JT32
62

To the Graduate Council:

I am submitting herewith a thesis written by Clyde Baker Hoskins entitled "Recovery of Clostridium Perfringens in the Presence of an Oxygen-Reducing Membrane Fraction." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

P. Michael Davidson

P. Michael Davidson

We have read this thesis and recommend its acceptance:

John R Mount

Frances A. Draughon

Accepted for the Council:

Lew Minkel

Vice Provost
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature 

Date 4 March 1987

RECOVERY OF *CLOSTRIDIUM PERFRINGENS* IN THE PRESENCE OF AN OXYGEN-REDUCING MEMBRANE FRACTION

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Clyde Baker Hoskins

March 1987

AG-VET-MED.
Thesis
87
.H66

DEDICATION

To my Mother in hopes that some day I will be as wise as she.

ACKNOWLEDGMENTS

I extend my most sincere appreciation to Dr. Mike Davidson for his guidance, assistance and patience throughout the course of this study. Without his inspiration and advice, I would never have been able to begin this endeavor. My appreciation is also expressed to Dr. Ann Draughon and Dr. John Mount for their assistance in serving as committee members and to Dr. Howard Adler and Dr. Weldon Crow for their cooperation and guidance in this project. Dr. Adler also provided the oxygen-reducing membrane fraction that was used throughout this study.

My sister, Susan, spent many tedious hours converting my script into typing, a task for which I will always be grateful. A special thanks is extended to my office mates Margaret, Eric, Laure and Sherry for their understanding and patience with my moods and to Emi and Mo for teaching me how to not be dangerous in a micro lab.

Finally, all my family and friends deserve a part of this degree. Without their support, no one would be reading this.

ABSTRACT

The recovery and growth rates of pure cultures of C. perfringens were evaluated on media to which an oxygen-reducing membrane fraction (ORMF) had been added. C. perfringens ATCC 12917 reached stationary phase more rapidly in fluid thioglycollate medium (FTM) plus ORMF than in FTM alone. Three strains of C. perfringens were incubated in tryptose sulfite cycloserine (TSC) agar with and without ORMF. Recoveries were greater in media incubated aerobically with ORMF than in other formulations incubated aerobically or anaerobically.

Recovery of the organism from inoculated and uninoculated foods was also evaluated. In inoculated foods, the organism was usually recovered in greater numbers using TSC+ORMF and aerobic incubation than with TSC and anaerobic incubation. Of thirty-five raw meat samples tested, C. perfringens was recovered from twenty-two samples using TSC+ORMF compared with eighteen using TSC alone. Organisms inoculated into beef stew were subjected to heat and cold stress for various periods. In all cases TSC+ORMF effected a greater recovery of viable C. perfringens.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION.	1
II.	REVIEW OF THE LITERATURE.	3
	Clostridium Perfringens	3
	Anaerobic Techniques in Microbiology.	16
	Development of an Oxygen-Reducing Membrane Fraction . . .	18
III.	MATERIALS AND METHODS	21
	Test Organism	21
	Membrane Fraction	21
	Media	22
	Procedures.	23
	Statistical Analysis of Data.	28
IV.	RESULTS AND DISCUSSION.	29
	Oxygen Reduction in Tryptose Sulfite Cycloserine Agar . .	29
	Recovery Rate of Pure Cultures.	29
	Growth Rate of Pure Cultures.	34
	Recovery of Pure Cultures Using a Hydrophobic Grid Membrane Filter	36
	Recovery from Inoculated Foods.	38
	Recovery of Organisms Exposed to Abuse Temperatures . . .	42
	Recovery from Uninoculated Foods.	46
V.	CONCLUSIONS	49
LIST OF REFERENCES.		51
APPENDIXES.		60
APPENDIX A. ENUMERATION DATA FOR FIGURES 3-6		61
APPENDIX B. ENUMERATION DATA, BY STRAIN, FOR ALL EXPERIMENTS EXCEPT HGMF		65
VITA.		75

LIST OF TABLES

TABLE		PAGE
1.	Tryptose sulfite cycloserine agar.	22
2.	Medium, overlay and incubation condition for plates used in Test 1	25
3.	Enumeration of Clostridium perfringens in media with and without oxygen-reducing agents	31
4.	Enumeration of Clostridium perfringens in tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction at 24 and 48 hours.	33
5.	Comparison of Clostridium perfringens recovery using pour plates and Hydrophobic Grid Membrane Filters (HGMF) with and without an oxygen-reducing membrane fraction. . . .	37
6.	Incidence of Clostridium perfringens in raw meat samples from local retail sources.	47
7.	Enumeration of Clostridium perfringens grown in inoculated food products during a 12-hour time period	62
8.	Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of Clostridium perfringens from beef stew . . .	63
9.	Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of Clostridium perfringens from beef stew heated at various temperatures for 1 hour	64
10.	Enumeration of Clostridium perfringens by strain in media with and without oxygen-reducing agents.	66
11.	Enumeration of Clostridium perfringens by strain in tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction at 24 and 48 hours	67
12.	Enumeration of Clostridium perfringens grown in chicken gravy for 12 hours	68
13.	Enumeration of Clostridium perfringens grown in beef gravy for 12 hours	69

TABLE PAGE

14. Enumeration of Clostridium perfringens grown in chicken a la king for 12 hours 70

15. Enumeration of Clostridium perfringens grown in beef stew for 12 hours. 71

16. Enumeration of Clostridium perfringens by strain grown in inoculated foods for 12 hours 72

17. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of individual strains of Clostridium perfringens from beef stew 73

18. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of individual strains of Clostridium perfringens from beef stew heated at various temperatures for 1 hour. 74

LIST OF FIGURES

FIGURE		PAGE
1.	Rate of reduction of dissolved oxygen in tryptose sulfite cycloserine agar at 23°C.	30
2.	Rate of growth of Clostridium perfringens ATCC 12917 in fluid thioglycollate medium (FTM) with and without an oxygen-reducing membrane fraction (ORMF)	35
3.	Enumeration of Clostridium perfringens grown in (A) chicken gravy; (B) beef gravy; (C) chicken a la king; and (D) beef stew for 12 hours	40
4.	Enumeration of Clostridium perfringens grown in inoculated food products for 12 hours.	41
5.	Enumeration of Clostridium perfringens grown in beef stew and then stored at (A) 4°C and (B) -15°C.	43
6.	Enumeration of Clostridium perfringens grown in beef stew and then heated to various temperatures for one hour.	44

CHAPTER I

INTRODUCTION

Clostridium perfringens is classified as a Gram-positive, anaerobic, sporeforming bacterium that produces an enterotoxin which is one of the most common causes of foodborne illness. Foods which are frequently implicated as vehicles for *C. perfringens* include meat and poultry based stews, sauces and gravies that have been held at improper temperatures prior to ingestion by humans (Hatheway et al., 1980). In order to confirm *C. perfringens* as the cause of a foodborne illness, the organism must be isolated from the food product and quantified. This is often a challenging problem for the food microbiologist. Potential contamination of the food by other microflora necessitates the use of a selective, differential medium and anaerobic incubation for the recovery of *C. perfringens*. The solid medium of choice for the organism in the United States is tryptose sulfite cycloserine (TSC) agar (Meade, 1985). As with most other selective agars, TSC has selective agents which may be inhibitory to *C. perfringens* as well as potential contaminating microflora. Current anaerobic techniques employing anaerobe jars may also tend to decrease the recovery rate of *C. perfringens* since there is a lag time of one hour or more before aerobic conditions are established. These jars have the additional disadvantage of being inconvenient and expensive.

Adler and Crow (1981) and Adler et al. (1983) first reported on the use of an oxygen-reducing membrane fraction (ORMF) from *Escherichia*

coli to stimulate the growth of anaerobic bacteria in nutrient broth and agar. If their material could be used in media normally used to isolate *C. perfringens*, the detection of this microorganism in foods could be greatly facilitated.

The objectives of this study were to determine: (a) if the addition of ORMF to TSC would allow *C. perfringens* growth under aerobic conditions; (b) the effect of ORMF on the growth rate of *C. perfringens*; (c) the effect of ORMF on the recovery of *C. perfringens* from inoculated and uninoculated foods; and (d) the effect of ORMF on recovery of temperature-stressed *C. perfringens* cells.

CHAPTER II

REVIEW OF THE LITERATURE

A. *CLOSTRIDIUM PERFRINGENS*

Classification and Characteristics

Clostridium perfringens is a gram-positive, square- or blunt-ended, non-motile bacillus with the ability to form spores. Cells vary from 2-6 μm long and 0.8-1.5 μm wide and usually occur singly but can be seen as short chains in actively growing cultures (Duncan, 1976). The organism has been referred to by a number of different names throughout its recorded history including *Bacillus aerogenes capsulatus*, *Bacillus phelegmones emphysematosae*, *Bacillus emphysematosus*, *Bacillus perfringens*, *Bacillus welchii*, *Clostridium welchii* and currently *Clostridium perfringens* (Duncan, 1976). In some literature, particularly medical journals, it is still occasionally referred to as *Clostridium welchii* (Keusch and Weinstein, 1975).

C. perfringens is divided into five toxigenic subtypes designated A, B, C, D and E. This division is based on the type of toxin produced by each of the strains (Hatheway et al., 1980; Hauschild, 1973). Type A is the most common type implicated in foodborne disease outbreaks with the symptoms being relatively mild and short-term (Hatheway et al., 1980; Craven et al., 1981). Strains of this type are most frequently found in soil and have spores that may survive temperatures of 100°C for an hour or more (Hobbs et al., 1953). Types B, C, and D are

associated with enterotoxemia in livestock. Type C is also known to cause a severe and sometimes lethal type of necrotizing enteritis in man (Sterne and Warrack, 1964) and neonatal livestock (Howard-Martin et al., 1986). Type E is reported by some to cause enterotoxemia in calves (Hobbs, 1969) while others feel the pathogenicity of this type is questionable (Hatheway et al., 1980).

C. perfringens is intermediate within the clostridial species in its tolerance of oxygen. C. novyi type B and C. difficile are strict anaerobes while C. histolyticium and C. tertium are aerotolerant and will form small colonies on the surface of blood agar when incubated aerobically (Keusch and Weinstein, 1975). C. perfringens will tolerate exposure to oxygen for short periods of time during laboratory processing or normal environmental conditions. However, anaerobic incubation is required if it is to be isolated and enumerated from foods (Harmon and Duncan, 1984). The fact that C. perfringens prefers anaerobic conditions was supported by Shoemaker and Pierson (1976) when they demonstrated that the organism grew better under other adverse conditions, such as elevated temperatures, when strict anaerobiosis was observed.

In addition to atmosphere, the temperature, pH and water activity (a_w) of the growth medium have been found to affect C. perfringens growth. The interaction of these variables has been studied by Meade (1969), Strong et al. (1970) and Bartsch and Walker (1982). Suitable ranges for each of these parameters have been reported by many

researchers. Hobbs (1969) reported growth of vegetative cells at temperatures of 12-50°C although activity was slow at the extreme values. Growth of some heat-resistant strains has been reported at 52.3°C (Shoemaker and Pierson, 1976). An optimum growth range of 35-45°C is supported by numerous researchers. Minimum water activity permitting growth of the organism has been reported as 0.93 (Silliker, 1980), 0.95 (Craven, 1980) and 0.97 (Duncan, 1976). The difference in these values is most likely due to the influence of the solutes used to adjust the a_w of the growth medium. Sodium chloride, potassium chloride, glucose, sucrose and glycerol are frequently used and have different limiting effects on C. perfringens' growth (Troller, 1973). Growth in the pH range of 5.0-8.5 represents the extreme limits reported (Silliker, 1980; Jay, 1986). An optimum pH of 7.2-7.4 is commonly accepted.

Generally C. perfringens requires a relatively complex mixture of amino acids and vitamins for growth. Minimal media support neither good growth nor toxin production (Duncan, 1976). A carbohydrate source is also necessary for energy production. The organism reduces sulfite to sulfide and nitrates to nitrites. It ferments lactose, liquifies gelatin and produces the enzyme lecithinase. All these biochemical characteristics are important in determinative identification of colonies isolated from samples.

History and Epidemiological Aspects

The strains of Clostridium perfringens that are commonly implicated in food poisoning are ubiquitous in nature. This probably contributes

to the facility with which this organism can find its way into foods. From a historical standpoint, C. perfringens is perhaps best recalled for its role in causing gas gangrene in man and other animals. However, of greater significance to public health in the United States today, is the ability of C. perfringens to cause foodborne illnesses. In 1981 and 1982 in the U.S., it was the third most common bacterial pathogen implicated in foodborne disease outbreaks (28 outbreaks (11.2%) in 1981; and 22 outbreaks (10%) in 1982) (CDC, 1983b and 1984). In both years it ranked behind only Salmonella and Staphylococcus aureus in number of outbreaks.

A manuscript by Klein in 1895 first implicated C. perfringens as a cause of human food poisoning. While many reports suggested that C. perfringens was responsible for foodborne illnesses, the first confirmed incidence attributed to this organism was in 1945 (McClung). Despite this finding, it was not until the mid-1960's that C. perfringens was accepted in the U.S. as a major food poisoning organism (Duncan, 1976). Extensive research was conducted in Europe in the 1940's and 1950's on C. perfringens toxins and their relationship to foodborne disease, but there was little interest in the organism in the U.S. This oversight has been attributed by Hobbs (1969) to a controversial study by Dack et al. in 1954 in which C. perfringens-laded foods were fed to 32 human subjects with negative results. In spite of the slow start, C. perfringens food poisoning has been a reportable disease to the Center for Disease Control since 1967. From then through 1982, there have

been 258 confirmed C. perfringens foodborne disease outbreaks in the U.S. (Hatheway et al., 1980; CDC, 1981-1984).

Foods most commonly implicated in C. perfringens outbreaks have been meat- and poultry-based dishes that have been cooked and held for some time before consumption (Zottola, 1971). The list of specific foods involved is extensive. In addition to cooked foods, C. perfringens has been found in 47% of raw ground beef (Ladiges et al., 1974) and 30% of raw boneless beef (Bryan and Kilpatrick, 1971) samples obtained from commercial sources.

