EFFECT OF PROTEIN SOURCES ON THE GROWTH OF CLOSTRIDIUM PERFRINGENS

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ABSTRACT

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The effects of various vegetable protein sources on the growth of Clostridium perfringens were studied. Two strains of C. perfringens, one with heat-sensitive spores (ATCC 3624) and one with heat-resistant spores (NCTC 8238) were used in this investigation. Growth media containing 2.0% trypticase (or other protein source on a percent protein basis), 0.25% sodium chloride, 0.15% dipotassium phosphate, 0.006% sodium thioglycollate, 0.004% L-cystine, 0.002% sodium sulfite, and 0.005% agar were prepared. The growth media were inoculated with C. perfringens organisms at concentrations of approximately 10^2 cells per ml. Flasks containing the inoculated growth media were placed in a constant-temperature water bath maintained at 45 $\pm$ 1 C and samples were taken at appropriate intervals to determine the extent of the lag and log growth phases. Nitrogen gas was introduced into the headspace of the growth vessels during sampling to maintain anaerobic conditions in the growth media.

Numbers of C. perfringens were determined by plating in a freshly prepared tryptose-sulfite-cycloserine (TSC) agar and incubating at 37 C in a nitrogen atmosphere for 24 hours. Generation times were calculated by using linear regression and measuring the slopes of the growth curves during exponential growth.

The results of this investigation indicated that the addition of vegetable protein sources as soy flour, texturized soy flour (Bontrae), and cottonseed flour (Proflo and Pharmamedia) had neither a stimulatory nor an inhibitory effect on the growth of the organism as compared to the addition of trypticase. A difference between strains was shown, however, as strain ATCC 3624 had significantly longer generation times in these media than did strain NCTC 8238. These results suggest a difference between strains in ability to utilize the substrate.

When strain ATCC 3624 was grown in media containing meat and vegetable protein sources, results showed that the addition of cottonseed protein in the form of Proflo and Pharmamedia did have an inhibitory effect on the growth of the organism as compared to the growth rate in media containing meat (beef, chicken, or turkey) or meat and soy flour. The addition of soy flour did not affect the growth of the organism as compared to the growth in media containing meat as the sole protein source.

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Clostridium perfringens strain ATCC 3624 was grown in media containing meat and meat with cottonseed flour protein or carbohydrate fractions. The addition of Proflo or Pharmamedia carbohydrate fractions to media containing meat exerted an inhibitory effect on the growth of the organism. One or more factors inhibitory to the growth of *C. perfringens* may be present in the carbohydrate fraction of the cottonseed products used in this investigation.

Clostridium perfringens strain ATCC 3624 was also grown in media containing turkey and Pharmamedia carbohydrate fraction which had been treated with glucose oxidase. Results of these studies suggested that glucose was not responsible for the inhibitory effect of the carbohydrate fraction of Pharmamedia.

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INTRODUCTION

Foodborne disease caused by Clostridium perfringens has become a major concern in the food industry. Cooked meat products that have been improperly handled are the major vehicles of transmission. In the United States in 1973, Clostridium perfringens was responsible for about 7% of the outbreaks and about 19% of the cases of confirmed foodborne disease of known etiology. Also, 25% of the total outbreaks of unknown etiology (or 45 outbreaks) were epidemiologically similar to Clostridium perfringens foodborne illness (USDHEW, 1974).

Food incriminated in outbreaks invariably has been held at room temperature or improperly refrigerated in large masses for several hours, often overnight or longer. Outbreaks frequently follow banquets or meals prepared at hospitals and schools where large amounts of meat or poultry are involved. Thus, Clostridium perfringens foodborne illness is a disease closely associated with the food service industry (Bryan, 1969).

Public acceptance of vegetable protein as a base in fabricated synthetic meats as well as of vegetable protein supplements in meat systems is increasing (Horan,

1974). Institutional markets, including schools and hospitals, are the major markets for synthetic meats. The trend in the United States toward more away-from-home eating also increases the potential use of these products (USDA, 1971). This increase in growth of the food service industry also increases the possibilities of food contamination and abuse. Since natural meats are major vehicles of Clostridium perfringens foodborne disease and since vegetable protein supplementation of meat systems is increasing, an evaluation of the behavior of Clostridium perfringens in these new products is appropriate.

The objective of the study was to determine the effects of various animal and vegetable protein sources on the growth of Clostridium perfringens.

