1.70	<2.0	<1.70	<2.0	5.44est	6.81	4.99	4.68	ND	ND
	4-7	9	ND	<2.0	ND	<2.0	ND	6.05	ND	4.91	ND	ND
	Unrefrigerated[g]											
	4-21	52	<1.70	<2.0	<1.70	<2.0	4.65	4.31	4.59	4.56	4.60	5.69

Table 6 Continued

Restaurant	Location and Date (1981)	Temp. (°C)	B. cereus[a]		C. perfringens[b]		Aerobic Plate Count		Anaerobic Plate Count		Lactics[e]	
			0hr	5 hr	0hr	5hr	0hr	5hr	0hr	5hr	0hr	5hr
E	Steam Table											
	4-7	MD	<1.70	<2.0	<1.70	<2.0	5.18	8.04[h] est	5.33	6.91	ND	ND
	4-21	65.5	<1.70	<2.0	<1.70	<2.0	4.64	5.06	4.89	4.79	4.91	5.61
	Refrigerator											
	4-7	39	<1.70	<2.0	<1.70	<2.0	5.98 est	7.40[h]	5.86 est	7.41	ND	ND
	Unrefrigerated[g]											
	4-21	57	<1.70	<2.0	<1.70	<2.0	4.97	3.67	4.74	3.83	4.00	4.56
F	Walk-In Refrigerator											
	4-21	6	<1.70	<2.0	<1.70	<2.0	4.80	3.58	4.75	4.70	4.84 est	4.64
	Microwaved											
	4-21	60	<1.70	<2.0	<1.70	<2.0	4.79	4.77	4.60	4.71	4.75	4.83

[a] PEMBA (Holbrook and Anderson, 1980) and MYP agar (Lancette and Harmon, 1980) treated as duplicate plates

[b] TSC agar (AOAC, 1980)

[c] Plate Count Agar (Difco)

[d] Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)

[e] Lactobacillus Selection Medium (BBL)

Table 6 Continued

---

[f]Missing Data

[g]Beans set out on counter

[h]Confirmed as coagulase-positive Staphylococcus aureus

[i]Not done

One bean dip sample (Restaurant A) and one batch of beans (Restaurant E) were found to contain large numbers of coagulase-positive Staphylococcus aureus (counts in excess of 100,000 per gram at 0 hour) (Tables 5; 6). Since these strains of S. aureus may produce enterotoxin, this is cause for concern. This toxin is extremely heat stable and is not destroyed by normal reheating procedures. Thus, toxin production in food may result in an outbreak of S. aureus intoxication. A current study in our laboratory indicates that S. aureus does have the ability to produce enterotoxin in cooked mashed, pinto beans.

The total aerobic and anaerobic counts varied from a count of less than 100 to an estimated 100,000,000 (see Tables 5; 6). Some of the variability in these counts may be due to the difficulty involved in counting the extremely minute colonies on these plates. Examination of the gram stains of these colonies revealed gram positive, tiny thin rods; cocci; short thick rods; and some larger rods with inclusion bodies. These organisms appear to be members of the genus Lactobacillus. Therefore, later samples were also plated on Lactobacillus Selective Medium (LBS, BBL). Counts on this medium were similar to the counts obtained for the total aerobic and anaerobic counts.

It should be noted that counts for the microwave-heated samples from restaurants C and D were relatively low; <10,000 for all samples. It must be remembered that the counts obtained from these samples may not reflect the safety of the food since heat stable toxins will remain after exposure to microwave heating.

The dip samples which had not been microwave-heated generally

had higher aerobic and anaerobic plate counts than did the refried bean samples and the bean burrito filling. These bean dip samples had a mean temperature of 45.3°C; the refried beans, 56.6°C, and 53°C for restaurants A and B, respectively (Table 5). Some cooling had occurred in the short interval of transporting the samples; however, aseptic procedures were necessary so temperatures were not recorded at time of purchase. A mean bean dip temperature of 45.3°C is within the optimum range for C. perfringens and the maximum range for B. cereus. These bean dip samples were obtained from the restaurant which holds bean dip over a double boiler to keep it "warm" for serving. It appears that this method of holding the dip maintains the temperature of the bean mixture within the "danger" zone (4-60°C) for growth of pathogenic bacteria. Bryan (1981) stressed that "hot-holding" of foods can be a very hazardous operation in a food service establishment and referred to it as a critical control point. He stated that most foods should be heated to 60°C or higher before they are put into hot-holding devices and as soon as their temperature falls below 55°C they should be served, reheated, or chilled and later thoroughly reheated. This practice is not observed in at least two of the restaurants sampled.

Another factor which may contribute to the higher aerobic and anaerobic counts on bean dip is the quantity of seasonings added to this product. The bean dips contained a much larger amount and a wider variety of seasonings than the mashed beans served at the local restaurants. One should keep in mind, however, that spices still constitute only a small portion of the bean product. These seasonings

were added after the beans were cooked. A preliminary microbiological investigation of the seasonings was therefore added.

Microbiology of Seasonings

Examination of seasonings commonly used in Mexican-style foods revealed large numbers of aerobic and anaerobic organisms. The total aerobic and anaerobic counts were close to the counts obtained on LBS agar (Table 7). Gram stains of the organisms revealed gram positive tiny cocci, small thick rods, and long rods.

The growth of lactic acid bacteria in foods results in lactic acid production accompanied by a lowering of the pH. This lower pH may inhibit growth of pathogenic bacteria, thus acting as a "safety factor" in spiced foods (Daly et al., 1972). Two restaurant managers mentioned batches of beans, which appeared to have fermented during storage. Much gas production and an off-odor were noticeable. This bean spoilage may have been due to the presence of lactic acid bacteria. Both restaurants discarded the spoiled beans.