In order for C. perfringens to be confirmed as the etiologic agent in a foodborne disease outbreak by the CDC, one of the following conditions must be met. First, organisms of the same serotype must be isolated from the incriminated food and the stool of a sick individual. Secondly, organisms of the same serotype must be isolated from the stool of the majority of sick individuals. Finally, at least 100,000 C. perfringens organisms per gram must be isolated from the suspect food (Hatheway et al., 1980). If laboratory confirmation by any of the above methods is not possible, a presumptive diagnosis of C. perfringens food poisoning is made if the incubation period of the illness is 8-14 hr (CDC, 1983a). Due to the short incubation time, low mortality ratio and relatively short recovery time from symptoms, there are almost certainly more outbreaks of C. perfringens food intoxication than are reported or confirmed. As laboratory procedures are simplified, more testing can be done at local and state levels which will

likely stimulate increased awareness of this already important etiologic agent (Hatheway et al., 1980).

Mechanism of Action in Enterotoxication

Enterotoxemia in which C. perfringens Type A is the etiologic agent is characterized by profuse diarrhea, abdominal cramps, and flatulence. Usually there is no nausea, vomiting, or fever and the symptoms generally subside in 18-24 hr. Type C enteritis has the additional symptoms of vomiting and necrotic ileitus and a 35-40% mortality rate. The typical scenario which takes place in C. perfringens illness is as follows (Hauschild, 1973):

1. Large numbers of vegetative cells are ingested;
2. these cells multiply and sporulate in the intestines;
3. enterotoxin is produced by the sporulating cells and is released during cell lysis; and
4. the enterotoxin causes an accumulation of fluid in the intestinal lumen resulting in diarrhea.

Even though the symptoms of enterotoxemia produced by different strains are varied, the enterotoxins have been found to be serologically and biologically similar (Skjelkvale and Duncan, 1975). Since Type A is, by far, the most commonly implicated in foodborne illness, it has received the most attention in studies addressing the mechanism of action of the toxin. The enterotoxin is a protein consisting of a single polypeptide chain of approximately 35,000 MW (Hauschild and Hilsheimer, 1971). The role of the toxin in the C. perfringens

organism is unclear, but it has been postulated that it is the result of the overproduction of a protein synthesized for some specific function (Bartholomew and Stringer, 1984).

The fact that production of enterotoxin is closely related to sporulation of cells is well established (Labbe, 1980). Spores and enterotoxin can occur in contaminated foods, but the most common cause of disease is believed to be the ingestion of large numbers of vegetative cells. Sporulation does occur quite readily in the intestines (Hauschild, 1973) but is rare in common growth media. In fact, considerable effort has been expended to develop media and incubation conditions to encourage sporulation of C. perfringens in vitro. This has facilitated study of the enterotoxin (Bartholomew and Stringer, 1984).

The mechanism by which enterotoxin causes the particular symptoms of the disease has not been fully established. Early research in this area proposed that phospholipase C, the alpha toxin produced by all types of C. perfringens, hydrolyzed lecithin to phosphocholine, which could cause diarrhea within 8-10 hr (Nygren, 1962). More recent research indicates that the mode of action is associated with direct cell membrane damage. This leads to an alteration of the membrane transport system in the intestines (McDonel, 1979, 1980). More specifically, studies on the effects of Type A enterotoxin on Vero (African green monkey kidney) cells demonstrated that the toxin could bind to specific sites on the cell membranes. Blebs and eventually holes were created in the membranes allowing leakage of some substances (McDonel and McClane, 1979).

In a subsequent study by McClane and McDonel (1981), a model for the mechanism of action of C. perfringens enterotoxin was proposed. The toxin binds to susceptible cells, damages the cell membrane and alters the transport functions. The influx of sodium and calcium ions to the intestinal lumen increases, with water following in an effort to correct the electrolyte imbalance. The increased intraluminal fluid causes diarrhea. This model is supported by the fact that osmotic stabilizers such as sucrose, polyethelene glycol, dextran and bovine serum albumin decreased the effect of C. perfringens enterotoxin in Vero cells (McClane and McDonel, 1981). Later McDonel and Demers (1982) demonstrated that the effect of the enterotoxin was restricted to the upper intestine. There was no alteration in cell membrane transport occurring in the colon even though binding of the enterotoxin to intestinal cells did take place there.

Abdominal cramps associated with the enterotoxemia are related not only to the increased intraluminal fluid, but also to gas production of the organisms in the gut. Gas production is also associated with the flatulence seen in the disease.

Growth in Food Systems

Although Clostridium perfringens is an anaerobe and has specific requirements for a complex mixture of nutrients, many foods prepared every day provide excellent growth conditions for this pathogen. If proper cooking procedures are followed, vegetative cells and any preformed enterotoxin are usually destroyed. Most disease outbreaks occur

in foods that are either contaminated after cooking or contaminated with spores that survived cooking (Sutton et al., 1972). In either instance, improper holding temperatures are implicated in a majority of cases (Zottola, 1971; Craven, 1980).

Since food systems are typically exposed to dynamic temperature gradients, extensive research has been conducted regarding the effect of variable temperatures on C. perfringens. Canada et al. (1964) conducted some of the early work in this area. They stated that temperatures of -17.7 and 7.1°C for 48 hr or 100°C for 10 min were adequate to kill most vegetative cells, a though many spores survived both temperature extremes.

Since that early work, many researchers have established that there is considerable variation in temperature sensitivity among strains of the organism. Craven et al. (1981) and Ando et al. (1985) investigated the relationship between heat resistance, sporulation, and germination in several different C. perfringens strains. They agreed that there was considerable strain variation and generally concluded that heat resistant spores could produce toxin in food systems (cooked chicken) as well as sporulation media within 8-12 hr following exposure to temperatures as high as 95°C. Al-Obaidy et al. (1985) demonstrated that enterotoxin was produced after only 2 hr incubation at 45°C in chicken meat inoculated with vegatative cells. They also conducted sensory evaluation of odor, color, and texture. Significant differences in scores of these attributes between inoculated and uninoculated

samples were detected within two hours after inoculation. Their sensory research offers one explanation as to why most C. perfringens foodborne disease outbreaks result from the ingestion of vegetative cells rather than preformed toxin.

It has, however, been established that intake of preformed toxin has the potential to cause disease (Skjelkvale and Uemura, 1977; Naik and Duncan, 1977), although the public health significance of this is unresolved (Bradshaw et al., 1982). That preformed toxin can cause disease prompted interest in the microbiological safety of long-time, low-temperature cooking methods associated with electric slow cookers. These slow cookers usually involve the preparation of a food product under conditions of a constantly rising temperature. Ritter et al. (1979), Foegeding and Busta (1980) and Roy et al. (1981) all established that enterotoxin could be formed under these conditions. However, they generally concluded that terminal temperatures were usually adequate to effect inactivation of the toxin. Roy et al. (1981) further determined that with at least two strains of C. perfringens (NCTC 8238 and 8798) a constantly-rising temperature was better at preventing development of heat resistance in the organism than was cooking at a higher constant temperature. The work by Bradshaw and his coworkers (1982) revealed that 72.8 min were required to inactivate C. perfringens enterotoxin at 59°C, while only 1.5 min were required for the same effect at 65°C. This agreed well with the data presented by Babajimopoulos and Mikolajcik (1981), who determined that, under certain conditions, destruction of alpha toxin was rapid at 65°C.

In addition to the effects of high temperatures on both C. perfringens organisms and enterotoxin, refrigeration and freezing temperatures also have detrimental effects. Trakulchung and Kraft (1977) inoculated various foods with both vegetative cells and spores and then held the samples at -29°C for up to 42 days. They determined that approximately 75% of the vegetative cells were killed during the storage period, while the spore counts remained fairly constant. They also established that thawing at low temperatures (5°C) for 48 hr caused a greater reduction in vegetative cells than did more rapid thawing at room temperatures. In a later study, White and Hall (1984) observed a C. perfringens survival rate of 33-60% after 6 months frozen storage. These higher survival rates could be attributed to the fact that their samples were thawed at 27°C.

Isolation and Quantitation

To isolate and accurately quantitate Clostridium perfringens from a food product has proven to be a challenging problem for the food microbiologist. Several different solid media have been proposed in the last 25 years. Most are differential based on the ability of the organism to reduce sulfites to sulfides. These sulfides, in the presence of an appropriate iron salt, result in the precipitation of black ferrous sulfide around individual colonies (Meade, 1985). In attempting to make the medium selective, researchers have added antimicrobials to the medium and varied the incubation temperature. The problem with many of the selective agents used has been that they were

not specific enough for C. perfringens or caused inhibition of the organism.

One of the early media advocated for C. perfringens was sulfite polymixin sulfadiazine (SPS) medium (Angelotti et al., 1962). However, the authors themselves reported that other clostridial species could grow in the medium. Shahidi and Ferguson (1971) later demonstrated that SPS not only allowed the growth of some facultative anaerobes but also inhibited some strains of C. perfringens. In an effort to overcome these shortcomings of SPS, Marshall et al. (1965) suggested the use of tryptose sulfite neomycin (TSN) agar and incubation at 46°C. This medium was also shown to allow the growth of several facultative anaerobes (Harmon et al., 1971b) and to inhibit some strains of C. perfringens (Handford, 1974).

Both SPS and TSN contain sodium sulfite as the sulfite source which was determined to be inhibitory to C. perfringens (Handford and Cavett, 1973). Sodium metabisulfite was substituted for sodium sulfite by Shahidi and Ferguson (1971) when they produced Shahidi-Ferguson perfringens (SFP) medium. This partially overcame the inhibition problem, but their medium was still not adequately selective against facultative anaerobes.

There are currently two solid media that are promoted as the best for C. perfringens isolation. Oleandomycin polymixin sulfadiazine perfringens agar (OPSPA), developed by Handford and Cavett (1973), is the medium of choice in the United Kingdom. In the United States, tryptose sulfite cycloserine (TSC) agar, developed by Harmon et al.

(1971a) and modified by Hauschild and Hilsheimer (1974a, b; Hauchild et al., 1974), is the medium of choice. Both TSC and OPSPA employ the same basal media formulations. The difference in the agars is in the selective agents. D-cycloserine is used in TSC, while OPSPA utilizes oleandomycin, polymixin B and sulfadiazine. Which medium is better depends upon the literature reviewed. One report comparing the two media found no difference in total counts when plating skin samples from chicken carcasses (Adams and Meade, 1980). TSC is in the first official action of the Association of Official Analytical Chemists (AOAC, 1984). It is not 100% selective for C. perfringens, and confirmatory tests are required. Harmon and Kautter (1978) recommended lactose-gelatin medium and a modified nitrite-motility medium for confirmation of the organism. These tests have been adopted by the AOAC (1984).

A number of researchers have looked at the ability of different plating media to recover temperature-stressed C. perfringens cells. Barach et al. (1974) found TSC to be superior to other commonly-used media in the recovery of heat-injured C. perfringens cells. Apiluktivongsa and Walker (1980) showed that D-cycloserine was less inhibitory than other conventional selective agents on cold-stressed cells. Adams and Meade (1980) found no difference in the ability of TSC and OPSPA to recover cold-stressed cells. In contrast to previous researchers, Labbe and Norris (1982) concluded that the heat treatment applied to the organism and the incubation temperature of the medium

were more important to the level of recovery of heat-injured cells than was the type of medium used.

Some of the liquid media advocated for use in enumerating C. perfringens include rapid perfringens medium (RPM) (Erickson and Deibel, 1978), lactose sulfite broth (LSB) (Beerens et al., 1982) and iron milk medium (IMM) (St. John et al., 1982). Both RPM and LSB were reported to be more selective than TSC (Erickson and Deibel, 1978; Neut et al., 1985). No significant difference between selectivity of IMM and TSC was noted, however, IMM was promoted as less expensive and easier to prepare (Abeyta et al., 1985). While various advantages of each media were reported, all shared the disadvantage of employing the more labor-intensive and less accurate most probable number (MPN) method of enumerating the organism (Meade, 1985).

B. ANAEROBIC TECHNIQUES IN MICROBIOLOGY

Development and improvement of methods for the cultivation of anaerobic bacteria have long received attention from microbiologists. Methods recommended for creating an anaerobic system can be divided into physical/mechanical, chemical, and biological types (Anderson and Fung, 1983).

The first category includes techniques such as boiling solutions to remove oxygen (Hall, 1929), using a vacuum system to remove oxygen from a container or replacing oxygen with an inert gas (Hunziker, 1902). The latter two methods have been combined and modified in developing

some of the anaerobe jars and glove boxes used today. Another process has been the use of overlays to prevent or retard the diffusion of oxygen into the incubation media. Agar, paraffin, vaseline, and mineral oil are commonly used.

The use of chemical reducing agents dates back to 1886 when Liborius determined that anaerobic growth was stimulated by glucose (Hunziker, 1902). Currently, sodium thioglycollate and sodium metabisulfite are common reducing agents.

Pasteur has been credited with being the first to observe that aerobic bacteria could utilize all of the oxygen in a medium and create an environment suitable for the growth of anaerobes. Other biological agents have been proposed as potential oxygen reducers, but few have proven useful. Smith and Hungate (1958) reported the successful cultivation of Methanobacterium ruminantium in a medium that had been reduced by the growth of Escherichia coli and subsequently pasteurized. Anderson and Fung (1983) felt that this method was impractical for large numbers of cultures. More recently, Adler and Crow (1981) reported the production of a membrane fraction of E. coli that could remove oxygen from several different biological media. This provided a suitable growth environment for a number of different anaerobic bacteria.

Another method of classifying anaerobic techniques is based on the degree of oxygen sensitivity exhibited by the organism in question (Barnes, 1985). She recommends dividing the techniques into three

categories. The first is the Hungate technique used for obligate anaerobes (Hungate, 1950, 1969) in which all procedures are carried out in an anaerobic environment. Secondly, prereduced agar plates are inoculated and incubated in anaerobe jars. The third category is liquid media with reducing agents in capped bottles incubated aerobically.

In actual practice anaerobic methods used are often based on materials available and experience rather than the metabolic requirements of the organism to be cultivated. This is a less-than-ideal situation in food microbiology since total numbers of cells are often critical in supporting or refuting a diagnosis in foodborne disease outbreaks (Anderson and Fung, 1983; Harmon and Duncan, 1984). Most of the currently recommended anaerobic techniques in food microbiology are listed in the Compendium of Methods for the Microbiological Examination of Foods (Speck, 1984).