REVIEW OF LITERATURE

Clostridium perfringens

Clostridium perfringens (C. welchii) is a non-motile, encapsulated, short and thick bacillus with blunt ends. It occurs singly, in pairs, and occasionally in short chains. Filamentous cells are sometimes produced. Young cultures are gram-positive, but old cultures may appear gram-negative. Subterminal ovoid spores are produced. Vegetative cells sporulate readily in the human intestines, but rarely in cooked meat (Bryan, 1969).

Strains of C. perfringens have been divided into five toxicological types (A to E) on the basis of four major toxins (alpha, beta, episilon, iota) which may be produced (Sterne and Warrack, 1964). There is no evidence to associate these toxins with foodborne illness. Type A strains cause the typical mild cases of gastroenteritis associated with foodborne illness and also cause gas gangrene. Type C (formerly known as type F) strains have caused infrequent outbreaks of necrotic enteritis in Germany and in New Guinea (Bryan, 1969).

The major toxin produced by C. perfringens type A is alpha toxin, an enzyme (lecithinase C) which attacks

lecithin and similar chemical substances to liberate phosphorylcholine. Opaque zones of precipitate surrounding colonies of C. perfringens on egg yolk agar are evidence of lecithinase activity. Strains of C. perfringens type A may or may not be hemolytic; they do, however, form collagenase, hyaluronidase, and deoxyribonuclease. Type A strains of C. perfringens were originally divided into two subdivisions: gas gangrene strains and food poisoning strains (Hobbs et al., 1953). Organisms with heat-sensitive spores (D-values of less than 1 minute when heated in water at 100 C and 3 to 5 minutes at 90 C) that produced large amounts of lecithinase and theta toxin were considered as gas gangrene strains. Food poisoning strains had heat resistant spores (D-values of 4 to 17 minutes at 100 C), produced little lecithinase, and no theta toxin. Today, however, it is believed that it is not necessary for a particular Type A strain to possess a specific biochemical characteristic in order to produce foodborne illness. Thus, there is no such thing as a "food poisoning strain of C. perfringens" as such (Hall and Dowell, 1967; Hobbs, 1969).

Growth of the Organism

Temperature. Growth is most rapid near 45 C for almost all strains of C. perfringens. Boyd et al. (1948) reported an optimum of 45 C for the highly alpha-toxigenic

strain BP6K, and Sasarman and Horodniceau (1961) found the same optimum temperature an important aid in devising selective procedures for isolating C. perfringens because it is above the temperature at which most other bacteria readily grow. Growth of C. perfringens below 20 C is slow but may even extend to 5 C for a few strains. However, growth at any appreciable rate stops at 15 C for most strains. The majority of strains will grow at 50 C even though most of the cells introduced as inocula will perish within the first few hours. The survivors, however, will start multiplying at very nearly their maximum rate and will continue to do so until nutrients are exhausted. This curious phenomenon was first noted by Collee et al. (1961) who gave it the name "Phoenix phenomenon." Although the temperature optimum for growth is clearly in the 43 to 46 C range, this is not necessarily the optimum temperature for production of enzymes or toxins by C. perfringens. For example, the temperature optimum for the production of phospholipase C (alpha toxin) was found to vary, among different strains, from 30 to 46 C (Nakamura et al., 1969), while that for epsilon toxin production was 30 to 33 C. Spore production by C. perfringens has a temperature optimum of 37 C (Kim et al., 1967).

Several observations have been made in regard to the effect of frozen and refrigerated storage on C. perfringens. Traci and Duncan (1974) found that at 4 C

96% of an initial population of exponential-phase cells was killed upon cold shock and 95% of the remaining population was killed within 90 minutes of continued exposure at 4 C. In general, stationary-phase cells were more resistant to cold shock than exponential-phase cells. Canada et al. (1964) studied the effects of lowered temperatures on four strains of C. perfringens. After exposure to freezing or refrigeration temperatures (-17.7 and 7.1 C, respectively), only small numbers of the vegetative cells were recovered. After similar treatment, 16 to 58% of the spores were recovered.

Nutritional Requirements. According to Smith (1971), strains of C. perfringens are rather demanding in their nutritional needs, requiring 13 to 15 amino acids and 5 to 7 additional growth factors (Table 1). These needs can be fulfilled by ordinary bacteriological media.