Although spices are a common source of B. cereus only one sample yielded a typical B. cereus (Table 6) colony on the 10^2 dilution on PEMBA. Spices are also a common source of C. perfringens. One sample, cayenne pepper, yielded typical colonies on TSC agar (total TSC count 800/g). The 10^2 dilution was the only one plated for both selective media. Since B. cereus spores have been found to be present in numbers ranging from 100 to 1000 per gram in other studies (Speck, 1976, Powers et al., 1976, Kim and Goepfert, 1976, and Ayres et al., 1980) and C. perfringens in numbers ranging from 10 to several hundred per gram (Speck, 1976; Nakamura and Kelly, 1968); a lower dilution

Table 7
Microorganisms (log viable cells per gram) in seasonings commonly used in Mexican foods

Seasoning	*B. cereus*[a]	*C. perfringens*[b]	Aerobic Plate Count[c]	Anaerobic Plate Count[d]	Lactics[e]
Fresh onion	<2.00	<2.00	5.04	5.73	5.93
Fresh garlic (peeled)	<2.00	<2.00	5.19	5.00	6.93
Fresh garlic (unpeeled)	<2.00	<2.00	5.79	5.88	7.02
Hot Sauce	<2.00	<2.00	6.88	6.05	7.49 est
Black Pepper A (Ground)	<2.00	<2.00	7.25	6.84est	7.17
Black Pepper B (Ground)	<2.00	<2.00	7.18	6.78est	6.71
Black Pepper C (Ground)	<2.00	<2.00	7.11	6.61	6.60
Chili Powder A	<2.00	<2.00	6.97	6.05	6.25
Chili Powder B	<2.00	<2.00	5.96	6.10	6.96
Chili Powder C	<2.00	<2.00	6.76	5.91	6.90
Cumin (ground)	<2.00	<2.00	6.85	6.17	6.89
Cumin (ground)	<2.00	<2.00	6.86	5.89	7.07
Cumin Seed	<2.00	<2.00	6.94	6.32	7.00
Garlic powder	<2.00	<2.00	6.81	6.09	6.92
Cayenne	<2.00	2.90	7.01	6.16	6.94
Onion powder	<2.00	<2.00	6.57	5.85	6.83
Crushed Red Pepper	<2.00	<2.00	7.13	6.56est	6.97
Chilies	<2.00	<2.00	5.99	5.92	6.75

[a] PEMBA (Holbrook and Anderson, 1980)
[b] TSC agar (AOAC, 1980)
[c] Plate Count Agar (Difco)
[d] Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL)
[e] Lactobacillus Selection Medium

of the sample would have permitted a more definitive study of contamination of spices with these two species.

The total aerobic, anaerobic and lactic counts on the black pepper samples were similar to those reported by Draughton et al. (1981). However, the chili powder yielded counts 10 to 100 times greater and the cumin, 1000 times greater than those found in their study.

Restaurant Practices

Interviews were conducted with the managers of the four local restaurants which specialize in Mexican food, to determine methods used to cook beans and common holding practices. In restaurants B and D, beans were cooked under pressure (10 lb. pressure for 1 hour 40 minutes and 12 lb. pressure for 45 minutes, respectively). In the other two restaurants, A and C, the beans were boiled without pressure (2 hours on medium high heat and 9 hours on low heat, respectively). In all restaurants salt was added; in B and D, lard (B) or shortening (D) was also added before cooking. Fresh onion, garlic powder, spices, and spice mixes were also included by some of the restaurant cooks during cooking.

Most restaurant cooks mashed or whipped the beans using a drill-like instrument with a beater-like attachemnt, after allowing them to cool slightly. Additional seasonings were added to some beans at this time. Generally beans were held in a walk-in refrigerator overnight or longer. One restaurant practice, however, was to serve the beans the same day after simmering the beans, or, if mashed, to

hold them over a steam table until required.

The Benton County Sanitarian (Kathy Morris, personal communication) reported that during several visits she noticed deep tubs of beans (10-18" deep) set out on the counter to cool in two restaurants. In both cases the beans had been cooked in the morning and were still sitting out at 2:30-3:00 P.M. In one instance, the beans were near an open screen door which might allow for contamination through dust or airborne bacterial spores.

Improper cooling is the most frequent factor contributing to foodborne disease outbreaks according to Bryan (1981). Cooling is therefore the most critical control point in food service. He stated that most outbreaks of bacterial foodborne illness are due to cooked foods being left at room temperature for several hours before being refrigerated. Solid and semisolid food should be cooled rapidly and should not be placed in pans deeper than four inches (Bryan, 1981). Many of the tubs used to store beans in the refrigerator were large and deeper than this recommendation (6-8"). This results in a long cooling period until the beans reach the refrigeration temperature. During this period the beans remain at ambient temperatures, thereby allowing possible growth of bacteria. A large mass of food should be cooled rapidly in water or ice baths or by other means prior to refrigeration.

Methods of reheating the beans before serving varied. Restaurant B cooks heated the beans over direct heat until they reached approximately 160°F (71°C) after removal from the walk-in refrigerator. The beans were then placed on the steam table maintained at 160°F. In

restaurants C and D, beans were put from the refrigerator directly onto the steam table to heat. These two restaurants had microwave ranges and heated all meat, bean, or cheese dishes immediately before serving. Restaurants A and C serve bean dip. In restaurant A, leftover beans from the previous day are seasoned with cumin, oregano, garlic, tomatoes, and peppers. The dip is heated over a double boiler until "hot." When the dip is "hot," the heat is lowered so the bean dip can be served "warm." Restaurant C cooks add pepper, onion, spices, jalapeños, and cheese to beans taken from the steam table. The dip is then held in a crock pot and heated in a microwave range before being served.