C. DEVELOPMENT OF AN OXYGEN-REDUCING MEMBRANE FRACTION

For a number of years Adler and coworkers conducted research on irradiation and its effect on growth, division and recovery of bacteria with particular emphasis on Escherichia coli (Adler and Hardigree, 1964, 1965; Adler et al., 1966; Adler et al., 1981). The ability of injured E. coli cells to replicate is related to their ability to form septa rather than long mononucleate filaments. DeLaporte (1956)

observed that injured cells had a greater propensity to replicate when the cells were closer together and called this phenomenon "neighbor restoration." It was noted that this neighbor restoration occurred only in recipient cells that were mutated at the lon locus (Adler and Hardigree, 1964, 1965; Adler et al., 1966).

Septation and recovery of colony-forming ability in lon mutants is stimulated by several different agents (Adler and Hardigree, 1965; Alper and Gillies, 1960). One of the most effective of these agents is a particulate fraction obtained from some microorganisms and other cells (Adler et al., 1981). Evidence is provided that demonstrates that the fraction is from the cytoplasmic membrane of donor cells. Preparation of the membrane fraction with appropriate modifications has been previously described (Schnaitman, 1970; Adler et al., 1981). The membrane fraction was termed "P_2" in honor of the fermentation vat in which donor cells are produced (Adler, 1985). That these fractions promote septation is attributed to the ubiquinone-cytochrome-dependent respiratory activity of the membrane preparations (Adler et al., 1981). During the course of determining that the cytochrome oxidase system was involved in promoting septation, measurements of the activity of the system were made. One of the measurements was the rate of reduction of oxygen to water in nutrient broth. It was observed that essentially all of the oxygen in the medium was removed in approximately 5 min (Adler et al., 1981).

In an observation unrelated to their previous research, it was concluded that this oxygen removal from media might provide a new

approach to enhancing the growth of anaerobic microorganisms (Adler and Crow, 1981). To determine the feasibility of this supposition, 18 species of anaerobic bacteria were grown in liquid and solid media to which the oxygen-reducing membrane fraction (ORMF) had been added at a rate of 10-20 µl/ml of medium. Plates or tubes were incubated aerobically, and the plates had a 5 ml overlay of medium without ORMF (Adler and Crow, 1981; Adler et al., 1983). In all cases, growth of the organisms was achieved. In some instances, the growth was to a greater level than observed with anaerobic incubation and no ORMF. This increased growth has been attributed to two factors. First, ORMF effects a more rapid removal of oxygen from the system than does conventional anaerobic methodology. Secondly, particles of ORMF cannot penetrate bacterial cell walls which apparently restricts their activity to the reduction of oxygen to water (Adler and Crow, 1981). Consequently, the membrane fraction possesses little or no toxicity for the organism being grown.

During the course of their work Adler and Crow (1981) observed that the oxygen-reducing ability of ORMF was concomitant to the presence of an oxygen donor in the media being evaluated. Their research was restricted to the use of Difco nutrient broth and agar. For their technique to be applicable to the varied specialized branches of microbiology, the oxygen-reducing capability of ORMF in complex differential and selective media must be examined.

CHAPTER III

MATERIALS AND METHODS

A. TEST ORGANISM

Three strains of Clostridium perfringens (ATCC 12917, NCTC 10239 and NCTC 8798) were obtained from the Food Technology and Science Microbiological Laboratory. Through the course of the study, the organisms were maintained in fluid thioglycollate medium (FTM) (BBL, Cockeysville, MD) and subcultured biweekly. Immediately prior to each test, the organisms were transferred to fresh FTM and incubated for 18 hr at 42°C.

B. MEMBRANE FRACTION

Frozen oxygen-reducing membrane fraction (ORMF) was obtained from the Biology Division, Oak Ridge National Laboratories, Oak Ridge, TN. Isolation and purification of ORMF has been previously described (Adler et al., 1981; Schnaitman, 1970). After recepit, the ORMF was thawed, dispensed in 1.5 ml plastic vials and stored at -15°C. Immediately prior to use, the approximate amount of ORMF required was thawed at 37°C and added at a rate of 20 µl/ml melted agar medium. Unused portions of ORMF could be refrozen once for later use with no apparent loss in oxygen-reducing capacity.

C. MEDIA

Tryptose sulfite cycloserine (TSC) agar without egg yolk enrichment was used for all enumerative plating. Most of the TSC was prepared as described by Schwab et al. (1984) without modification (Table 1).

Table 1. Tryptose sulfite cycloserine agar.

Ingredient	Amount
Tryptose	15 g
Soytone	5 g
Yeast extract	5 g
Sodium bisulfite	1 g
Ferric ammonium citrate	1 g
Agar	20 g
Distilled water	1 l
D-cycloserine[a]	10 ml

[a]4% filter-sterilized added after agar cooled to 50°C.

In some of the preliminary work using pure cultures, TSC was prepared without the reducing agent sodium bisulfite. This was done to determine if there were any inhibitory effects of sodium bisulfite on C. perfringens.

The reducing ability of ORMF in the media was evaluated by measuring dissolved oxygen levels with a Chemical Microsensor System (Transidine General Corporation, Ann Arbor, MI) and a miniature Clark-style electrode.

The electrode was placed in an oxygen uptake chamber containing 1 ml of medium initially equilibrated with air at 23-25°C. ORMF was then added to the medium in the uptake chamber and the rate of reduction of dissolved oxygen was measured.

TSC or TSC without sodium bisulfite (TSC-sb) was dispensed into 150 ml media bottles, sterilized at 121°C for 15 min and stored at room temperature after the addition of D-cycloserine. ORMF was not added until plates were poured. With the exception of the experiment involving recovery rates of pure cultures, all plates with ORMF were incubated aerobically, and those without ORMF were incubated anaerobically in a Gas-Pak Anaerobic System (BBL, Cockeysville, MD).

Confirmatory tests for C. perfringens in uninoculated foods involved the use of lactose-gelatin and nitrate-motility medium. These were prepared as described by Schwab et al. (1984) and are the confirmatory tests for C. perfringens recommended by Harmon and Duncan (1984) and AOAC (1984).

Dilutions in all experiments were accomplished with 0.1% (w/v) peptone water.

D. PROCEDURES

Recovery Rates of Pure Cultures

Two separate tests were performed. In the first, freshly subcultured organisms were diluted and pour plated on the following media: TSC, TSC-sb and TSC-sb+ORMF. One-half of the plates were overlaid with the same medium. One-half of the plates without ORMF were incubated

anaerobically and the remainder were incubated aerobically. All plates with media containing ORMF were incubated aerobically. Incubation was at 42°C for 48 hr. The procedure was replicated twice (see Table 2).

In the second test, organisms were pour plated on TSC and TSC+ORMF. One-half of each were overlaid with TSC. Incubation was as described in the first test. Plates were enumerated at 24 and 48 hr. The procedure was replicated 3 times.

Growth Rate of Pure Cultures

A 0.5 ml portion of fresh subculture (ATCC 12917) was added to each of 48 sterile tubes. One set of 24 tubes contained FTM while the other set contained FTM+ORMF. All tubes were placed in a 42°C incubator and one tube from each set was removed every 30 min. After agitation for 10 sec in a Vortex-Genie (Scientific Industries, Springfield, MA) turbidity was determined spectrophotometrically at 535 nm (Spectronic 20, Bausch and Lomb, Rochester, NY). A control tube containing FTM+ORMF only was measured every 30 min along with the sample tubes. The procedure was replicated twice.

Recovery of Pure Cultures on a Hydrophobic Grid Membrane Filter

Plates containing TSC and TSC+ORMF were prepared and air-dried for 15 min in a Biological Cabinet (BBL, Cockeysville, MD) with both the UV light and air flow in operation. Peptone water (100 ml) containing a C. perfringens culture diluted 1:100,000 was placed in a hydrophobic

Table 2. Medium, overlay and incubation condition for plates used in Test 1.

Medium[a]	Sodium bisulfite	ORMF	Overlay	Incubation Condition
TSC	no	yes	yes	Aerobic
TSC	no	yes	no	Aerobic
TSC	no	no	yes	Anaerobic
TSC	no	no	no	Anaerobic
TSC	no	no	yes	Aerobic
TSC	no	no	no	Aerobic
TSC	yes	no	yes	Anaerobic
TSC	yes	no	no	Anaerobic
TSC	yes	no	yes	Aerobic
TSC	yes	no	no	Aerobic

[a]TSC=tryptose sulfite cycloserine.

grid membrane filtration (HGMF) unit (Iso-Grid, QA Laboratories, Ltd., Toronto, Canada) and filtered. Filters were then placed on the pre-dried TSC and TSC+ORMF plates and overlaid with TSC and TSC+ORMF, respectively. At the same time, 1 ml from each dilution bottle containing the C. perfringens was pour plated into TSC and TSC+ORMF. All plates were overlaid, incubated at 42°C for 16 hr and most probable numbers (MPN) of colony-forming units were determined (Anonymous, 1985). The procedure was replicated 4 times.

Recovery from Inoculated Foods

Canned beef gravy, chicken gravy, chicken a la king and beef stew were procured from a local commercial source. Cans were aseptically opened and the contents mixed in a Stomacher Lab-Blender (Seward Laboratory, London, England) for 2 min. A portion (100 g) of each product was placed in each of 3 sterile 250 ml beakers. The absence of any interferring microorganisms from the sample prior to inoculation was confirmed by pour plating a 1:10 dilution of each product on TSC and TSC+ORMF and incubating at 42°C 24 hr. A fresh subculture (0.2 ml) from each test strain was added to each of the three beakers containing chicken gravy while 0.1 ml of each test strain was added to the 3 beakers containing each of the other products. Inoculated products were mixed by stirring with a sterile pipette for 30 sec and then incubated aerobically at 50°C. Samples were collected at 0, 2, 4, 6, 8, 10 and 12 hr and pour plated on TSC and TSC+ORMF. All plates were overlaid with TSC, incubated at 42°C for 24 hr and counted to determine

if differences existed between treatments. The procedures were replicated 3 times.

Recovery of Cold-Stressed Organisms

One of 3 cans of beef stew (425 g each) was inoculated with 1 ml of each C. perfringens test strain. Inoculated samples were mixed in a Stomacher apparatus for 2 min and transferred to separate 600 ml sterile beakers. Samples were incubated at 42°C for 18 hr. Control samples were then pour plated at TSC and TSC+ORMF to determine initial microbial levels. Five 25 g samples were removed from each beaker, placed in separate 25 x 85 mm screw cap tubes and stored at -15°C. The sample remaining in each beaker was stored at 4°C for 10 days. Refrigerated samples were analyzed daily for C. perfringens while a frozen sample was analyzed weekly. Comparative platings on TSC and TSC+ORMF were conducted. All plates were overlaid with TSC and incubated at 42°C for 24 hr. Three replications were performed.

Recovery of High-Temperature Injured Organisms

One of 3 beef stew samples (600 g each) was inoculated with 1 ml of each C. perfringens test strain. Inoculated samples were mixed in a Stomacher apparatus for 2 min, placed in sterile 1000 ml beakers and incubated at 42°C for 18 hr. Separate 100 g samples were removed and held in a Shaker Water Bath Model 127 (Fisher Scientific, Pittsburgh, PA) at 55, 60, 65 and 70°C for 1 hr. Control samples (no heat treatment) and heat treated samples were pour plated on TSC and TSC+ORMF. Overlays and incubation conditions were as above. Three replications were performed.

Recovery from Uninoculated Foods

Thirty-five raw ground meat samples and 10 ready-to-eat beef and chicken products were obtained from local commercial establishments. Raw samples were placed on ice inside a styrofoam box while the ready-to-eat samples were placed in an empty styrofoam box. All samples were transported to the laboratory and 1:10 dilutions were pour plated on TSC and TSC+ORMF. After overlaying with TSC, plates were incubated at 42°C for 24 hr and comparative counts were made. Where possible, 5 representative black colonies from each positive plate were stab inoculated into lactose-gelatin and nitrate-motility media. When less than 5 black colonies were present on a plate, all black colonies present were evaluated.

E. STATISTICAL ANALYSIS OF DATA

Analysis of variance was used to evaluate comparative data. Data presented are the means of all replications of each experiment. Where appropriate, significant differences ($P < 0.05$) among means were separated using Duncan's Multiple Range Test.

CHAPTER IV

RESULTS AND DISCUSSION

A. OXYGEN REDUCTION IN TRYPTOSE SULFITE CYCLOSERINE AGAR

When ORMF was added to TSC, the dissolved oxygen level was reduced to zero within approximately 3 min (Figure 1). Injection of air into the medium resulted in a small, short-lived increase in the oxygen level.

B. RECOVERY RATE OF PURE CULTURES

The first objective of this experiment was to determine if the addition of ORMF to TSC would allow C. perfringens to be grown under aerobic conditions. In the first test, TSC from which sodium bisulfite, a known reducing agent, had been removed was used for enumeration. This was done to establish that oxygen removal from aerobically incubated plates was due to ORMF alone. The presence of ORMF, overlay and incubation environment were varied to evaluate the effect of all combinations of these parameters for future studies.