Hydrogen Ion Concentration. Almost all strains of C. perfringens grow most rapidly somewhat below neutrality, generally in the range pH 6.3 to 6.5. No growth was observed below pH 4.9 in unbuffered medium with glucose as the fermentable carbohydrate. On the alkaline side, pH 8.3 marked the limit for the strains of type A and type D that have been studied. Just as the temperature optimum for toxin production differs from that for growth, so the hydrogen ion optimum for toxin production

Table 1. The amino acid and growth factor requirements of *Clostridium perfringens*.[1]

Arginine	Serine[2]
Leucine	Tyrosine
Isoleucine	Alanine[2]
Methionine	Aspartic acid
Phenylalanine	Biotin
Threonine	Pantothenate
Valine	Pyridoxal
Tryptophane	Riboflavin[2]
Histidine	Adenine
Cystine	Uracil[2]
Glutamic acid	Nicotinamide

[1]Modified from Boyd *et al.* (1948) and Fuchs and Bonde (1957).

[2]Variable.

differs from that for growth. The pH optimum for production of phospholipase is slightly below 7.0 and that for production of beta toxin is 7.5. The pH optimum for production of epsilon toxin is 7.0 to 7.2 (Smith, 1971).

Oxidation-Reduction Potential. Finding a suitable oxidation-reduction potential for C. perfringens is not generally a problem. However, on minimal media, in which the nutrient supply is just adequate to provide for barely visible colonies, the oxidation-reduction potential can be important. The toxic effect of oxygen on the organisms is nullified if an active reducing agent is added to the medium. The larger the inoculum and the more active its metabolic state, the higher can be the oxidation-reduction potential. The endogenous metabolism of the cells in the inoculum increases the electron density in the neighborhood of the inoculum and provides a nidus of low potential and low pH from which active growth can progress (Smith, 1971).

Growth in Meat. The effect of various holding temperatures on the growth of C. perfringens in laboratory-prepared beef cubes in natural gravy was studied by Hall and Angelotti (1965). At 46 C less than 4 hours were required to produce a 100-fold increase over the inoculum. When raw chicken was cooked to obtain a temperature of 85 to 90 C in the breast muscle, a C. perfringens spore

inoculum was reduced from 1.0 x 10^4 spores per gram to 1.5 per gram. Upon incubation of the cooked chicken at 45 C, a lag period was observed for about 4 hours and the growth then became logarithmic. After 14.7 hours, 1.0 x 10^7 cells were present. When cooked chicken was inoculated with 1.0 x 10^3 vegetative cells, they multiplied to a total of 1.0 x 10^7 cells in 6.3 hours at 45 C (Pivnic et al., 1968).

The generation time of C. perfringens can be as short as 8.5 minutes (median of 12 minutes for 22 strains) in broth cultures incubated at 46 C and it is about 20 minutes at 37 C (Bryan, 1969). In various poultry and meat stock soups, the generation times ranged from 24-32 minutes at 45 C (Smith, 1962). Vegetative cells of C. perfringens survived for over 6 hours when roast turkey slices in broth were held at conditions of steam table storage of 68 C (Strong and Rip, 1967). When boiled lamb, inoculated with 2 x 10^5 vegetative cells, was stored in gravy on a hot plate at 40 to 50 C for 3 hours, the count increased to 4.6 x 10^7 cells of C. perfringens per slice. When meat was stored at the same temperature and time without gravy, about half as many organisms were present. Meat stored at room temperature for 3 hours showed no increase of organisms (Hobbs, 1957).

A study conducted by Schroder and Busta (1970) showed generation times of 12.5 minutes and 10.8 minutes

in beef loaves at 45 C. Busta and Schroder (1970) reported generation times of 14.3 minutes for C. perfringens in liquid media containing beef and 19.5 minutes in media containing turkey.

Growth in Vegetable Proteins. Effects of various soybean proteins used in meat loaf formulations on the growth of two strains of C. perfringens were studied by Schroder and Busta (1970). Their findings indicate that beef products were excellent growth media for the organism and that the addition of soybean proteins had little effect on the growth rates at 45 C. The addition of soy protein concentrate to beef in meat loaf formulations gave average generation times of 11.5 and 9.9 minutes. The addition of soy protein isolate gave generation times of 11.5 and 9.3 minutes. Textured soy flour, when added to the beef loaf formulation, resulted in generation times for C. perfringens of 11.7 and 9.6 minutes. The addition of soy grits gave average generation times of 11.0 minutes and 9.0 minutes.

In another study by Busta and Schroder (1970), liquid media containing meat and meat plus vegetable protein supplements were evaluated for stimulatory or inhibitory effects on some strains of C. perfringens. When compared to trypticase, some soy proteins had stimulative effects on the growth of the organism, whereas sodium caseinate and some soy proteins were inhibitory. In

liquid media in which meat or soy meat was the source of protein, there was a marked stimulation by beef, chicken, and soy beef. Soy chicken supported growth at a rate less than observed with trypticase.