It should be noted that the concepts of spore survival and heat stable enterotoxins were not well understood by the managers interviewed. In one instance, a manager mentioned that if a tub of beans had inadvertently been left at room temperature overnight, it was thoroughly reheated, and used the following day. The reheating may kill vegetative cells but not inactivate the heat stable toxins of S. aureus or the emetic toxin of B. cereus (Fung and Cunningham, 1980).

Restaurant personnel should be careful not to gain a false sense of security through use of the microwave range. It is important that the food have microbial counts as low as possible before being heated by microwaves. The exposure times recommended by manufactures may not be adequate to result in uniform heating to temperatures necessary for destruction of high levels of vegetative bacteria in foods. In addition, spores are more resistant to heat than vegetative cells and different species have different survival abilities.

One must also keep in mind that preformed heat-stable toxins such as those of S. aureus and the emetic toxin of B. cereus will not be inactivated by this amount of heating before serving. Thus, the holding conditions of the food before microwave heating are of extreme importance in determining the final microbiological condition and safety of the food.

Recommendations for Further Research and Application

There are further areas in which research is needed. B. cereus is responsible for two distinct syndromes of foodborne illness. Both syndromes result from the ingestion of preformed toxin. Since almost all strains produce the diarrheal toxin as a metabolite during vegetative growth, it would have been of value to test for this toxin in the growth studies. When the study was planned an agreement was made with E. J. Schantz of Food Research Institute to provide antiserum, which was soon to be available. However, the antiserum is still not available. None of the animal assays were practical for our laboratory situation.

Since the strain of B. cereus used in this study, Bc 4810/73, was originally isolated from vomitus of a patient, it is capable of production of the emetic toxin. This toxin has reportedly been produced in large quantities only in rice (Turnbull, 1976). Growth of B. cereus in rice is stimulated by the addition of both egg and Casamino acids (with B-complex vitamins) (Morita and Woodburn, 1977). Beans contain more protein (23%) than does rice (6.7%) whereas rice contains more starch (80%) than do beans (45-50%) when comparing

dry weights (Watt and Merrill, 1975). The toxin has been produced in small amounts in both tryptone soy broth and tryptone soy agar (Turnbull et al., 1979). B. cereus strain 4810/73 had a similar growth pattern in beans to that of B. cereus strain 158/73 (isolated from rice implicated in an emetic outbreak of B. cereus food poisoning) in rice with added egg protein (Morita and Woodburn, 1977). The maximum level of 100,000,000 organisms per gram of rice was reached in approximately 8 hours at 43°C and 8 hours in beans at 37°C. It would be useful to examine the possibility of emetic toxin production in beans. Since presently monkey feeding trials are the only effective method to test for this toxin (Turnbull, 1976), this was not possible in our laboratory situation. Further research and development of immunoassays to measure both emetic and diarrheal toxin production by B. cereus in foods are needed.

The presence of lactic acid bacteria in many of the commercial bean samples may serve as a safety factor in several ways. Lactic acid bacteria may inhibit the growth of pathogenic bacteria in foods either directly or through the production of lactic acid (Daly et al., 1972). In addition, changes resulting from growth of these bacteria may result in an unacceptable product so that the food would be discarded. Thus if a cook does store beans improperly, the beans will ferment and not be served; whereas, if pathogenic organisms made up the major flora of the beans, they may not appear spoiled.

Although the restaurant samples tested did not reveal large numbers of either B. cereus or C. perfringens, this does not indicate that these two organisms are not involved in foodborne illness related

to Mexican foods. It would be of interest to examine additional samples of beans and other Mexican foods, such as meat dishes and rice, for the presence of these two organisms as well as other foodborne pathogenic bacteria. It is extremely important that future outbreaks of foodborne illness involving Mexican foods be reported and investigated thoroughly. Leftover food and fecal samples from outbreaks of foodborne illness should be analyzed for the presence of B. cereus and C. perfringens as well as other foodborne pathogens.

Restaurant practices essential for prevention of growth of pathogens must be stressed. Although this study was limited to two organisms of public health concern, outbreaks of other types involving Mexican food have been identified. Between 1972 and 1978 four outbreaks traced to S. aureus were reported to the Center for Disease Control (Center for Disease Control, 1972b; Center for Disease Control 1973; Center for Disease Control, 1974; Center for Disease Control, 1975b; Center for Disease Control, 1976; Center for Disease Control, 1977; Centers for Disease Control, 1978b). Salmonella and Shigella have also been implicated in foodborne illness resulting from consumption of Mexican food. It should be noted that the presence of most pathogens in a food does not necessarily result in a foodborne illness outbreak. In most cases there must be opportunity for growth of the organism to sufficient numbers to cause illness. Restaurant personnel must be educated to apply the principles of food safety involving rapid cooling and thorough reheating before "hot-holding" of foods in order to maintain the safety of their products. The importance of this may be accepted if they are presented with a foundation of research findings.