Media to which ORMF had been added supported the best growth while presence or absence of an overlay did not significantly affect growth in TSC-sb+ORMF (Table 3). There was no difference in recovery of C. perfringens on plates incubated anaerobically or TSC-sb plates incubated aerobically with an overlay. TSC plates incubated aerobically

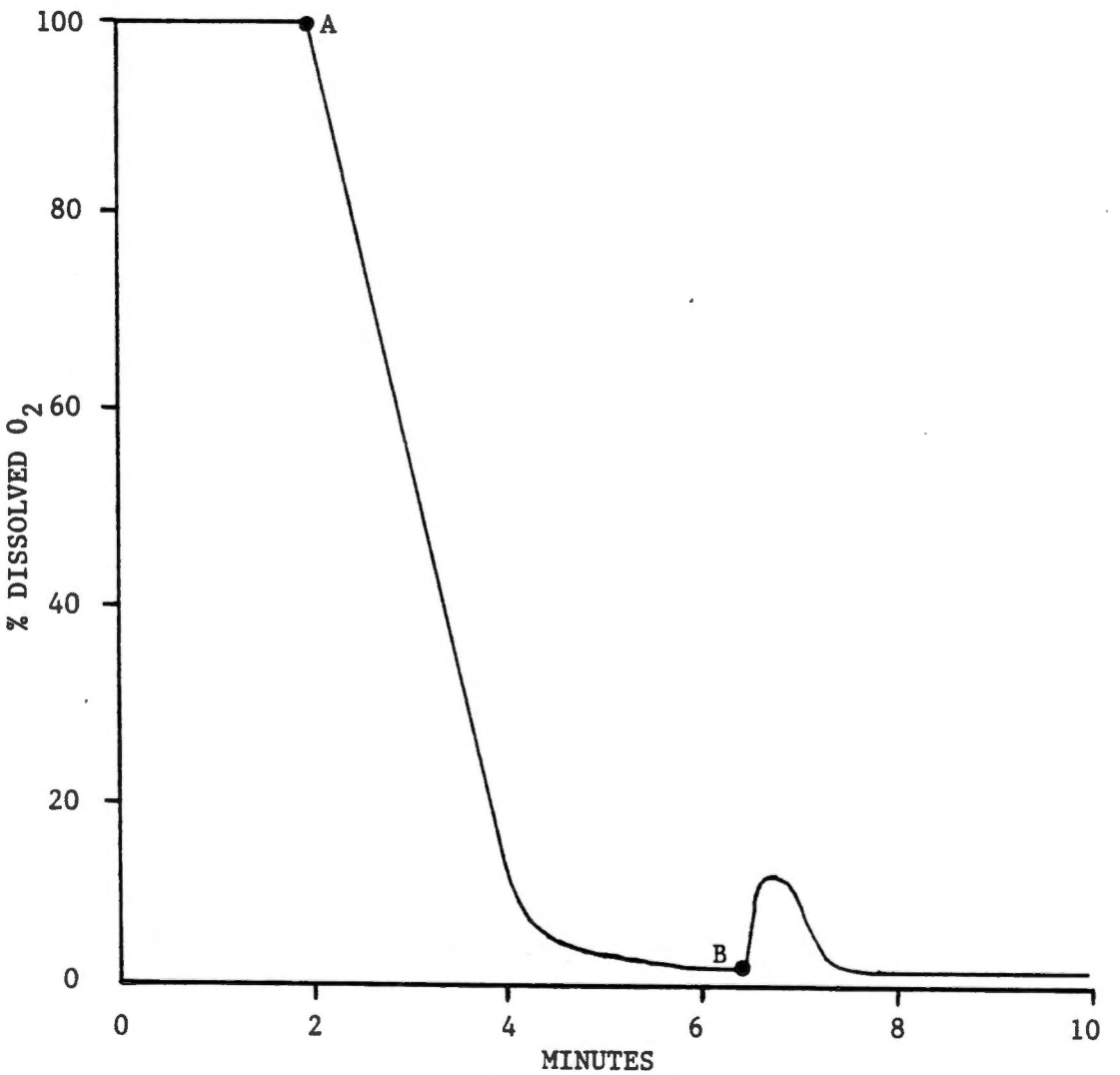


Figure 1. Rate of reduction of dissolved oxygen in tryptose sulfite cycloserine agar at 23°C. Point A: oxygen-reducing membrane fraction injected into the medium. Point B: air injected into the medium.

Table 3. Enumeration of Clostridium perfringens in media with and without oxygen-reducing agents.

Medium[a]	Overlay	Incubation Condition	Log_{10} CFU/ml[b]
TSC-sb+ORMF	yes	Aerobic	7.39[c]
TSC-sb+ORMF	no	Aerobic	7.39[c]
TSC-sb	yes	Anaerobic	6.89[d]
TSC-sb	no	Anaerobic	6.81[d]
TSC-sb	yes	Aerobic	6.73[d]
TSC	yes	Anaerobic	6.66[d]
TSC	no	Anaerobic	6.68[d]
TSC	yes	Aerobic	5.97[e]
TSC-sb	no	Aerobic	5.41[f]
TSC	no	Aerobic	<5.00[f,g]

[a]TSC=tryptose sulfite cycloserine; sb=sodium bisulfite; ORMF=oxygen-reducing membrane fraction.

[b]Numbers shown are means of 2 replications and 3 strains.

[c-f]Means with different superscripts are significantly different ($P<0.05$).

[g]For analysis of variance and mean separations a value of log_{10} CFU/ml=5.00 was used.

and TSC-sb plates with no overlay incubated aerobically supported very poor or no growth when compared to other plates.

No difference in counts between TSC and TSC-sb incubated anaerobically suggested that sodium bisulfite was not a significant inhibitor of C. perfringens in this medium. Since sodium bisulfite contributes to the differential properties of TSC, it is important that it be a component of the medium when evaluating food products for C. perfringens.

The primary objective of Test 2 was to determine the effect of ORMF on growth of C. perfringens in complete TSC. A secondary objective was to determine if differences in numbers of colony forming units (CFU) existed when plates were counted at 24 or 48 hr. Results showed that C. perfringens incubated aerobically in TSC+ORMF with an overlay yielded the highest counts ($P < 0.05$) (Table 4). There was no difference in counts among the remaining TSC+ORMF and TSC treatments incubated anaerobically. TSC plates incubated aerobically supported very little or no growth. There was no difference in number of CFU's when plates were counted at 24 or 48 hr.

The results of the tests showed that the addition of ORMF to TSC allowed the growth of C. perfringens under aerobic conditions. Incubating TSC+ORMF plates anaerobically was redundant and did not yield any higher counts. Incubating TSC plates aerobically supported only very poor growth. Consequently both of these conditions were omitted from future plating experiments within this project. Use of an overlay yielded variable results in that counts were either higher or there was

Table 4. Enumeration of Clostridium perfringens in tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction at 24 and 48 hours.

Medium[a]	Overlay	Incubation Condition	Log_{10} CFU/ml[b]	
			24 hr	48 hr
TSC+ORMF	yes	Aerobic	7.89[c]	7.89[c]
TSC+ORMF	yes	Anaerobic	7.71[d]	7.71[d]
TSC+ORMF	no	Aerobic	7.69[d]	7.69[d]
TSC+ORMF	no	Anaerobic	7.68[d]	7.69[d]
TSC	yes	Anaerobic	7.62[d]	7.63[d]
TSC	no	Anaerobic	7.61[d]	7.62[d]
TSC	yes	Aerobic	6.54[e]	6.54[e]
TSC	no	Aerobic	<6.00[f,g]	<6.00[f,g]

[a]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[b]Numbers shown are means of 3 replications and 3 strains.

[c-f]Means with different superscripts are significantly different ($P<0.05$).

[g]For analysis of variance and mean separations a value of log_{10} CFU/ml=6.00 was used.

no difference when one was used. Since an overlay was recommended in the pour plating technique described by Adler et al. (1983), it was used in future experiments. In summary, all subsequent plating experiments used TSC with an overlay incubated anaerobically and TSC+ORMF with an overlay incubated aerobically.

C. GROWTH RATE OF PURE CULTURES

The objective of this experiment was to determine whether ORMF had any effect on the growth rate of pure cultures of C. perfringens in fluid thioglycollate medium (FTM). Strain ATCC 12917 reached stationary growth phase in approximately 8 hr in FTM+ORMF while over 11 hr was required in FTM without ORMF (Figure 2). Turbidity of the culture in FTM was approximately 30% of maximum at the same time maximum turbidity in FTM+ORMF was achieved. In the uninoculated control containing FTM+ORMF, some turbidity developed. The absorbance of this control was subtracted from the readings for the inoculated samples containing FTM+ORMF.

The increased growth rate noted here is similar to that observed by Adler and Crow (1981) when they grew a number of anaerobic organisms in either FTM or nutrient broth to which ORMF had been added. They attributed the increase to two factors. First, ORMF removes oxygen from media more rapidly than other common reducing agents. Secondly, ORMF does not appear to have any toxic side effects on the microorganisms being grown.

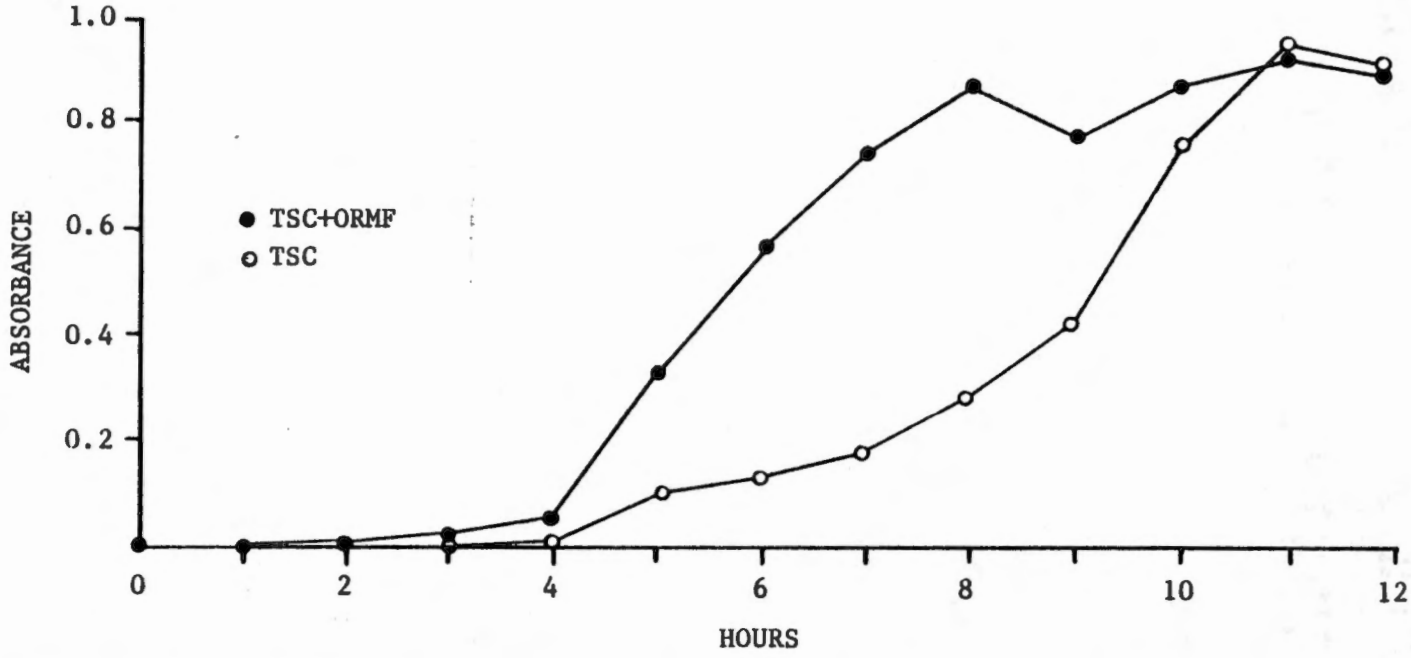


Figure 2. Rate of growth of Clostridium perfringens ATCC 12917 in fluid thioglycollate medium (FTM) with and without an oxygen-reducing membrane fraction (ORMF). Points represent the mean of two replications.

D. RECOVERY OF PURE CULTURES USING A HYDROPHOBIC GRID MEMBRANE FILTER

The objectives of this experiment were twofold: (a) to establish the feasibility of adapting a hydrophobic grid membrane filtration (HGMF) technique to the enumeration of C. perfringens in the presence of ORMF and (b) to compare the recovery rate of C. perfringens using the HGMF technique and a pour plating method.

In all three strains, recovery of C. perfringens was significantly greater using pour plates than the HGMF technique (Table 5). The highest counts were obtained when organisms were pour plated with TSC+ORMF. In all strains there was no difference in using TSC or TSC+ORMF when the HGMF technique was used. When all replications and strains were averaged, the recovery rate using HGMF was 49% of the pour plate technique on TSC medium. On TSC+ORMF, HGMF recovery was 62% of the pour plate value. Part of the discrepancy between HGMF and pour plates can be explained by the fact that the organisms are exposed to large volumes of oxygen during the filtering process.

Although it was possible to recover C. perfringens organisms using the HGMF, there was some difficulty involved in adapting the process. The Iso-Grid Methods Manual (Anonymous, 1985) does not recommend application of an overlay when using HGMF's. However, when no overlay was used, no growth occurred on plates that were incubated aerobically even in the presence of ORMF. Absence of growth was probably due to the fact that the filter lies on the surface of the medium and is

Table 5. Comparison of Clostridium perfringens recovery using pour plates and Hydrophobic Grid Membrane Filters (HGMF) with and without an oxygen-reducing membrane fraction.

Medium[a]	Technique	Strain[b] (Log_{10} CFU/ml)		
		ATCC12917	NCTC10239	NCTC8798
TSC+ORMF	Pour Plate	6.85[c]	6.63[c]	6.98[c]
TSC	Pour Plate	6.70[d]	6.46[d]	6.84[c]
TSC+ORMF	HGMF	6.47[e]	6.31[e]	6.73[d]
TSC	HGMF	6.41[e]	6.20[e]	6.71[d]

[a]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[b]Numbers shown represent means of 4 replications.

[c-e]Means in columns with different superscripts are significantly different ($P<0.05$).

continuously exposed to oxygen. In order to obtain growth with aerobic incubation, an overlay of TSC+ORMF was needed.

The overlay itself also created problems. Gas produced by C. perfringens tended to raise the overlay from the surface of the HGMF. Once this occurred, the black precipitate, which is formed by the organism reacting with sulfite and iron in the medium, spread over the surface of the HGMF very rapidly. This made the grids impossible to count. It was determined that if low numbers of C. perfringens were filtered and if incubation times were reduced to 16 hours, this problem could usually be eliminated. One additional difficulty was encountered. If the overlay was not poured very gently over the surface of the filter, organisms tended to be washed off and colonies would grow outside the boundaries of the filter. This problem arose periodically throughout the conduct of the experiment necessitating additional replications.

E. RECOVERY FROM INOCULATED FOODS

Once it was determined that TSC+ORMF would support growth of C. perfringens under aerobic conditions a logical progression was to determine if this technique could be applied to practical uses in food microbiology. The objective of this experiment was to determine if C. perfringens could be recovered from food products inoculated with the organism but otherwise commercially sterile. After inoculation, the food products were incubated at 50°C to simulate steam table conditions. Recovery rates using aerobic and anerobic incubation were compared.