Incidence of the Organism

Clostridium perfringens is a normal inhabitant of the intestinal tract of man and animals. The organisms exist in sewage and soil in both spore and vegetative states. Taylor and Gordon (1940) reported that 190 of 196 samples of soil examined contained C. perfringens, mostly type A. Eighty-five percent of dust samples from a commercial kitchen environment contained C. perfringens isolates (McKillop, 1959).

Strong et al. (1963) isolated C. perfringens from 16% of 122 samples of raw meat, fish, and poultry. Hall and Angelotti (1965) examined 262 samples of retail cuts of meat for the presence of C. perfringens. Of this total, 167 samples were raw unprocessed beef, veal, lamb, pork, and chicken and 101 were processed raw samples of meat and meat dishes. Clostridium perfringens was isolated from 58% of the raw samples and from 20% of the cooked samples. The difference in values reported by these studies could be due to the use of enrichment methods by Hall and Angelotti (1965).

Foodborne Illness

When several million viable cells of C. perfringens are ingested, diarrhea and abdominal pain accompanied by large volumes of gas in the intestine occur after an incubation period of from 4 to 22 hours, with an average of about 12 hours. Nausea, vomiting, and fever are rare (Hobbs et al., 1953). The vegetative cells multiply and sporulate in the intestine and produce an enterotoxin in situ. The enterotoxin is released with lysis of the sporangium upon release of the mature spore and causes increased capillary permeability, vasodilation, and excess fluid movement into the intestinal lumen resulting in diarrhea (Hauschild, 1971).

Purified C. perfringens enterotoxin has a UV absorption spectrum characteric for proteins, and is essentially free of nucleic acids, lipids, and reducing sugars. Its apparent molecular weight is 36,000 $\pm$ 4,000 and its isoelectric point is pH 4.3 (Hauschild, 1971). The toxin is sensitive to heat (Duncan and Strong, 1969) and its specific toxicity is about 2,000 mouse MLD/mg N (Hauschild and Hilsheimer, 1971).

Media and Methods for Isolation

Selective Media. Several selective media have been developed for isolating C. perfringens from material containing other bacteria. Almost all of the media

contain sulfite, since it has been demonstrated that this substance is not only inhibitory to many other bacteria, but does not inhibit the clostridia (Mossel, 1959; Mossel et al., 1956). The most common solid media also contain iron and allow the sulfite-reducing clostridia to produce black colonies. Of these media, the following are currently being used: SPS (sulfite-polymyxin-sulfadiazine) (Angelotti et al., 1962), TSN (tryptose-sulfite-neomycin) (Marshall et al., 1965), SFP (Shahidi-Ferguson perfringens) (Shahidi and Ferguson, 1971), and TSC (tryptose-sulfite-cycloserine) agars (Harmon et al., 1971b).

SPS agar selectively inhibits growth or interferes with the formation of black colonies by the sulfite-reducing Enterobacteriaceae and Achromobacteriaceae. It also inhibits growth of most other facultative anaerobes and members of the genera Pseudomonas, Bacillus, and Lactobacillus (Angelotti et al., 1962; Harmon et al., 1971a).

However, low recoveries of C. perfringens in commercial SPS agar have been reported (Shahidi and Ferguson, 1971). In one preparation, the cause was traced to a particular lot of yeast extract. It appears that the selective ingredients of this agar are at a level where a slight adverse change in the medium may result in a drastic inhibition of C. perfringens (Hauschild and Hilsheimer, 1974).

TSN agar has not been used as extensively as SPS agar but reports indicate that TSN is inhibitory to a number of C. perfringens strains (Handford and Cavett, 1973; Harmon et al., 1971a).

While SFP agar allows quantitative recovery of C. perfringens (Handford and Cavett, 1973; Harmon et al., 1971a; Harmon et al., 1971b), it does not prevent growth of a large number of facultative anaerobes, some of which are sulfite-reducing (Harmon et al., 1971b; Shahidi and Ferguson, 1971). The elaborate preparation involved in the use of SFP agar is another disadvantage since it requires addition of fresh egg yolk, surface plating, and pouring of an agar overlay.

Harmon et al. (1971a) evaluated SFP, TSN, and SPS agars to determine which medium was most selective for C. perfringens and most inhibitory to other microorganisms. It was found that while SFP gave significantly higher recoveries of spores and vegetative cells than the other two, it was the least selective. TSN was found to be most selective. SFP agar was later modified (Harmon et al., 1971b) by replacing polymyxin B and kanamycin with 0.04% D-cycloserine. This antibiotic had been shown to selectively inhibit growth of many of the common facultative anaerobes (Fuzi and Csukas, 1969). The use of this new modified medium (TSC) was further improved by

elimination of the egg yolk and by using a pour-plate technique (Harmon et al., 1971b).