SUMMARY

Before planning the study, practices related to the production and safety of beans and bean products were determined through personal interviews with the managers of local Mexican restaurants. Laboratory experiments were designed to determine the effects of varying temperatures, duration of incubation, and the location in the product, as it might affect aeration, on growth of B. cereus and C. perfringens, as single and mixed inocula, in cooked, mashed pinto beans. Pinto beans were cooked under pressure and then beaten aseptically for 10 minutes. Immediately following this, the inocula prepared from spore suspensions of B. cereus strain 4810 and C. perfringens type A strain 232 were added to the beans. The beans were incubated at 23°C and 37°C. Growth of B. cereus was very rapid at 37°C with significantly larger numbers found in the top, aerobic portion of the beans; the size of the difference varied with temperature and time of incubation (significant interaction of location with time and with temperature $\leq .01$). Goepfert et al. (1972) reported that a level of 10,000 to 100,000 B. cereus per gram is the minimum for causing illness. This level was reached in the beans incubated at 37°C after 4 hours while it took between 6 and 8 hours to reach this level in the beans at 23°C in both top and bottom locations. Spoilage of the beans incubated at 37°C was evident after 12 hours and much more pronounced after 24 hours incubation. Obvious signs of spoilage were not observed in the beans incubated at 23°C after 24 hours.

C. perfringens growth was rapid in the beans incubated at 37°C with the maximum level of 980,000,000 organisms per gram reached by 8

hours. At 24 hours, C. perfringens incubated at 23°C was still in its log phase of growth. C. perfringens gastroenteritis usually results from the ingestion of between 100,000,000 and 1,000,000,000 organisms per gram of food. This level was reached at 37°C after 6 to 8 hours of incubation; although, at 23°C this level had not been reached at 24 hours. At both temperatures and in both top and bottom portions of the beans, obvious spoilage had occurred by the time the danger level was reached.

During the mixed inoculum studies, bacterial growth patterns were similar to those in the individual B. cereus and C. perfringens growth studies. Improved growth of C. perfringens, however, did occur in the beans incubated at 23°C with a similar response in both the top and bottom portions of the beans. Spoilage resembled that observed in the C. perfringens growth study.

Sporulation of B. cereus and C. perfringens was examined during the individual growth studies. Sporulation of B. cereus appears to be dependent on aerobic conditions since sporulation was greater in the top portion of the beans at 37°C. Sporulation of C. perfringens was detected in only one sample in which a count of 8300 spores per gram was obtained after 24 hours. It has been reported that C. perfringens does not sporulate readily in foods (Genigeorgis, 1975; Hobbs, 1979).

A total of 42 commercial samples of beans and bean dip were analyzed for the presence of B. cereus and C. perfringens, as well as total aerobic and anaerobic plate populations. The total aerobic and anaerobic plate counts varied from less than 100 to an estimated 100,000,000. Lactic acid bacteria appeared to predominate and their

presence may be related to seasoning ingredients. Examination of seasoning commonly used in Mexican-style food revealed large numbers of aerobic and anaerobic organisms. These counts were close to those obtained on LBS; thus lactics appear to constitute the major flora of the seasonings. The bean dip samples generally had higher total aerobic and anaerobic counts than the mashed beans which may be due to the larger amount and wider variety of seasonings added to this product.

B. cereus was found in low numbers in 2 commercial bean samples. C. perfringens was not isolated from any of the 42 samples. Two samples revealed large numbers (>100,000 per gram) of coagulase positive S. aureus. These results do not indicate that B. cereus and C. perfringens are not involved in foodborne illness related to Mexican foods. It is extremely important that future outbreaks of foodborne illness involving Mexican foods be reported and investigated thoroughly. Leftover food and fecal samples should be analyzed for the presence of B. cereus and C. perfringens.

Two critical control points where problems may originate were identified: failure to cool large quantities of beans rapidly and failure to reheat beans thoroughly before "hot-holding." The concepts of spore survival and heat stable enterotoxins were not well understood by managers. Restaurant personnel need to understand and enforce the principles of food safety in order to maintain the safety of their products.

REFERENCES

Association of Official Analytical Chemists. 1980. Clostridium perfringens Official Final Action, p. 829-830 in William Horwitz (ed.) Official methods of analysis of the Association of Official Analytical Chemists, 13th ed., Washington, D.C.

Ayres, J. C., Mundt, J. O., and Sandine, W. E. 1980. Microbiology of foods. W. H. Freeman and Company, San Francisco. p. 252.

Banwart, George J. Basic Food Microbiology. 1979. The AVI Publishing Co., Westport, Conn. p. 325-327.

Beckers, V. J. 1976. The bacteriological quality of boiled and fried rice from Chinese restaurants in relation to Bacillus cereus food poisoning. Archiv FÜr Lebensmittelhygiene 27: 41-80.

Beebe, D., Bergdoll, M. S., and Schantz, E. J. 1978. Diarrheagenic toxin purification. In Food Research Institute Annual Report, 1978, University of Wisconsin-Madison, p. 424.

Blakey, L. J. and Priest, F. G. 1980. The occurrence of Bacillus cereus in some dried foods including pulses and cereals. J. Appl. Bacteriol. 48: 297-302.

Bodnar S., 1962. Ueber durch Bacillus cereus verusachte Lebensmittelvergiftungen. Z. Ges. Hyg. Grenzeb 8:368 (Zentralbl. Bakteriol. I Ref 187:218, 1963) as cited in Goepfert, J. M., Spira, W. M. and Kim, H. U. 1972, Bacillus cereus: food poisoning organism. A review. J. Milk Food Technol. 35: 213-227.

Boyd, M. J., Logan, M. A., and Tjtell, A. A. 1948. The growth requirements of Clostridium perfringens. J. Biol. Chem. 174:1013-1035.