In all products tested, recovery of C. perfringens was possible using both TSC (anaerobic incubation) and TSC+ORMF (aerobic incubation). In most cases, counts were significantly higher on TSC+ORMF throughout the 12 hours of the study for individual products as well as all products combined (Figures 3 and 4 and Table 7, Appendix A). In those instances where counts were not higher, there was no statistical difference between counts on TSC and TSC+ORMF. The results also indicated that C. perfringens grew quite readily in all products except chicken gravy where there was a tenfold decrease in the number of organisms during the test period.

Growth at typical steam table temperatures in different meat based products has been noted by other researchers (Hall and Angelotti, 1965; Brown and Twedt, 1972). Prior to the growth phase in products tested in this experiment, a decrease in viable organisms was usually seen at 2 and sometimes 4 hours. It is interesting to note that the largest differences in counts between TSC and TSC+ORMF also occurred at 2 or 4 hours for all products (Figures 3 and 4 and Table 7, Appendix A). A probable explanation for the early decrease in numbers is that the organisms underwent some type of stress or injury when they were first inoculated into the food product. The larger numbers of organisms recovered during this period on TSC+ORMF could be interpreted to mean that the mechanisms by which ORMF removes oxygen from a growth medium provides some type of protective effect for stressed organisms. It could also be that the rapid oxygen removal and the lack of toxic

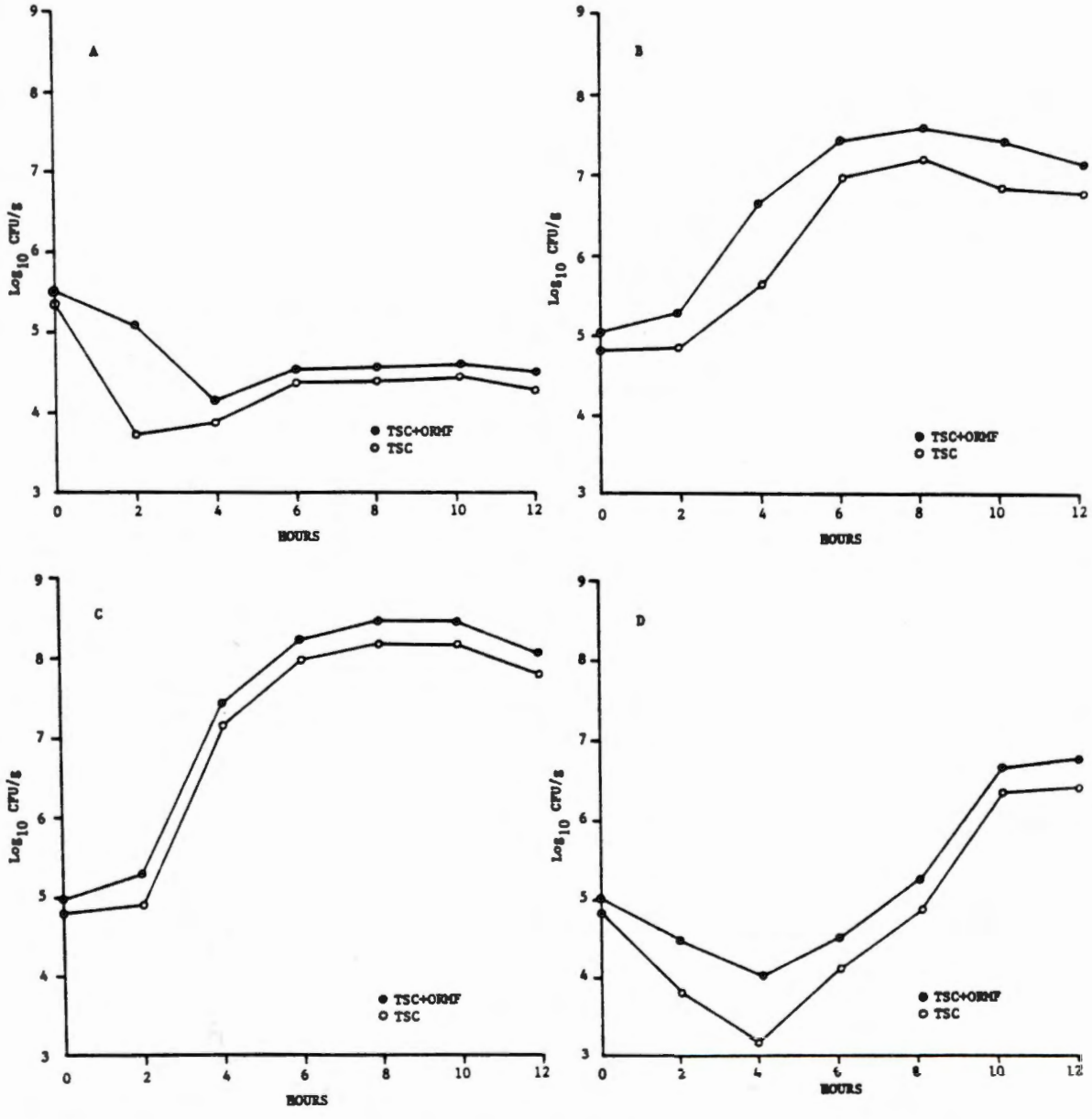


Figure 3. Enumeration of Clostridium perfringens grown in (A) chicken gravy; (B) beef gravy; (C) chicken a la king; and (D) beef stew for 12 hours. Each point represents the mean of three strains and three replications. TSC = tryptose sulfite cycloserine; ORMF = oxygen-reducing membrane fraction.

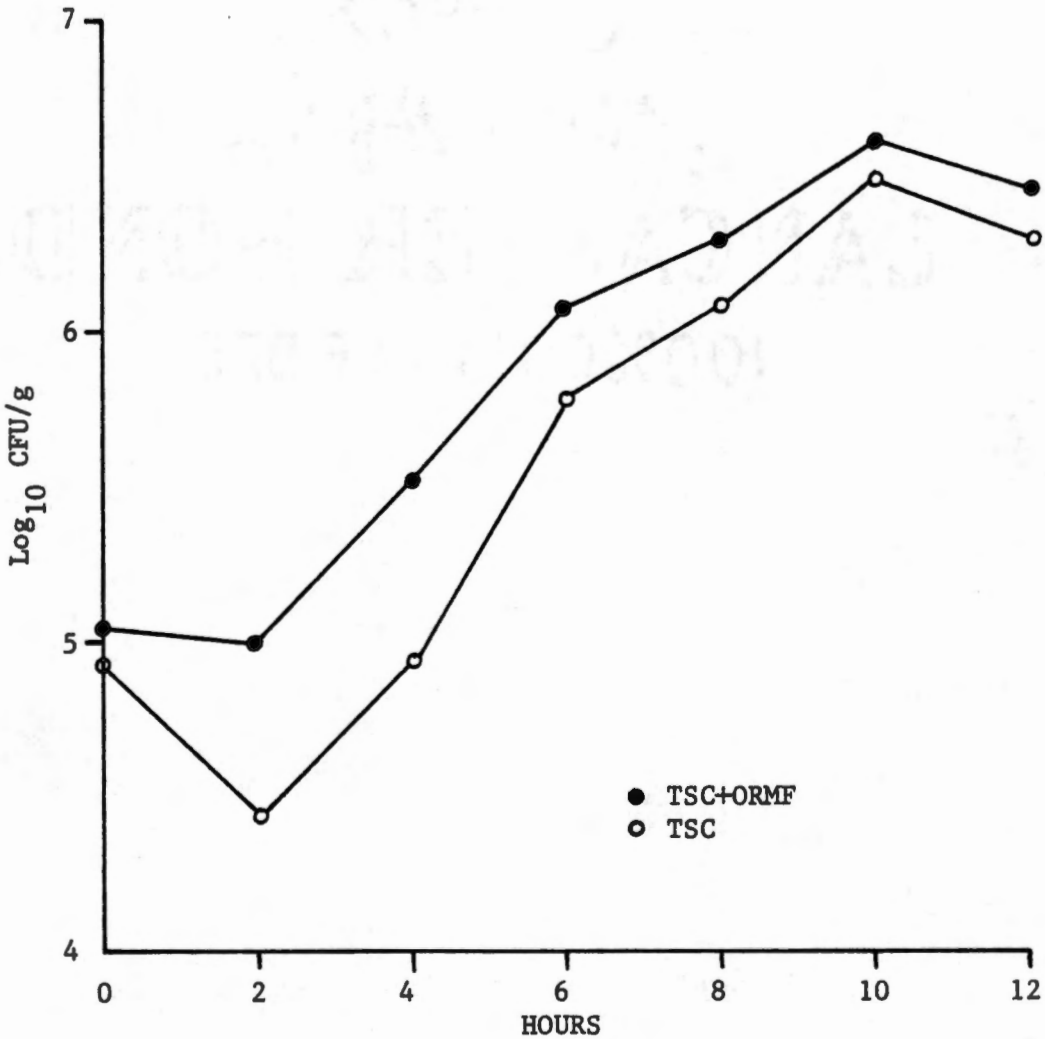


Figure 4. Enumeration of Clostridium perfringens grown in inoculated food products for 12 hours. Each point represents the mean of four products, three strains and three replications. TSC = tryptose sulfite cycloserine; ORMF = oxygen-reducing membrane fraction.

side effects postulated by Adler and Crow (1981) become more apparent when dealing with stressed organisms.

F. RECOVERY OF ORGANISMS EXPOSED TO ABUSE TEMPERATURES

A type of stress to which C. perfringens organisms are frequently subjected is unfavorable temperature conditions. Refrigeration, freezing and heating to above 50°C all have a detrimental effect on the organism. Vegetative cells are most susceptible, but spores are not immune to temperature injury. In separate experiments, C. perfringens was exposed to low and high temperatures to induce injury. The organisms were then recovered on TSC and TSC+ORMF to determine if differences in recovery rates existed.

Food samples inoculated with C. perfringens and stored at 4°C for 10 days showed significant differences in counts between samples plated on TSC and those plated on TSC+ORMF (Figure 5A and Table 8, Appendix A). On all days higher counts were found on TSC+ORMF. Media containing ORMF also yielded higher counts than did TSC from samples stored at -15°C (Figure 5B and Table 8, Appendix A).

Control samples as well as those heated to 55, 60 and 65°C had higher counts on TSC+ORMF (Figure 6 and Table 9, Appendix A). However, no organisms were recovered in either medium when samples were heated to 70°C for an hour. On cursory examination, it might be concluded that the counts on TSC+ORMF were higher for heat stressed organisms

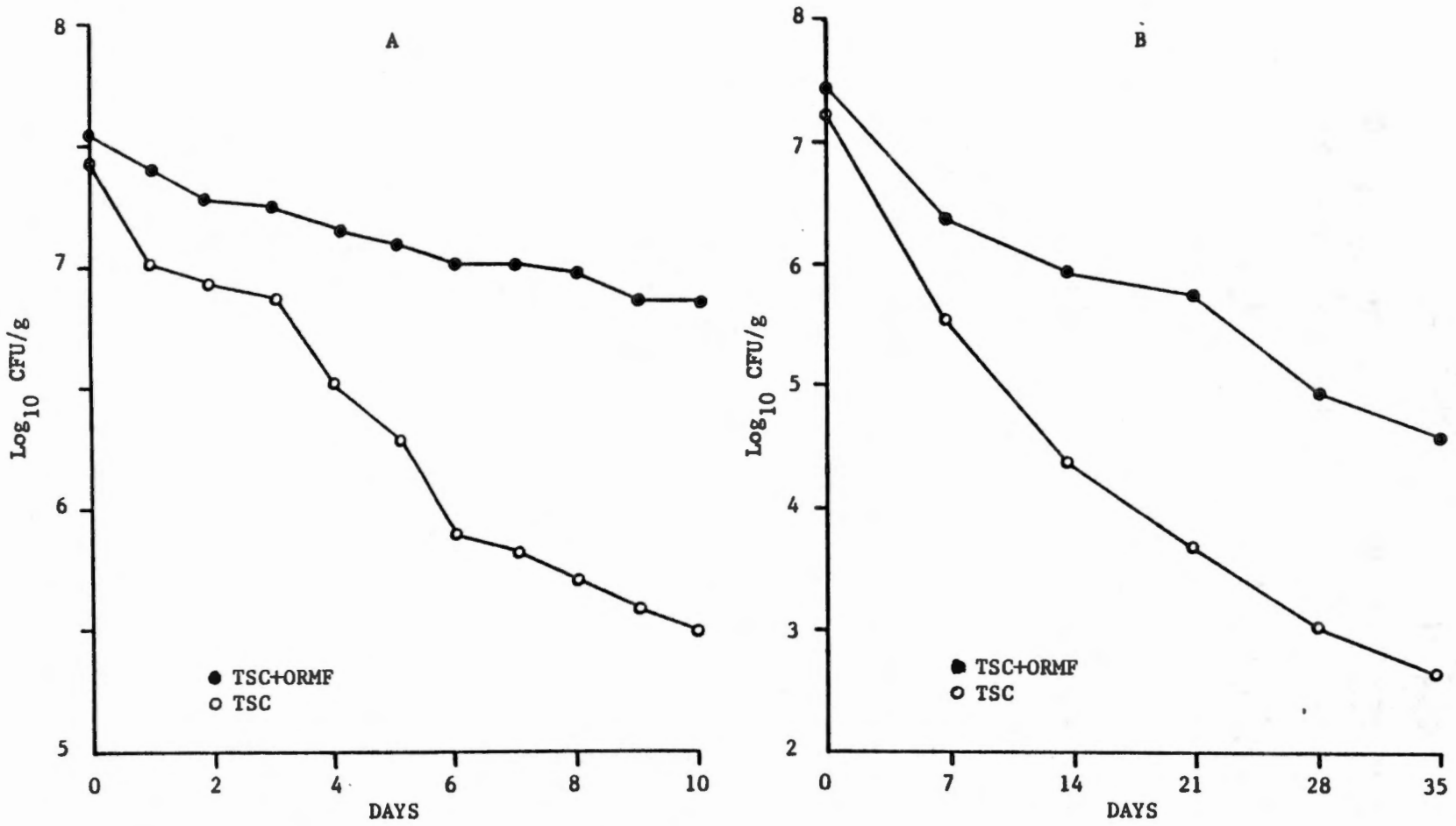


Figure 5. Enumeration of Clostridium perfringens grown in beef stew and then stored at (A) 4°C and (B) -15°C. Each point represents the mean of three strains and three replications. TSC = tryptose sulfite cycloserine; ORMF = oxygen-reducing membrane fraction.