Diluents. Ordinary buffered salt solutions often contain trace amounts of heavy metals that are lethal or bacteriostatic to C. perfringens when the concentration of bacteria is relatively low. Viable counts were three or four times higher when 0.1% peptone solution was used as the diluting fluid instead of phosphate buffer (Hauschild et al., 1967).

Method of Plating. The difference between the pour plate count and the roll tube count is significant only when a minimal medium is used and is insignificant with complete media. Viable counts are about the same with both methods. Similarly, there is little difference in viable counts when the plates are surface-inoculated or when the inoculum is added to the agar before pouring (Smith, 1971).

Vegetable Proteins

At the present time, soybeans are the major source of edible oil in the United States and soybean meal provides an important source of protein for animal feeds. Although food use of soybean protein in the form of defatted flour began in the 1930s, the market for these products has developed slowly. With the exception of certain textured vegetable protein products (such as

meat-flavored dice, chunk, and crumble products), the current uses of these soy proteins are mainly as ingredients to give desired changes in structure, texture, and composition at a reasonable price. Soy protein products are, in many cases, used to supplement, extend, or replace more costly ingredients derived from animal protein sources, such as meat, eggs, and milk (Wolf, 1970a; Wolf 1970b).

Forms of Soybean Proteins

Flours and Grits. Forms of soybean proteins presently used as raw materials in the food industry are classified into three groups based on protein content. The least refined forms are flours and grits which have varying fat contents, particle sizes, textures, and degrees of heat treatment. Flours are prepared by grinding soybean flakes to 100-mesh or finer, whereas grits are coarser than 100-mesh. Minimum protein contents of these materials range from 40 to 50%, depending on the fat content. Defatted flours usually contain close to 50% protein (Wolf, 1970b). Proteins, carbohydrates, and ash are the major components of the defatted flour with about one-half of the flour carbohydrates being oligosaccharides--sucrose, stachyose, and raffinose--while the other half is made up of polysaccharides, which are insoluble in water or alcohol (Aspinall et al., 1967).

Concentrates. Soybean protein concentrates are more refined than flours and grits and contain 70% or more protein on a dry basis. The protein concentrates are generally prepared from defatted flakes or flour by removing the oligosaccharides, part of the ash, and some of the minor components by alcohol extraction (Mustakas et al., 1962), acid leach, or moist heat followed by a water wash (Wolf, 1970b). The physical properties of the concentrates vary according to the method of their preparation (Meyer, 1966).

Isolates. The isolates are the most refined forms of soybean proteins. They contain 90% or more protein and are prepared by removing the water-insoluble polysaccharides, as well as the oligosaccharides and other low molecular weight components (Wolf, 1970b).

Chemical and Physical Properties of Soybean Proteins

Amino acid composition of the soybean proteins determines the nutritional value. Table 2 lists the essential amino acid compositions for the three major forms of soybean proteins. The proteins are high in lysine and so are useful as supplements for cereals, which tend to be low in this amino acid. Methionine, however, is the first limiting amino acid in soy proteins and this should be considered when the proteins are added for nutritional

Table 2. Essential amino acid content of soybean proteins.[1]

Amino Acid	Meal g/16g N	Concentrate g/16g N	Isolate g/16g N
Lysine	6.9	6.6	5.7
Methionine	1.6	1.3	1.3
Cystine	1.6	1.6	1.0
Tryptophan	1.3	1.4	1.0
Threonine	4.3	4.3	3.8
Isoleucine	5.1	4.9	5.0
Leucine	7.7	8.0	7.9
Phenylalanine	5.0	5.3	5.9
Valine	5.4	5.0	5.2

[1]Table from Wolf (1970b).

purposes rather than merely for functional purposes (Wolf, 1970).

The major soybean proteins are globulins, which are insoluble at their isoelectric points. The proteins are, however, soluble in water or dilute salt solutions at pH values above or below the isoelectric point (Wolf, 1970b).

Soy proteins bind fat, are hydrophylic, and have film-forming and adhesive qualities. Although the functional properties of soy products are usually attributed to the protein fraction, when the less refined products are used, other constituents may also contribute to the effect. For example, in flours, grits, and concentrates

the polysaccharides as well as the proteins absorb water (Wolf, 1970b).