Bryan, F. L. 1969. What the sanitarian should know about Clostridium perfringens foodborne illness. J. Milk Food Technol 32:383-389.

Bryan, F. L. 1981. Hazard analysis of food service operations. Food Technol. 35:78-87.

Buchanan, R. E. and Gibbons, N. E. (ed) 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins Company, Baltimore, Md.

Centers for Disease Control. 1971. Foodborne Disease Outbreaks Annual Summary 1971, issued October, 1972.

Centers for Disease Control. 1972a. Clostridium perfringens gastroenteritis-Washington. Morbidity and Mortality Weekly Report 22:3.

Center for Disease Control. 1972b. Foodborne Outbreaks Annual Summary 1972. Issued November, 1973.

Center for Disease Control. 1973. Foodborne and Waterborne Disease Outbreaks Annual Summary 1973. Issued December, 1974.

Center for Disease Control. 1974. Foodborne and Waterborne Disease Outbreaks Annual Summary 1974. Issued January 1976.

Center for Disease Control. 1975a. Bacillus cereus food poisoning-Wisconsin. Morbidity and Mortality Weekly Report 24:306.

Center for Disease Control. 1975b. Foodborne and Waterborne Disease Outbreaks Annual Summary 1975. Issued September, 1976.

Center for Disease Control. 1976. Foodborne and Waterborne Disease Outbreaks Annual Summary 1976. Issued October, 1977.

Centers for Disease Control. 1977. Foodborne Disease Outbreaks Annual Summary, 1977. Issued August, 1979.

Centers for Disease Control. 1978a. Clostridium perfringens food poisoning-California. Morbidity and Mortality Weekly Report 27:164-165.

Centers for Disease Control. 1978b. Foodborne Disease Outbreaks Annual Summary 1978. Revised. Reissued, February 1981.

Centers for Disease Control. 1981. Outbreak of illness due to Clostridium perfringens-California. Morbidity and Mortality Weekly Report 30(14):171-172.

Collee, J. G. 1974. Clostridium perfringens (Cl. welchii) in the human gastrointestinal tract, p. 205-216. In F. A. Skinner and J. G. Carr (ed.), The normal microbial flora of man. Academic Press, New York.

Daly, C., Sandine, W. E., and Elliker, P. R. 1972. Interactions of food starter cultures and food-borne pathogens: Streptococcus diacctilactis versus food pathogens. J. Milk Food Technol. 35:349-357.

Draughton, F. A., Elahi, M. and McCarty, I. E. 1981. Microbial spoilage of Mexican-style sauces. J. Food Protect 44:284-287.

Duncan, C. L., Strong, D. H., and Sebald, M. 1972. Sporulation and enterotoxin production by mutants of Clostridium perfringens. J. of Bacteriol. 110:378-391.

Duncan, C. L. 1973. Time of enterotoxin formation and release during sporulation of Clostridium perfringens Type A. J. Bacteriol. 113:932-936.

Fruin, J. T. 1977. Significance of C. perfringens in processed foods. J. Food Protect. 40:330-332.

Fuchs, A. R., and Bonde, G. J. 1957. The nutritional requirements of Clostridium perfringens. J. Gen. Microbiol. 16:317-329.

Fung, D. Y., and Cunningham, F. E. 1980. Effect of microwaves on microorganisms in foods. J. Food Protect. 8:641-650.

Genigeorgis, C. 1975. Public health importance of Clostridium perfringens. J. Am. Vet. Med. Assoc. 167:821-827.

Giannella, R. A., and Brasile, L. 1979. A hospital food-borne outbreak of diarrhea caused by Bacillus cereus: clinical, epidemiologic, and microbiologic studies. J. Infect. Dis. 139: 366-370.

Gilbert, R. J., Stringer, M., and Peace. 1974. The survival and growth of B. cereus in boiled and fried rice in relation to outbreaks of food poisoning. J. Hyg. (Camb) 73:433-444.

Gilbert, R. J. 1976. Survey on colony counts and predominant organisms in samples of boiled and fried rice from Chinese and Indian restaurant and take-away shops. Environ. Health 84:141-143.

Gilbert, R. J., and Parry, J. M. 1977. Serotypes of Bacillus cereus from outbreaks of food poisoning and from routine foods. J. Hyg. (Camb) 78:69-74.

Gilbert, R. J. 1979. Bacillus cereus gastroenteritis, p. 495-518. In H. Riemann and F. L. Bryan (ed.), Foodborne infections and intoxications, 2nd ed. Academic Press Inc., San Francisco.

Goepfert, J. M., Spira, W. M., and Kim, H. V. 1972. Bacillus cereus: Food poisoning organism. A review. J. Milk Food Technol. 35: 213-227.

Gordon, R. E., Haynes, W. C., Pang, C. H. 1973. The Genus Bacillus, p. 23-25. In Agric. Handbook No. 427 USDA, Washington, D.C.

Gough, B. A., and Alford, J. A. 1965. Effect of curing agents on the growth and survival of food-poisoning strains of Clostridium perfringens. J. Food Sci. 30:1025-1028.

Hall, H. E., Angelotti, R., Lewis, K. H., and Foter, M. J. 1963. Characteristics of Clostridium perfringens strains associated with food and food-borne disease. J. Bacteriol. 85:1094-1103.

Harmon, S. M., and Duncan, C. L. 1980. Clostridium perfringens: enumeration, identification, and enterotoxin detection, Chap. XV. In Food and Drug Administration, 1978, Bacteriological Analytical Manual, 5th ed. (revised section). Association of Official Analytical Chemists, Washington, D.C.