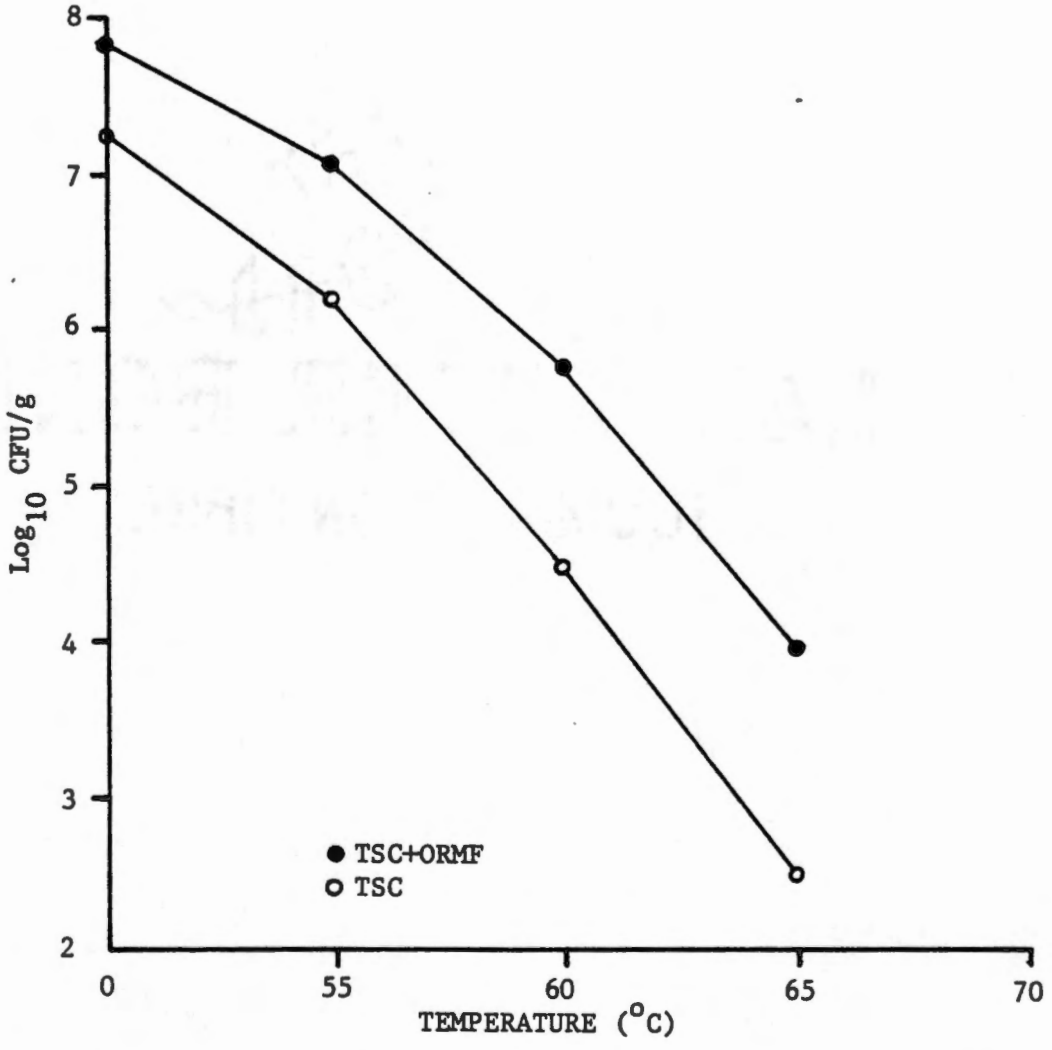


Figure 6. Enumeration of Clostridium perfringens grown in beef stew and then heated to various temperatures for one hour. Each point represents the mean of three strains and three replications. TSC = tryptose sulfite cycloserine; ORMF = oxygen-reducing membrane fraction.

because the control samples had higher counts on TSC+ORMF. This is not the case. Each level of heat treatment was an independent sample taken from the original incubated sample. Consequently, differences in counts should be attributed to different media conditions rather than number of organisms in the sample.

It has been well established that a decrease in the number of viable C. perfringens organisms takes place when they are exposed to abuse temperatures (Barach et al., 1974; Canada et al., 1964; Trakulchang and Kraft, 1977; White and Hall, 1984). The rate at which decreases occur is dependent on a number of conditions such as temperature, rate of heating or cooling, strain of the organism, recovery method and others. The parameters in the present experiments induced stress similar to that used by other researchers. They noted decreasing numbers of viable organisms when length or severity of exposure to unsuitable temperatures was increased.

This same decreasing numbers with increasing abuse was seen in the current study. However, further examination of the data presented here reveals that, for samples stored at 4°C, the rate of decrease in numbers of organisms was greater for TSC than for TSC+ORMF. Not only was the count for TSC+ORMF significantly higher on Days 1 through 10 but the difference in recovery rates between the two media was also greater as the days progressed. A similar relationship held true for the samples maintained at -15°C and those heated to 55, 60 and 65°C. The relative increase in number of organisms recovered by TSC+ORMF as

stress increases appears to support the hypothesis that ORMF may be efficient in aiding recovery of stressed organisms.

G. RECOVERY FROM UNINOCULATED FOODS

Previous phases of this study have dealt with laboratory strains either in pure form or artificially inoculated into food products. As with any other technique developed for food microbiology, this one must be applicable to food samples that have become naturally contaminated during some stage of processing or storage. The objective of this experiment was to determine if TSC+ORMF could be used to recover C. perfringens from naturally contaminated foods and, if so, to determine if ORMF has an effect on the recovery rate.

Of the 35 raw ground meat samples tested, C. perfringens was detected in 22 (Table 6). In the positive samples, the average count using TSC+ORMF was 224 CFU/g and 135 CFU/g in TSC. Both TSC and TSC+ORMF were able to detect the organism in 18 of the samples. In all cases except one, the counts were higher using TSC+ORMF. In the exception, the counts were equal. In the remaining 4 samples, C. perfringens was detected by only the TSC+ORMF. In no cases were C. perfringens organisms detected on TSC alone. All black colonies that were evaluated by confirmatory tests were determined to be C. perfringens.

Ten ready-to-eat food samples were plated on TSC and TSC+ORMF with negative results. The samples evaluated were beef stew (2), beef gravy (2), salisbury steak (2), ground beef patties with gravy, meat loaf with gravy, meat balls with gravy and cube steak with gravy.

Table 6. Incidence of Clostridium perfringens in raw meat samples from local retail sources.

Sample	Source	TSC	TSC+ORMF
Ground Beef	A	<10	<10
Ground Beef	B	2180	2430
Ground Chuck	B	40	70
Ground Sirloin	B	280	720
Ground Sirloin	C	<10	10
Ground Beef	C	<10	<10
Ground Beef	D	<10	60
Ground Beef	E	820	1110
Ground Beef	F	<10	<10
Ground Beef	G	<10	<10
Chopped Beef	H	<10	<10
Chopped Sirloin	I	<10	<10
Ground Beef (Bulk)	J	360	480
Ground Beef	J	2480	2610
Ground Turkey	K	<10	<10
Ground Beef (Bulk)	K	100	110
Ground Beef	K	10	10
Ground Turkey (Bulk)	K	20	30
Ground Beef	L	<10	<10
Ground Beef	M	<10	<10
Chopped Beef	N	4800	5300
Ground Beef (Bulk)	N	3900	4900
Cubed Steak	O	<10	<10
Ground Beef	O	50	60
Ground Beef	P	<10	<10
Ground Beef (Bulk)	P	<10	110
Chicken Livers	P	<10	<10
Ground Beef	Q	<10	<10
Chopped Beef	R	1890	2470
Ground Beef	S	30	40
Ground Beef	T	<10	<10
Ground Beef	U	<10	<10
Ground Beef	V	<10	40
Cubed Beef	W	<10	<10
Ground Beef	X	<10	<10

Recovery from 51.4% of the raw samples using TSC agreed well with the data presented by Ladiges et al. (1974) in which C. perfringens was recovered from 47% of raw ground beef samples obtained from a commercial source. The 62.8% recovery rate using TSC+ORMF was somewhat higher. That 4 samples were positive for C. perfringens on TSC+ORMF and negative on TSC is most important. It indicates that, under certain conditions, TSC+ORMF is a more sensitive test for the presence of the organism in a food product.

CHAPTER V

CONCLUSIONS

In the first part of this study, it was shown that the normally anaerobic microorganism, Clostridium perfringens, could be grown under aerobic conditions when an ORMF was added to TSC. These results were important for 3 reasons. Firstly, the need for using cumbersome and expensive anaerobe jars and associated materials was eliminated. Secondly, petri plates could be monitored for growth periodically without interfering with anaerobic conditions. Thirdly, a potential was demonstrated for this technique to be adapted to the recovery of other anaerobes.

Another important finding of the study was that C. perfringens recovery was generally better and the growth rate more rapid in TSC+ORMF than in TSC alone. This observation exceeded the original expectations for ORMF and gave rise to several interesting considerations. First, a more rapid growth rate could allow for shorter laboratory incubation times and, consequently, shorter sample analysis. Secondly, ORMF, or its mechanism of reducing oxygen, appeared to have some protective effect on stressed organisms. This was seen in cells exposed to temperature abuse and foods that were sampled within 2 hours of inoculation. Finally, the results indicated that this technique had a greater probability for recovering C. perfringens from food samples in which very low numbers of the organism were present. This is supported by the fact that four

naturally contaminated samples were negative for C. perfringens on TSC and positive on TSC+ORMF.

Considering the difficulty encountered in adapting the TSC+ORMF technique for use with a hydrophobic grid membrane filter, and the fact that no improvement in counts was achieved, additional work in this area is not recommended.

LIST OF REFERENCES

LIST OF REFERENCES

Abeyta, Jr., C., Wekell, M. M. and Peeler, J. T. 1985. Comparison of media for enumeration of Clostridium perfringens in food. J. Food Sci. 50:1732.

Adams, B. W. and Meade, G. C. 1980. Comparison of media and methods for counting Clostridium perfringens in poultry meat and further processed products. J. Hyg., Camb. 84:151.

Adler, H. I. 1985. Personal communication. Oak Ridge National Laboratories, Oak Ridge, TN.

Adler, H. I., Carrasco, A., Crow, W. and Gill, J. S. 1981. Cytoplasmic membrane fraction that promotes septation in an Escherichia coli lon mutant. J. Bacteriol. 147:326.

Adler, H. I. and Crow, W. 1981. A novel approach to the growth of anaerobic organisms in "Biotechnology and Bioengineering Symposium," No. 11, p. 533. J. Wiley and Sons, Inc., New York.

Adler, H. I., Crow, W. D., Hadden, C. T., Hall, J. and Machanoff, R. 1983. New techniques for growing anaerobic bacteria: Experiments with Clostridium butyricum and Clostridium acetobutylicum. In "Biotechnology and Bioengineering Symposium," No. 13, p. 153. J. Wiley and Sons, Inc., New York.

Adler, H. I., Fisher, W. D., Hardigree, A. A. and Stapleton, G. E. 1966. Repair of radiation-induced damage to the cell division mechanism of Escherichia coli. J. Bacteriol. 91:737.

Adler, H. I. and Hardigree, A. A. 1964. Analysis of a gene controlling cell division and sensitivity to radiation in E. coli. J. Bacteriol. 87:720.

Adler, H. I. and Hardigree, A. A. 1965. Post-irradiation growth, division and recovery in bacteria. Radiat. Res. 25:92.

Al-Obaidy, H. M., Khan, M. A. Blaschek, H. P. and Klein, B. P. 1985. Early detection of Clostridium perfringens enterotoxin and its relationship to sensory quality of cooked chicken. J. Food Safety 7:43.

Alper, T. and Gillies, N. E. 1960. The relationship between growth and survival after irradiation of Escherichia coli strain B and two resistant mutants. J. Gen. Microbiol. 22:113.

Anderson, K. L. and Fung, D. Y. C. 1983. Anaerobic methods, techniques and principles for food bacteriology. J. Food Prot. 46:811.

Ando, Y., Tzuzuki, T., Sunagawa, H. and Oka, S. 1985. Heat resistance, spore germination and enterotoxigenicity of Clostridium perfringens. Microbiol. Immunol. 29(4):317.

Angelotti, R., Hall, H. E., Foter, M. J. and Lewis, K. H. 1962. Quantitation of Clostridium perfringens in foods. Appl. Microbiol. 10:193.

Anonymous. 1985. "The Iso-Grid Methods Manual," 2nd. ed. QA Laboratories Limited, Toronto, Canada.

AOAC. 1984. "Official Methods of Analysis," 14th ed. Association of Official Analytical Chemists, Washington, D.C.

Apiluktivongsa, P. and Walker, H. W. 1980. Influence of selective agents on recovery of cold-stressed cells in Clostridium perfringens. J. Food Sci. 45:574.

Babajimopoulos, M. and Mikolajcik, E. M. 1981. Heat inactivation and reactivation of alpha toxin from Clostridium perfringens. J. Food Prot. 44:899.

Barach, J. T., Adams, D. M. and Speck, M. L. 1974. Recovery of heated Clostridium perfringens type A spores on selective media. Appl. Microbiol. 28:793.

Barnes, E. M. 1985. Isolation methods for anaerobes in foods. Int. J. Food Microbiol. 2:97.

Bartholomew, B. A. and Stringer, M. F. 1984. Clostridium perfringens enterotoxin: A brief review. Biochem. Soc. Trans. 12(2):195.

Bartsch, A. G. and Walker, H. W. 1982. Effect of temperature, solute, and pH on the tolerance of Clostridium perfringens to reduced water activities. J. Food Sci. 47:1754.

Beerens, H., Romond, C., Lepage, C. and Criqueloin, J. 1982. A liquid medium for the enumeration of Clostridium perfringens in food and feces. In "Isolation and Identification Methods for Food Poisoning Organisms," ed. J. E. L. Corry, D. Roberts and F. A. Skinner, p. 137. Academic Press, London.

Bradshaw, J. G., Stelma, G. N., Jones, V. I., Peeler, J. T., Wimsatt, J. C., Corwin, J. J. and Twedt, R. M. 1982. Thermal inactivation of Clostridium perfringens enterotoxin in buffer and in chicken gravy. J. Food Sci. 47:914.