Cottonseed Protein

It was recognized early in the history of the processing of cottonseed that the cottonseed could be a valuable source of both protein and oil (Richardson, 1917). However, the presence of the chemically reactive antinutritional factor--gossypol (Berardi and Goldblatt, 1969)--prevented the rapid development of edible protein products from the cottonseed. Gossypol is a highly reactive yellow polyphenolic binaphthaldehyde. In the metabolically active or "free" form, it has adverse physiological effects when ingested by monogastric animals (Berardi and Goldblatt, 1969). It also may impart an undesirable color to the cottonseed protein product. Gossypol is contained in relatively large intercellular structures called pigment glands. These glands and, hence, the gossypol, have been reduced through breeding (McNichael, 1959; Miravalle, 1969). This development plus the development of the Liquid Cyclone method (Gastrock et al., 1969) for processing glanded cottonseed have created new interest in the potential of edible cottonseed products.

Forms of Cottonseed Proteins

Flour. Cottonseed flour is the finely ground material produced from dehulled and defatted seed.

Cottonseed, when essentially completely dehulled and defatted to a lipid content of 1% or less, provides a product which contains 60% protein (N x 6.25) and 3% or less fiber. Solvent extraction operations using heat and mechanical pressure are commonly used commercially. However, these means must be used judicially or the protein constituent will be affected. Denaturation effects due to the defatting operation can be adequately estimated by the nitrogen solubility of the flour. The standard procedures developed for soy protein products are not applicable to cottonseed protein products. The cottonseed proteins, unlike soy proteins, are not readily dispersible in water. Alkaline conditions are needed to solubilize the major proteins of the cottonseed (Martinez et al., 1970).

Concentrates. Further concentration of the proteins of the cottonseed can be accomplished by either wet or dry processing of the defatted flour. Ninety percent ethanol gave optimum extraction of residual lipid and sugars with minimum removal of nitrogen (Berardi et al., 1968). Ethanol extraction increased the protein content from 66 to 72%. Aqueous extraction at essentially neutral pH (pH 6.3 to 6.8) is another wet procedure for the preparation of cottonseed protein concentrates. Water or dilute divalent cationic salt solutions can also be used.

Isolates. Further concentration of the cottonseed protein requires extraction of the protein from the defatted seed. A two-step extraction procedure has been developed by Berardi et al. (1968) that provides the selective separation of low and high molecular weight proteins from the nondenatured flours. The flour is extracted with water (Step I) and then with 0.01N NaOH (Step II).

Physical and Chemical Properties of Cottonseed Proteins

Amino acid composition of cottonseed flour and isolates is listed in Table 3. Glandless cottonseed flours, when properly processed, were equal in nutritive value to the casein control when tested in three separate trials using the standard AOAC procedure for protein efficiency ratio (PER) evaluation (Hopkins, 1967). Lysine is the first limiting amino acid in cottonseed. Therefore, the air-classified concentrates, which were slightly lower in lysine than the parent flours, were significantly lower in PER, 2.3 rather than 2.5 (casein set at 2.5). However, the residues from the air classification process were equal in PER to the parent flours (Hopkins, 1967).

On the basis of extractability characteristics, the proteins of the cottonseed can be divided into two groups, water-soluble and water-insoluble. The water-soluble proteins are low in molecular weight and many in number. They have minimum solubility at pH 4. The

Table 3. Amino acid composition of cottonseed flour and isolates.[1]

Amino Acid	Glandless Flour g/16g N	Isolate I g/16g N	Isolate II g/16g N
Lysine	4.3	6.0	3.0
Histidine	2.8	2.6	3.0
Arginine	12.1	10.4	11.3
Aspartic	8.8	6.7	8.4
Threonine	3.0	2.9	2.7
Serine	4.0	3.4	4.5
Glutamic	19.8	21.8	18.9
Proline	3.4	3.1	3.1
Glycine	4.0	3.2	3.7
Alanine	3.6	3.2	3.5
Valine	4.5	3.3	4.1
1/2 Cystine	---	2.6	0.3
Methionine	1.2	1.7	1.0
Isoleucine	3.3	2.6	3.1
Leucine	5.6	5.1	5.8
Tyrosine	3.0	3.3	2.6
Phenylalanine	5.3	3.7	6.3

[1]Martinez *et al.*, 1970.

water-insoluble proteins are high in molecular weight and are few in number. They have a minimum solubility at pH 7 (Martinez et al., 1970).