Hauschild, A. H. W. 1971. Clostridium perfringens enterotoxin. J. Milk Food Technol. 34:596-599.

Hobbs, B. C. 1979. Clostridium perfringens gastroenteritis, p. 131-171. In H. Riemann and F. L. Bryan (ed.), Food-borne infections and intoxications. 2nd ed. Academic Press, Inc., San Francisco.

Hodge, J. E., and Osmond, E. 1976. Carbohydrates, p. 41-50. In Fennema O. "Principles of Food Science." Marcel Dekker, Inc., New York City.

Holbrook, R., and Anderson, J. M. 1980. An improved selective and diagnostic medium for the isolation and enumeration of Bacillus cereus in foods. Can. J. Microbiol. 26:753-759.

Jephcott, A. E., Barton, B. W., Gilbert, R. J., and Shearer, C. W. 1977. An unusual outbreak of food-poisoning associated with meals-on-wheels. The Lancet 2:129-130.

Kang, C. K., Woodburn, M., Pagenkopf, A., and Cheney, R. 1969. Growth, sporulation, and germination of Clostridium perfringens in media of controlled water activity. Appl. Microbiol. 18: 798-805.

Kim, H. U., and Goepfert, J. M. 1971a. Occurence of Bacillus cereus in selected dry food products. J. Milk Food Technol. 34:12-15.

Kim, H. U., and Goepfert, J. M. 1971b. Enumeration and identification of Bacillus cereus in foods. I. 24-hour presumptive test medium. Appl. Microbiol. 22:581-587.

Labbe, R. G., and Duncan, C. L. 1974. Sporulation and enterotoxin production by Clostridium perfringens Type A under conditions of controlled pH and temperature. Can. J. Microbiol. 20:1493-1501.

Labbe, R. G., and Rey, D. K. 1979. Raffinose increases sporulation and enterotoxin production by Clostridium perfringens Type A. Appl. Environ. Microbiol. 37:1196-1200.

Lancette, G. A., and Harmon, S. M. 1980. Enumeration and confirmation of Bacillus cereus in foods: collaborative study. J. Assoc. Off. Anal. Chem. 63:581-586.

McDonel, J. L., and Duncan, C. L. 1977. Regional localization of activity of Clostridium perfringens Type A enterotoxin in the rabbit ileum, jejunum, and duodenum. J. Infect. Dis. 136:661-666.

McDonel, J. L. 1979. The molecular mode of action of Clostridium perfringens enterotoxin. Am. J. Clin. Nutr. 32:210-218.

Melling, J., Capel, B. J., Turnbull, P. C. B., and Gilbert, R. J. 1976. Identification of a novel enterotoxigenic activity associated with Bacillus cereus. J. Clin. Path. 29:938-940.

Midura, T., Gerbert, M., Wood, R., and Leonard, A. R. 1970. Outbreak of food poisoning caused by Bacillus cereus Public Health Rep. 85:45-48.

Morita, T., and Woodburn, M. 1977. Stimulation of Bacillus cereus growth by protein in cooked rice and combinations. J. Food. Sci. 42:1232-1235.

Naik, H. S., and Duncan, C. L. 1976. Enterotoxin formation in foods by Clostridium perfringens Type A, p. 314-316. In Food Research Institute Annual Report, 1976. University of Wisconsin-Madison.

Nakamura, M., and Kelly, K. D. 1968. Clostridium perfringens in dehydrated soups and sauces. J. Food Sci. 33:424-426.

Niilo, L. 1971. Mechanism of action of the enteropathogenic factor of Clostridium perfringens Type A. Infect. Immun. 3:100-106.

Ormay, L., and Novotny, T. 1969. Significance of food poisoning in Hungary, p. 279-285. In E. H. Kampelmacher, M. Ingram and D. A. A. Mossell (ed.), The Microbiology of dried foods. Proc. 6th Int. Symposium Food Microbiol. Int. Assoc. Microb. Society Haarlem, The Netherlands.

Pelczar, M. J., Reid, R. D., and Chan, E. C. S. 1977. Microbiology. 4th ed. p. 706-713. McGraw-Hill Book Company, San Francisco.

Portnoy, B. L., Goepfert, J. M., and Harmon, S. M. 1976. An outbreak of Bacillus cereus food poisoning resulting from contaminated vegetable sprouts. Am. J. Epidemiol. 103:589-594.

Powers, E. M., Lah, T. G., and Brown, T. 1976. Incidence and levels of B. cereus in processed spices. J. Milk Food Technol. 38:668-670.

Raevouri, M., Kiutamo, T., and Niskanen, A. 1977. Comparative studies of B. cereus strains isolated from various foods and food poisoning outbreaks. Acta. Vet. Scand. 18:397-407.

Raevouri, M., Kiutamo, T., Niskanen, A., and Salminen, K. 1976. An outbreak of Bacillus cereus food-poisoning in Finland associated with boiled rice. J. Hyg. (Camb.):319-327.

Rey, C. R., Walker, H. W., and Rohrbugh, P. L. 1975. The influence of temperature on growth, sporulation, and heat resistance of spores of six strains of Clostridium perfringens. J. Milk Food Technol. 38:461-465.

Robert, T. A. 1968. Heat and radiation resistance and activation of spores of Clostridium welchii. J. Appl. Bacteriol. 31:133-144.

Rockland, L. B., Gardiner, B. L., and Pieczarka, D. 1969. Stimulation of gas production and growth of Clostridium perfringens Type A (no. 3624) by legumes. J. Food Sci. 34:411-414.