Brown, D. F. and Twedt, R. M. 1972. Assessment of the sanitary effectiveness of holding temperatures on beef cooked at low temperature. Appl. Microbiol. 24:599.

Bryan, F. L. and Kilpatrick, E. G. 1971. Clostridium perfringens related to roast beef cooking, storage and contamination in a fast food service restaurant. Am. J. Public Health 61:1869.

Canada, J. C., Strong, D. H. and Scott, L. G. 1964. Response of Clostridium perfringens spores and vegetative cells to temperature variation. Applied Microbiol. 12(3):273.

CDC. 1981. "Foodborne Disease Surveillance; Annual Summary 1979," U.S. Department of Health and Human Services, Center for Disease Control, Publication No. CDC 81-8185.

CDC. 1983a. "Foodborne Disease Outbreaks; Annual Summary 1980," U.S. Department of Health and Human Services, Center for Disease Control, Publication No. CDC 83-8185.

CDC. 1983b. "Foodborne Disease Outbreaks; Annual Summary 1981," U.S. Department of Health and Human Services, Center for Disease Control, Publication No. CDC 83-8185.

CDC. 1984. "Foodborne Disease Outbreaks; Annual Summary 1982," U.S. Department of Health and Human Services, Center for Disease Control, Publication No. CDC 84-8185.

Craven, S. E. 1980. Growth and sporulation of Clostridium perfringens in foods. Food Technol. 34:80.

Craven, S. E., Blankenship, L. C. and McDonel, J. L. 1981. Relationship of sporulation, enterotoxin formation and spoilage during growth of Clostridium perfringens type A in cooked chicken. Appl. Environ. Microbiol. 41:1184.

Dack, G. M., Sugiyama, H. Owens, F. J. and Kirsner, J. B. 1954. Failure to produce illness in human volunteers fed Bacillus cereus and Clostridium perfringens. J. Inf. Dis. 94:34.

DeLaporte, B. 1956. La "restauration par voisinage" chez des bacteria irradiees par des rayon X. Ann. Inst. Pasteur (Paris) 91:727.

Duncan, C. L. 1976. Clostridium perfringens. In "Food Microbiology: Public Health and Spoilage Aspects," ed. M. P. Defigueiredo and D. F. Splittstoesser, p. 170. AVI Publishing Co., Westport, CT.

Erickson, J. E. and Deibel, R. H. 1978. New method for rapid screening and enumeration of Clostridium perfringens in foods. Appl. Environ. Microbiol. 36:567.

Foegeding, P. M. and Busta, F. F. 1980. Production of phospholipase C by nine strains of Clostridium perfringens at 37°C and at a constantly rising temperature. J. Food Prot. 43:15.

Hall, I. C. 1929. A review of the development and application of physical and chemical principles in the cultivation of obligately anaerobic bacteria. J. Bacteriol. 17:255.

Hall, H. E. and Angelotti, R. 1965. Clostridium perfringens in meat and meat products. Appl. Microbiol. 13:352.

Handford, P. M. 1974. A new method for the detection and enumeration of Clostridium perfringens in foods. J. Appl. Bacteriol. 37:559.

Handford, P. M. and Cavett, J. J. 1973. A medium for the detection and enumeration of Clostridium perfringens (welchii) in foods. J. Sci. Food Agri. 24:487.

Harmon, S. M. and Duncan, C. L. 1984. Clostridium perfringens. In "Compendium of Methods for the Microbiological Examination of Foods," ed. M. L. Speck, 2nd ed., p. 483. American Public Health Assn., Washington, D.C.

Harmon, S. M. and Kautter, D. A. 1978. Media for confirming Clostridium perfringens from food and feces. J. Food Prot. 41:626.

Harmon, S. M., Kautter, D. A. and Peeler, J. T. 1971a. Improved medium for enumeration of Clostridium perfringens. Appl. Microbiol. 22:688.

Harmon, S. M., Kautter, D. A. and Peeler, J. T. 1971b. Comparison of media for the enumeration of Clostridium perfringens. Appl. Microbiol. 22:922.

Hatheway, C. L., Whaley, D. N. and Dowell, Jr., V. R. 1980. Epidemiological aspects of Clostridium perfringens foodborne illness. Food. Technol. 34:77.

Hauschild, A. H. W. 1973. Food poisoning by Clostridium perfringens. Can. Inst. Food Sci. Technol. J. 6(2):106.

Hauschild, A. H. W. and Hilsheimer, R. 1971. Purification and characteristics of the enterotoxin of Clostridium perfringens type A. Can. J. Microbiol. 17:1425.

Hauschild, A. H. W. and Hilsheimer, R. 1974a. Evaluation and modifications of media for enumeration of Clostridium perfringens. Appl. Microbiol. 27:78.

Hauschild, A. H. W. and Hilsheimer, R. 1974b. Enumeration of food-borne Clostridium perfringens in egg-yolk free tryptose-sulfite-cycloserine agar. Appl. Microbiol. 27:521.

Hauschild, A. H. W., Hilsheimer, R. and Griffith, D. W. 1974. Enumeration of fecal Clostridium perfringens spores in egg yolk-free tryptose-sulfite-cycloserine agar. Appl. Microbiol. 27:527.

Hobbs, B. C. 1969. Clostridium perfringens and Bacillus cereus infections. In "Food-borne infections and intoxications," ed. H. Riemann, p. 131. Academic Press, New York.

Hobbs, B. C., Smith, M. E., Oakley, C. L., Warrack, G. H. and Cruickshank, J. C. 1953. Clostridium welchii food poisoning. J. Hyg. 51:75.

Howard-Martin, M., Morton, R. J., Qualls, C. W. and MacAllister, C. G. 1986. Clostridium perfringens type C enterotoxemia in a newborn foal. J. Am. Vet. Med. Assn. 189:564.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1.

Hungate, R. E. 1969. A roll tube method for the cultivation of strict anaerobes. In "Methods in Microbiology, Vol. 3B," ed. J. R. Norris and D. W. Ribbons, p. 117. Academic Press, London.

Hunziker, O. F. 1902. A review of the existing methods for cultivating anaerobic bacteria. J. Appl. Microscopy 5:1694, 1741, 1800, 1854.

Jay, J. M. 1986. "Modern Food Microbiology," 3rd ed. D. Van Nostrand Co., New York.

Keusch, G. T. and Weinstein, L. (Ed.). 1975. "Anaerobic Bacteria and Disease," The Upjohn Co., Kalamazoo, MI.

Klein, E. 1895. Uber einen pathogen anaeroben Darmbacillus, Bacillus enteritidis sporogens. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. I. Orig. 18:737.

Labbe, R. G. 1980. Relationship between sporulation and enterotoxin production in Clostridium perfringens type A. Food Technol. 34:88.

Labbe, R. G. and Norris, K. E. 1982. Evaluation of plating media for recovery of heated Clostridium perfringens spores. J. Food Prot. 45:686.

Ladiges, W. C., Foster, J. F. and Ganz, W. M. 1974. Incidence and viability of Clostridium perfringens in ground beef. J. Milk Food Technol. 37:622.

Marshall, R. S., Steenbergen, J. F. and McClung, L. S. 1965. Rapid technique for the enumeration of Clostridium perfringens. Appl. Microbiol. 13:559.

McClane, B. A. and McDonel, J. L. 1981. Protective effects of osmotic stabilizers on morphological and permeability alterations induced in Vero cells by Clostridium perfringens enterotoxin. Biochem. Biophys. Acta 641:401.

McClung, L. S. 1945. Human food poisoning due to growth of Clostridium perfringens (C. welchii) in freshly cooked chicken: Preliminary note. J. Bacteriol. 50:229.

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

McDonel, J. L. 1980. Mechanism of action of Clostridium perfringens enterotoxin. Food Tech. 34:91.

McDonel, J. L. and Demers, G. W. 1982. In vivo effects of enterotoxin from Clostridium perfringens type A in the rabbit colon: Binding vs. biologic activity. J. Inf. Dis. 145:490.

McDonel, J. L. and McClane, B. A. 1979. Binding versus biological activity of Clostridium perfringens enterotoxin in Vero cells. Biochem. Biophys. Res. Comm. 87:497.

Meade, G. C. 1969. Combined effects of salt concentration and redox potential of the medium of the initiation of vegetative growth of Clostridium welchii. J. Appl. Bacteriol. 32:468.

Meade, G. C. 1985. Selective and differential media for Clostridium perfringens. Int. J. Food Microbiol. 2:89.

Naik, H. S. and Duncan, C. L. 1977. Enterotoxin formation in foods by Clostridium perfringens type A. J. Food Safety 1:7.

Neut, C., Pathak, J., Romond, C. and Beerens, H. 1985. Rapid detection of Clostridium perfringens: Comparison of lactose sulfite broth with tryptose-sulfite-cycloserine agar. J. Assoc. Off. Anal. Chem. 68:881.

Nygren, B. 1962. Phsopholipase C producing bacteria and food poisoning: An experimental study on Clostridium perfringens and Bacillus cereus. Acta. Path. Microbiol. 2nd Suppl. 160:1.

Ritter, J., O'Leary, J. and Langlois, B. E. 1979. Fate of selected pathogens inoculated into foods prepared in slow cookers. J. Food Prot. 43:872.

Roy, R. J., Busta, F. F. and Thompson, D. R. 1981. Thermal inactivation and Clostridium perfringens after growth at several constant and linear rising temperatures. J. Food Sci. 46:1586.

Schnaitman, C. 1970. Protein composition of the cell wall and cytoplasmic membrane of E. coli. J. Bacteriol. 104:890.

Schwab, A. H., Leininger, H. V. and Powers, E. M. 1984. Media, reagents, and stains. In "Compendium for the Microbiological Examination of Foods," ed. M. L. Speck, 2nd ed., p. 788. American Public Health Assn., Washington, D.C.

Shahidi, S. A. and Ferguson, A. R. 1971. New quantitative, qualitative and confirmatory media for rapid analysis of food for Clostridium perfringens. Appl. Microbiol. 21:500.

Shoemaker, S. P. and Pierson, M. D. 1976. "Phoenix phenomenon" in the growth of Clostridium perfringens. Appl. Environ. Microbiol. 32:803.

Silliker, J. H. (Ed.). 1980. "Microbial Ecology of Foods," Vol. I. Academic Press, Inc., Orlando, FL.

Skjelkvale, R. and Duncan, C. L. 1975. Enterotoxin formation by different toxigenic types of Clostridium perfringens. Infect. Immunity. 11:563.

Skjelkvale, R. and Uemura, T. 1977. Experimental diarrhea in human volunteers following oral administration of Clostridium perfringens enterotoxin. J. Appl. Bacteriol. 43:281.

Smith, P. H. and Hungate, R. E. 1958. Isolation and characterization of Methanobacterium ruminantium N. sp. J. Bacteriol. 75:713.

Speck, M. L. (Ed.). 1984. "Compendium of Methods for the Microbiological Examination of Foods," 2nd ed. American Public Health Association, Washington, D.C.

Sterne, M. and Warrack, G. H. 1964. The types of Clostridium perfringens. J. Pathol. Bacteriol. 88:279.

St. John, W. D., Matches, J. R. and Wekell, M. M. 1982. Use of iron milk medium for enumeration of Clostridium perfringens. J. Assoc. Off. Anal. Chem. 65:1129.

Strong, D. H., Foster, E. F. and Duncan, C. H. 1970. Influence of water activity on the growth of Clostridium perfringens. Appl. Microbiol. 19:980.

Sutton, R. G. A., Kendall, M. and Hobbs, B. C. 1972. The effect of two methods of cooking and cooling on Clostridium welchii and other bacteria in meat. J. Hyg., Camb. 70:415.

Trakulchang, S. P. and Kraft, A. A. 1977. Survival of Clostridium perfringens in refrigerated and frozen meat and poultry items. J. Food Sci. 42:518.

Troller, P. A. 1973. The water relationship of food-borne bacterial pathogens. A review. J. Milk Food Technol. 36:276.

White, C. A. and Hall, L. P. 1984. The effect of temperature abuse on Clostridium perfringens and Bacillus cereus in raw beef and chicken substrates during frozen storage. Food Microbiol. 1:97.

Zottola, E. A. 1971. "Clostridium perfringens Food Poisoning," Agricultural Extension Service, University of Minnesota, Minneapolis, MN.

APPENDIXES

APPENDIX A

ENUMERATION DATA FOR FIGURES 3-6

Table 7. Enumeration of Clostridium perfringens grown in inoculated food products during a 12-hour time period.

Hour	Log_{10} CFU/gm[a]									
	Chicken Gravy		Beef Gravy		Chicken a la King		Beef Stew		All Products	
	TSC[d]	TSC+ ORMF[d]	TSC	TSC+ ORMF	TSC	TSC+ ORMF	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	5.22[b]	5.33[c]	4.72[b]	5.00[c]	4.83[b]	4.96[c]	4.80[b]	4.98[c]	4.89[b]	5.07[c]
2	3.74[b]	5.11[c]	4.83[b]	5.18[c]	4.93[b]	5.22[c]	3.85[b]	4.48[c]	4.34[b]	5.00[c]
4	3.90[b]	4.18[c]	5.57[b]	6.51[c]	7.17[b]	7.39[c]	3.12[b]	3.97[c]	4.94[b]	5.52[c]
6	4.33[b]	4.38[b]	6.92[b]	7.37[c]	7.86[b]	8.05[c]	4.09[b]	4.48[c]	5.80[b]	6.07[c]
8	4.35[b]	4.47[b]	7.15[b]	7.46[c]	8.05[b]	8.21[c]	4.85[b]	5.23[c]	6.10[b]	6.34[c]
10	4.46[b]	4.50[b]	6.88[b]	7.27[c]	8.14[b]	8.30[c]	6.38[b]	6.61[c]	6.46[b]	6.67[c]
12	4.22[b]	4.33[b]	6.74[b]	7.01[c]	7.69[b]	7.83[c]	6.50[b]	6.69[c]	6.29[b]	6.47[c]

[a]Numbers shown are means of 3 replications and 3 strains.

[b,c]Means in rows under the same product with different superscripts are significantly different ($P<0.05$).

[d]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

Table 8. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of Clostridium perfringens from beef stew.