METHODS AND MATERIALS

Source and Preparation of Cultures

Two strains of C. perfringens, one with heat-sensitive spores (ATCC 3624) and one with heat-resistant spores (NCTC 8238) were obtained from the culture collection of the Department of Food Science and Human Nutrition, Michigan State University. Spores were produced using the procedure of Duncan and Strong (1967). Active cultures were produced by inoculating 0.1 ml of the original cultures into 10 ml of fluid thioglycollate medium (Difco) which was then incubated at 37 C for 16 hours. Two subsequent transfers were made into 10 ml of fresh fluid thioglycollate medium, using 10% inocula and 4-hour incubation periods at 37 C. The entire contents of the last tube were inoculated into 100 ml of sporulation medium which had been freshly steamed and cooled to 37 C in a 250-ml screw-cap Erlenmeyer flask. The sporulation medium contained 0.4% yeast extract (Difco), 1.5% proteose peptone (Difco), 0.4% soluble starch (BBL), 0.1% sodium thioglycollate (Difco), and 1.0% $Na_2HPO_4 \cdot 7H_2O$ (Merck). A Petroff-Hausser Bacteria Counter (C. A. Hausser & Son, Philadelphia, Pa.) was used in conjunction with a Series 10

Microstar phase microscope (American Optical Corporation, Buffalo, N.Y. 14215) to estimate the percent sporulation in cultures after incubation for 40 hours in the sporulation medium. Aliquots of the cultures containing spores and vegetative cells were frozen and stored at -12 C and they served as stock cultures. As needed, cultures were removed from the freezer and thawed at room temperature. Active cultures were produced by inoculating 0.1 ml of the stock culture into 10 ml of fluid thioglycollate medium without dextrose (Difco) which was then incubated at 37 C for 24 hours. After four such transfers, less than 10 spores per ml were present. These cultures were used as inocula and were diluted in 0.1% peptone to achieve a concentration of approximately 10^2 cells per ml in the growth media.

Preparation of Growth Media

Various test protein sources were substituted for trypticase (Difco) in thioglycollate medium without dextrose (Busta and Schroder, 1970). Table 4 lists the ingredients of the basal growth medium and the protein sources used as variables. All of the meat used in these experiments was obtained from Food Stores, Michigan State University. The ground chicken and ground turkey were mixtures of 50% (w/w) light meat and 50% dark meat. The ground beef was lean ground round steak. United States Department of Agriculture (Anonymous, 1972) statistics were used to calculate

Table 4. Basal medium for Clostridium perfringens.

Constituent	Amount g/l
Trypticase or substitution by proteins[1] on a % protein basis	20.0
Sodium chloride	2.5
Dipotassium phosphate	1.5
Sodium thioglycollate	0.6
L-cystine	0.4
Sodium sulfite	0.2
Agar	0.5

[1]Proteins: Soy flour (Fern brand Low Fat Soya Powder), texturized soy flour (Bontrae), cottonseed flour (Proflo), cottonseed flour (Pharmamedia), ground beef, ground turkey, and ground chicken.

amounts of meat added to the growth media to obtain the same protein concentration (w/w) as the trypticase control (Table 5). The soy flour product used was Fern^{R} brand Low Fat Soya Powder (Richards Food Corp., Melrose Park, Illinois 60160). The texturized soy product was Bontrae^{R} (General Mills, Minneapolis, Minn. 55453) and the two cottonseed products used were Proflo^{R} and Pharmamedia^{R} (Traders Protein Div., Traders Oil Mill Co., Fort Worth, Texas). Manufacturers' specifications were used to calculate the amounts of vegetable protein substituted in the growth media to obtain the same protein concentration as the control (Table 5).

Table 5. Percent protein in protein constituents and amounts used in basal media for Series I.

Constituent	Percent Protein	Amount g/l
Soy flour	50.0	40.0
Bontrae	20.0	100.0
Proflo	60.0	33.3
Pharmamedia	56.8	35.2
Ground beef	19.0	106.0
Ground turkey	23.0	86.0
Ground chicken	22.0	90.0

Series I. A series of experiments was conducted using the basal growth medium (Table 4) containing a 2% level of protein. The protein sources tested in this series were:

a. trypticase (control)
b. soy flour
c. Bontrae
d. Proflo
e. Pharmamedia

Percent protein in the protein sources and the amounts used in the growth media are listed in Table 5.

Series II. A series of experiments was also conducted using the basal growth medium containing a 2% protein level from meat alone or meat plus vegetable protein. Combinations of meat (beef, chicken and turkey) plus vegetable protein used in this series were:

a. meat + soy flour
b. meat + Pharmamedia
c. meat + Proflo
d. meat

Table 6 lists the amounts of the protein sources used in the growth media.