Sgarbieri, V., Antunes, P., and Almeida, L. 1979. Nutritional evaluation of four varieties of dry beans (Phaeseolus vulgaris L.) J. Food Sci. 44:1306-1308.

Snedecor, G. W., and Cochran, W. G. 1967. Statistical Methods, p. 317-320. The Iowa State University Press. Ames.

Speck, M. L. (ed.) 1976. Compendium of methods for the microbiological examination of foods. Am. Public Health Assoc. Inc., Washington, D. C.

Spira, W. M., and Goepfert, J. M. 1975. Biological characteristics of an enterotoxin produced by Bacillus cereus. Can. J. Microbiol. 21:1236-1246.

Terranova, W., and Blake, P. A. 1978. B. cereus food poisoning. N. Engl. J. Med. 298:143-144.

Tong, J. L., Engle, H. M., Cullyferd, J. S., Shimp, D. J., and Love, C. E. 1962. Investigation of an outbreak of food poisoning traced to turkey meat. Am. J. Public Health 52:976-990.

Turnbull, P. C. B. 1976. Studies on the production of enterotoxins by Bacillus cereus. J. Clin. Path. 29:941-948.

Turnbull, P. C. B., Kramer, J. M., Jorgensen, K., Gilbert, R. J., and Melling, J. 1979. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of Bacillus cereus. Am. J. Clin. Nutr. 32:219-228.

Watt, B. K., and Merrill, A. L. 1975. Composition of Foods. . .raw, processed, prepared. Agriculture Handbook No. 8 USDA, Washington, D. C.

Yousten, A. A. 1975. Germination of Bacillus cereus endospores; a proposed role for heat-shock and nucleosides. Can. J. Micro. 21:1192-1197.

APPENDIX

Table 1

Growth of B. cereus strain 4810 (Log viable cells per gram[a]) in cooked mashed pinto beans at 23°C

Location in Beans	Time (hr)	Replications 1	2	3	Mean	Standard Deviation
Top	0	4.28	4.34	4.08	4.23	0.14
	2	ND	4.26	4.18	4.22	0.06
	4	4.08	4.23	4.18	4.16	0.08
	6	4.64	ND	ND	4.64	--
	8	5.20	4.82	4.88	4.97	0.20
	12	7.15	6.26	6.20	6.54	0.53
	24	8.65	8.23	8.48	8.45	0.21
Bottom	2	ND	4.18	3.99	4.09	0.13
	4	4.00	4.26	4.11	4.12	0.13
	6	4.15	ND	ND	4.15	--
	8	4.86	4.86	4.71	4.81	0.09
	12	6.15	6.04	5.66	5.95	0.26
	24	7.75	7.81	7.67	7.74	0.07

[a]PEMBA without Polymyxin B (Holbrook and Anderson, 1980) and Plate Count Agar (Difco) treated as duplicate plates

Table 2
Growth of B. cereus strain 4810 (log viable cells per gram[a]) in cooked mashed pinto beans at 37°C

Location in Beans	Time (hr)	Replications 1	2	3	Mean	Standard Deviation
Top	0	4.28	4.34	4.08	4.23	0.14
	2	ND	4.28	4.26	4.27	0.01
	4	6.18	5.92	6.09	6.06	0.13
	6	7.18	ND	ND	7.18	--
	8	8.18	8.34	8.15	8.22	0.10
	12	9.18	8.91	8.83	8.97	0.18
	24	9.08	8.88	9.15	9.04	0.14
Bottom	2	ND[b]	4.08	4.0	4.04	0.06
	4	5.81	5.30	MD[c]	5.56	0.36
	6	6.43	ND	ND	6.43	--
	8	6.98	7.04	6.43	6.82	0.34
	12	7.56	7.72	7.60	7.63	0.08
	24	8.26	8.38	8.40	8.35	0.08

[a] PEMBA without Polymyxin B (Holbrook and Anderson, 1980) and Plate Count Agar (Difco)

[b] Not done

[c] Missing data

Table 3
Growth of C. perfringens strain 232 (log viable cells per gram[a]) in cooked mashed pinto beans at 23°C

Location in Beans	Time[b] (hr)	Replications 1	2	3	Mean	Standard Deviation
Top	2	2.30	3.44	ND[c]	2.87	0.81
	4	2.90 est	2.78	3.38	3.02	0.32
	8	3.00	4.08	ND	3.54	0.76
	12	MD[d]	4.80	4.72	4.76	0.06
	24	6.00	6.41	7.34	6.58	0.69
Bottom	2	MD	3.20	MD	3.20	--
	4	2.30	2.90	3.38	2.86	0.54
	8	3.00	4.20	ND	3.60	0.84
	12	MD	5.08	5.04	5.06	0.03
	24	7.08	8.15	8.08	7.77	0.60

[a] SFP agar (Difco) and Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL) treated as duplicate plates

[b] 0 hour counts 3.15, 3.57, and 3.54 for replications 1, 2, and 3, respectively

[c] Not done

[d] Missing data

Table 4

Growth of C. perfringens strain 232 (log viable cells per gram[a]) in cooked mashed pinto beans at 37°C