Day	Storage Temperature (°C)	Log$_{10}$ CFU/gm[a]	
		TSC[b]	TSC+ORMF[b]
0	4	7.33[c]	7.50[d]
1	4	7.07[c]	7.39[d]
2	4	6.87[c]	7.27[d]
3	4	6.84[c]	7.25[d]
4	4	6.55[c]	7.17[d]
5	4	6.30[c]	7.14[d]
6	4	5.91[c]	7.04[d]
7	4	5.80[c]	7.01[d]
8	4	5.67[c]	6.96[d]
9	4	5.58[c]	6.89[d]
10	4	5.49[c]	6.86[d]
0	-15	7.33[c]	7.50[d]
7	-15	5.48[c]	6.34[d]
14	-15	4.38[c]	5.88[d]
21	-15	3.75[c]	5.72[d]
28	-15	3.02[c]	5.48[d]
35	-15	2.72[c]	5.12[d]

[a]Numbers shown are means of 3 strains and 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Numbers in rows with different superscripts are significantly different ($P<0.05$).

Table 9. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of Clostridium perfringens from beef stew heated at various temperatures for 1 hour.

Temperature (°C)	Log_{10} CFU/gm[a]	
	TSC[b]	TSC+ORMF[b]
Control[c]	7.41[d]	7.87[e]
55	6.24[d]	7.07[e]
60	4.47[d]	5.62[e]
65	2.43[d]	3.77[e]
70	0.00[d]	0.00[d]

[a]Numbers shown are means of 3 strains and 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c]Control samples were not exposed to any high temperatures prior to plating.

[d,e]Numbers in rows with different superscripts are significantly different ($P<0.05$).

APPENDIX B

ENUMERATION DATA, BY STRAIN, FOR ALL EXPERIMENTS EXCEPT HGMF

Table 10. Enumeration of Clostridium perfringens by strain in media with and without oxygen-reducing agents.

Medium[a]	Overlay	Incubation Condition	Log_{10} CFU/ml[b]		
			ATCC 12917	NCTC 10239	NCTC 8798
TSC-sb+ORMF	yes	Aerobic	7.39[c]	7.33[c]	7.46[c]
TSC-sb+ORMF	no	Aerobic	7.34[c]	7.38[c]	7.44[c,d]
TSC-sb	yes	Anaerobic	6.80[c,d]	6.79[c,d]	7.08[d,e]
TSC-sb	no	Anaerobic	6.66[d]	6.70[c,d]	7.06[e]
TSC-sb	yes	Aerobic	6.82[c,d]	6.26[d,e]	7.10[c,d,e]
TSC	yes	Anaerobic	6.66[d]	6.32[d,e]	7.01[e]
TSC	no	Anaerobic	6.70[d]	6.39[c,d,e]	6.96[e]
TSC	yes	Aerobic	5.87[e]	5.65[e,f]	6.39[f]
TSC-sb	no	Aerobic	6.24[d,e]	<5.00[f,h]	<5.00[g,h]
TSC	no	Aerobic	<5.00[f,h]	<5.00[f,h]	<5.00[g,h]

[a]TSC=tryptose sulfite cycloserine; sb=sodium bisulfite; ORMF=oxygen-reducing membrane fraction.

[b]Numbers shown are means of 2 replications.

[c-g]Means in the same columns with different superscripts are significantly different ($P<0.05$).

[h]For analysis of variance and mean separations a value of log_{10} CFU/ml=5.00 was used.

Table 11. Enumeration of Clostridium perfringens by strain in tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction at 24 and 48 hours.

Medium[a]	Overlay	Condition	Log_{10} CFU/ml[b]					
			ATCC 12917		NCTC 10239		NCTC 8798	
			24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
TSC+ORMF	yes	Aerobic	8.06[c]	8.05[c]	8.07[c]	8.08[c]	7.55[c]	7.55[c]
TSC+ORMF	yes	Anaerobic	7.96[c]	7.95[c]	7.84[c]	7.84[c]	7.34[c]	7.34[c]
TSC+ORMF	no	Aerobic	7.90[c]	7.88[c]	7.77[c]	7.76[c]	7.40[c]	7.41[c]
TSC+ORMF	no	Anaerobic	7.98[c]	7.99[c]	7.74[c]	7.75[c]	7.31[c]	7.31[c]
TSC	yes	Anaerobic	7.81[c]	7.81[c]	7.75[c]	7.75[c]	7.30[c]	7.32[c]
TSC	no	Anaerobic	7.77[c]	7.78[c]	7.73[c]	7.74[c]	7.34[c]	7.34[c]
TSC	yes	Aerobic	6.60[d]	6.61[d]	6.70[d]	6.70[d]	6.32[d]	6.32[d]
TSC	no	Aerobic	<6.00[d,f]	<6.00[d,f]	<6.00[e,f]	<6.00[e,f]	<6.00[d,f]	<6.00[d,f]

[a]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[b]Numbers shown are means of 3 replications.

[c-e]Means in columns with different superscripts are significantly different ($P<0.05$).

[f]For analysis of variance and mean separations a value of log_{10} CFU/ml=6.00 was used.

Table 12. Enumeration of Clostridium perfringens grown in chicken gravy for 12 hours.

	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
Hour	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	5.14[c]	5.41[d]	5.23[c]	5.25[c]	5.29[c]	5.32[c]
2	3.10[c]	5.02[d]	3.66[c]	5.13[c]	4.48[c]	5.19[d]
4	<3.00[c]	3.28[c]	3.82[c]	4.28[c]	4.89[c]	5.00[c]
6	<3.00[c]	<3.00[c]	4.30[c]	4.38[c]	5.69[c]	5.76[c]
8	<3.00[c]	<3.00[c]	4.26[c]	4.45[c]	5.80[c]	5.97[c]
10	<3.00[c]	<3.00[c]	4.44[c]	4.50[c]	5.94[c]	6.01[c]
12	<3.00[c]	<3.00[c]	4.20[c]	4.30[c]	5.46[c]	5.69[c]

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Means in rows under the same strain with different superscripts are significantly different ($P<0.05$).

Table 13. Enumeration of Clostridium perfringens grown in beef gravy for 12 hours.

Hour	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	4.76[c]	5.12[d]	4.80[c]	4.85[c]	4.62[c]	5.05[d]
2	4.63[c]	5.10[d]	4.67[c]	5.12[d]	5.17[c]	5.31[d]
4	5.30[c]	6.01[d]	5.68[c]	6.69[d]	5.74[c]	6.84[d]
6	6.76[c]	7.57[d]	7.09[c]	7.38[d]	6.92[c]	7.16[c]
8	7.07[c]	7.58[d]	7.33[c]	7.60[c]	7.05[c]	7.20[d]
10	7.03[c]	7.32[d]	6.50[c]	7.24[c]	7.12[c]	7.27[d]
12	6.44[c]	6.62[c]	6.20[c]	6.52[c]	7.60[c]	7.90[c]

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Means in rows under the same strain with different superscripts are significantly different ($P<0.05$).

Table 14. Enumeration of Clostridium perfringens grown in chicken a la king for 12 hours.

Hour	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	4.71[c]	5.10[d]	4.87[c]	4.87[c]	4.92[c]	4.90[c]
2	4.83[c]	5.26[d]	4.82[c]	5.16[d]	5.03[c]	5.25[d]
4	6.96[c]	7.44[d]	7.08[c]	7.11[c]	7.46[c]	7.61[d]
6	7.63[c]	8.19[d]	8.07[c]	7.96[c]	7.89[c]	7.99[c]
8	7.92[c]	8.12[d]	8.28[c]	8.30[c]	7.96[c]	8.20[d]
10	7.90[c]	8.18[d]	8.35[c]	8.44[c]	8.15[c]	8.27[d]
12	7.52[c]	7.53[c]	7.50[c]	7.74[c]	8.04[c]	8.22[d]

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Means in rows under the same strain with different superscripts are significantly different ($P<0.05$).

Table 15. Enumeration of Clostridium perfringens grown in beef stew for 12 hours.

	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
Hour	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	4.65[c]	5.10[d]	4.87[c]	4.91[c]	4.87[c]	4.93[c]
2	3.82[c]	4.66[d]	4.18[c]	4.58[c]	3.56[c]	4.21[c]
4	3.00[c]	3.97[d]	3.16[c]	3.99[d]	3.20[c]	3.97[c]
6	4.10[c]	4.50[c]	4.00[c]	4.20[c]	4.16[c]	4.74[c]
8	5.41[c]	5.94[c]	4.40[c]	4.70[c]	4.74[c]	5.04[c]
10	7.24[c]	7.35[c]	5.87[c]	6.16[c]	6.04[c]	6.33[c]
12	8.15[c]	8.34[c]	5.30[c]	5.55[c]	6.06[c]	6.17[c]

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Means in rows under the same strain with different superscripts are significantly different ($P<0.05$).

Table 16. Enumeration of Clostridium perfringens by strain grown in inoculated foods for 12 hours.

	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
Hour	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
0	4.81[c]	5.18[d]	4.94[c]	4.97[d]	4.92[c]	5.05[d]
2	4.09[c]	5.01[d]	4.36[c]	5.00[d]	4.56[c]	4.99[d]
4	4.56[c]	5.17[d]	4.94[c]	5.52[d]	5.32[c]	5.86[c]
6	5.37[c]	5.82[d]	5.87[c]	5.98[c]	6.16[c]	6.41[c]
8	5.85[c]	6.16[d]	6.07[c]	6.26[c]	6.39[c]	6.60[c]
10	6.30[c]	6.46[d]	6.29[c]	6.58[c]	6.81[c]	6.97[c]
12	6.28[c]	6.37[c]	5.80[c]	6.03[c]	6.79[c]	7.00[c]

[a]Numbers shown are means of 3 replications and 4 products.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Means in rows under the same strain with different superscripts are significantly different ($P<0.05$).

Table 17. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of individual strains of Clostridium perfringens from beef stew.

Day	Storage Temperature (°C)	Log$_{10}$ CFU/gm[a] ATCC 12917 TSC	ATCC 12917 TSC+ ORMF[b]	NCTC 10239 TSC	NCTC 10239 TSC+ ORMF	NCTC 8798 TSC	NCTC 8798 TSC+ ORMF
0	4	7.65^{c}	7.80^{c}	6.65^{c}	6.91^{c}	7.68^{c}	7.79^{c}
1	4	7.06^{c}	7.58^{c}	6.59^{c}	6.91^{c}	7.56^{c}	7.68^{c}
2	4	6.73^{c}	7.52^{c}	6.64^{c}	6.86^{c}	7.24^{c}	7.42^{d}
3	4	6.65^{c}	7.42^{d}	6.63^{c}	6.91^{c}	7.24^{c}	7.42^{d}
4	4	5.94^{c}	7.40^{c}	6.54^{c}	6.75^{c}	7.17^{c}	7.37^{d}
5	4	5.70^{c}	7.31^{d}	6.02^{c}	6.73^{d}	7.17^{c}	7.38^{d}
6	4	5.11^{c}	7.20^{d}	5.75^{c}	6.68^{d}	6.86^{c}	7.26^{c}
7	4	4.79^{c}	7.17^{d}	5.75^{c}	6.62^{d}	6.86^{c}	7.25^{c}
8	4	4.67^{c}	7.10^{d}	5.62^{c}	6.57^{d}	6.72^{c}	7.20^{d}
9	4	4.66^{c}	7.06^{d}	5.48^{c}	6.49^{d}	6.61^{c}	7.14^{d}
10	4	4.57^{c}	7.03^{d}	5.36^{c}	6.44^{d}	6.55^{c}	7.10^{d}
0	-15	7.65^{c}	7.80^{c}	6.66^{c}	6.91^{d}	7.68^{c}	7.79^{c}
7	-15	5.94^{c}	6.82^{d}	5.39^{c}	5.67^{d}	5.14^{c}	6.53^{d}
14	-15	5.28^{c}	6.61^{d}	4.26^{c}	4.70^{c}	3.61^{c}	6.32^{d}
21	-15	4.04^{c}	6.47^{d}	3.80^{c}	4.59^{d}	3.42^{c}	6.12^{d}
28	-15	3.25^{c}	6.01^{d}	2.69^{c}	4.46^{d}	3.14^{c}	5.96^{d}
35	-15	2.60^{c}	5.84^{d}	2.56^{c}	3.98^{c}	3.00^{c}	5.52^{d}

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c,d]Numbers in rows under each strain with different superscripts are significantly different ($P<0.05$).

Table 18. Comparison of tryptose sulfite cycloserine agar with and without an oxygen-reducing membrane fraction for the recovery of individual strains of Clostridium perfringens from beef stew heated at various temperatures for 1 hour.

Temperature (°C)	Log_{10} CFU/gm[a]					
	ATCC 12917		NCTC 10239		NCTC 8798	
	TSC	TSC+ ORMF[b]	TSC	TSC+ ORMF	TSC	TSC+ ORMF
Control[c]	7.17[d]	7.84[e]	7.26[d]	7.77[e]	7.60[e]	8.00[e]
55	5.69[d]	7.12[e]	6.50[d]	7.04[e]	6.51[e]	7.03[e]
60	4.91[d]	3.64[e]	4.74[d]	5.58[e]	3.96[e]	5.21[e]
65	1.77[d]	3.64[e]	2.26[d]	3.87[e]	3.27[e]	3.71[e]
70	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.00[e]	0.00[d]

[a]Numbers shown are means of 3 replications.

[b]TSC=tryptose sulfite cycloserine; ORMF=oxygen-reducing membrane fraction.

[c]Control samples were not exposed to any high temperatures prior to plating.

[d,e]Numbers in rows under each strain with different superscripts are significantly different ($P<0.05$).

VITA

Clyde Baker Hoskins was born on June 20, 1949 in Knoxville, Tennessee to Gomer and Katherine Hoskins. After graduation from Clinton High School, Clinton, Tennessee in May 1967, he entered the U.S. Military Academy. Upon graduation in June 1971 he began serving six years as an Air Defense Artillery officer in West Germany and Texas with the U.S. Army. In 1978 he was accepted to and entered the College of Veterinary Medicine at The University of Tennessee, Knoxville. The Doctor of Veterinary Medicine degree was awarded in June 1981 at which time active duty in the U.S. Army as a Veterinary Corps officer began. In September 1985, Clyde was given the opportunity to pursue a Master of Science degree in Food Technology and Science at The University of Tennessee, Knoxville. This degree was awarded in March 1987 after which military duties continued.

The author is a member of the Institute of Food Technologists, the American Veterinary Medical Association and Phi Kappa Phi.