Location in Beans	Time[b] (hr)	Replications 1	2	3	Mean	Standard Deviation
Top	2	3.72	3.67	ND[c]	3.70	0.04
	3	ND	5.62	ND	5.62	--
	4	MD[d]	7.18	7.18	7.18	0.00
	6	ND	8.61	ND	8.61	--
	8	8.69	8.82	ND	8.76	0.09
	12	MD	ND	8.87	8.87	--
	24	8.26	ND	8.10	8.18	0.11
Bottom	2	4.04	4.43	ND	4.23	0.28
	3	ND	4.92	ND	4.92	--
	4	MD	7.50	7.76	7.63	0.18
	6	ND	8.83	ND	8.83	--
	8	8.90	9.08	ND	8.99	0.13
	12	MD	ND	8.96	8.96	--
	24	ND	ND	6.48	6.63	0.21

[a] SFP agar (Difco) and Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL) treated as duplicate plates

[b] 0 hour counts 3.15, 3.57, and 3.54 for replications 1, 2, and 3, respectively

[c] Not done

[d] Missing data

Table 5
Growth of C. perfringens strain 232 (log viable cells per gram[a]) at 30°C in cooked mashed pinto beans

Location in Beans	Time (hr)	C. perfringens[b]
Top	0	3.54
	4	5.71
	12	8.26
	24	8.61
Bottom	4	6.15
	12	8.54
	24	8.99

[a]SFP agar (Difco) and Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL) treated as duplicate plates

[b]One replication was done

Table 6
Growth of B. cereus strain 4810
(log viable cells per gram[a]) in cooked mashed pinto beans
in competition with C. perfringens strain 232 at 23, 30 and 37°C

Location in Beans	Temp. (°C)	Time (hr)	Replications 1	2	3	4	Mean	Standard Deviation
		0	4.20	4.04	3.91	2.48	3.66	0.79
Top	23	6	4.61	4.72	4.49	ND[b]	4.61	0.22
		12	6.80	6.76	6.40	ND	6.65	0.22
		24	8.61	8.45	8.52	ND	8.53	0.08
Bottom	23	6	4.20	4.42	4.45	ND	4.36	0.14
		12	MD[c]	6.40	6.00	ND	6.20	0.28
		24	7.20	7.92	7.49	ND	7.54	0.36
Top	30	4	4.60	5.34	4.88	ND	4.94	0.37
		8	6.94	7.61	7.18	ND	7.24	0.34
		12	8.18	8.41	8.08	ND	8.22	0.17
Bottom	30	4	5.14	5.36	4.28	ND	4.93	0.57
		8	5.59	7.04	7.18	ND	6.60	0.88
		12	6.48	7.32	6.75	ND	6.85	0.43
Top	37	2	4.15	4.46	3.83	3.08	3.88	0.59
		4	5.73	6.15	5.34	4.99	5.55	0.50
		8	7.38	8.15	7.54	7.74	7.70	0.33
Bottom	37	2	3.93	4.34	3.85	2.93	3.76	0.60
		4	4.49	5.89	4.54	4.79	4.93	0.65
		8	5.53	7.00	5.00	7.04	6.14	0.60

[a] PEMBA (Holbrook and Anderson, 1980) and Plate Count Agar (Difco) treated as duplicate plates

[b] Not done

[c] Missing data

Table 7
Growth of C. perfringens strain 232 (log viable cells per gram[a]) in cooked mashed pinto beans in competition with B. cereus strain 4810 at 23, 30, and 37°C

Location in Beans	Temp. (°C)	Time (hr)	Replications 1	2	3	4	Mean	Standard Deviation
		0	4.08	4.11	3.89	2.60	3.67	0.72
Top	23	6	4.32	4.80	4.38	est ND[b]	4.50	0.26
		12	6.45	6.64	5.20	ND	6.09	0.78
		24	8.49	8.68	7.94	ND	8.37	0.38
Bottom	23	6	4.34	4.71	4.30	ND	4.45	0.23
		12	6.00	6.63	5.49	ND	6.04	0.57
		24	7.72	7.79	8.00	ND	7.84	0.15
Top	30	4	4.98	5.00	5.00	ND	4.99	0.01
		8	7.53	7.68	7.38	ND	7.53	0.15
		12	8.51	8.38	8.58	ND	8.49	0.10
Bottom	30	4	5.00	5.48	5.34	ND	5.27	0.25
		8	7.46	7.11	8.04	ND	7.53	0.47
		12	8.60	7.84	8.60	ND	8.35	0.44
Top	37	2	4.46	4.56	4.23	3.23	4.12	0.61
		4	7.30	6.15	6.46	5.11	6.26	0.90
		8	8.81	8.38	8.84	7.72	8.44	0.52
Bottom	37	2	4.34	4.62	4.08	2.90	4.01	0.76
		4	7.83	6.36	7.04	5.00	6.56	1.20
		8	8.99	8.48	8.89	7.84	8.55	0.52

[a] SFP agar (Difco) with Kanamycin and Polymyxin B and Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL) treated as duplicate plates

[b] Not done

Table 8
Growth of B. cereus strain 4810 and C. perfringens strain 232 (log viable cells per gram) in cooked mashed pinto beans in competition with each other at 43°C[a]

Location in Beans	Time (hr)	B. cereus[b]	C. perfringens[c]
Top	0	2.45	2.60
	2	3.13	2.78
	4	4.95	5.00
	8	7.51	7.78
	10	8.18	7.70
Bottom	2	2.93	2.70
	4	4.94	<5.00
	8	6.89	7.00
	10	7.23	7.70

[a] One replication was done

[b] PEMBA (Holbrook and Anderson, 1980) and Plate Count Agar (Difco) treated as duplicate plates

[c] SFP Agar (Difco) with Kanamycin and Polymyxin B and Plate Count Agar (Difco) incubated anaerobically using GasPak Anaerobic System (BBL) treated as duplicate plates