Series III. A series of experiments was also conducted using the basal growth medium containing a 1% level of meat protein plus protein and carbohydrate fractions prepared from cottonseed products, Proflo and Pharmamedia. The protein and carbohydrate fractions were substituted into the basal growth medium with each of the three types of meat at a level to give the same concentration (w/w) as a 1% protein level of the whole cottonseed flour. Combinations of meat (beef, chicken, and turkey) and vegetable protein sources used in this series were:

a. meat + Proflo protein fraction
b. meat _ Proflo carbohydrate fraction
c. meat + Pharmamedia protein fraction
d. meat + Pharmamedia carbohydrate fraction
e. meat

Table 7 lists amounts of protein and carbohydrate constituents used in the basal growth media.

Extraction of the Cottonseed Flour Fractions. Separation of the cottonseed flour into protein and carbohydrate fractions was accomplished by a modification

Table 6. Amounts of protein constituents used in basal media containing both meat and vegetable protein sources for Series II.

Constituent	Amount Used	
	Meat g/l	Veg. Protein g/l
Beef	106.0	
Beef and Soy Flour	53.0	20.0
Beef and Pharmamedia	53.0	17.0
Beef and Proflo	53.0	17.5
Chicken	90.0	
Chicken and Soy Flour	45.0	20.0
Chicken and Pharmamedia	45.0	17.0
Chicken and Proflo	45.0	17.5
Turkey	86.0	
Turkey and Soy Flour	43.0	20.0
Turkey and Pharmamedia	43.0	17.0
Turkey and Proflo	43.0	17.5

Table 7. Amounts of protein and carbohydrate constituents used in basal media for Series III.

Constituents	Amount Used g/l
Beef	45.0
Chicken	53.0
Turkey	43.0
Pharmamedia protein fraction[1]	12.1
Proflo protein fraction[2]	12.5
Pharmamedia carbohydrate fraction	1.8
Proflo carbohydrate fraction	1.8

[1]Contains 82.8% protein (N x 6.25).

[2]Contains 79.8% protein (N x 6.25).

of the extraction procedure described by Lillevik (1970). A 10-gram sample of the cottonseed product was placed in a 250-ml Erlenmeyer flask along with 150 ml of 50% ethanol and stirred for 3 hours with a Stir-Plate magnetic stirring apparatus (Thermolyne Corp., Dubuque, Iowa). The mixture was made to 250 ml with 95% ethanol, stirred, and filtered through No. 4 Whatman filter paper. The filtrate was then dried in a Thelco Precision Model vacuum oven (Precision Scientific Products, Chicago, Illinois) at 40 C and 25 inches of Hg for 24 hours. The protein content (N x 6.25) of the residue was determined by the micro-Kjeldahl method (Joslyn, 1970). The dried residue (referred to as

Pharmamedia protein fraction or Proflo protein fraction) was substituted into the basal growth media as previously described.

The extract produced by the extraction procedure was evaporated to dryness on a steam bath and the residue (referred to as Pharmamedia carbohydrate fraction or Proflo carbohydrate fraction) was substituted into the basal growth media as previously described.

The growth media were adjusted to pH 7.0 $\pm$ 0.1 with a glass electrode Beckman research pH meter and autoclaved at 121 C for 15 minutes. The media were steamed for 20 minutes before inoculation to insure a low oxidation-reduction potential.

Apparatus. The growth vessels consisted of 1000-ml screw-cap Erlenmeyer flasks for Series I and II and 250-ml screw-cap Erlenmeyer flasks for Series III. The flasks containing the growth media were placed in a constant-temperature water bath maintained at 45 $\pm$ 1 C. Samples were taken at appropriate intervals to determine the extent of the lag and log growth phases. Nitrogen gas was introduced into the headspace of the growth vessels during sampling to maintain anaerobic conditions in the growth media.

Enumeration of C. perfringens. Samples were serially diluted in 0.1% peptone dilution blanks (APHA,

1967) and numbers of C. perfringens were determined by plating in duplicate or triplicate in a freshly prepared plating medium, tryptose-sulfite-cycloserine (TSC) agar, consisting of the basal ingredients of sulfite-polymyxin-sulfadiazine (SPS) agar (Angelotti et al., 1962), with D-cycloserine (Sigma Chemical Co., St. Louis, Mo.) substituted for polymyxin B sulfate and sulfadiazine (Harmon et al., 1971b). The basal SPS medium contained 1.5% tryptone (Difco), 1.5% yeast extract (Difco), 0.05% ferric citrate (Baker Chemical Co., Phillipsburg, N.J.), 0.1% sodium metabisulfite (Mallincrodt Chemical Works, St. Louis, Mo.), and 20% agar (Difco). These ingredients were dissolved in 1 liter of distilled water and autoclaved at 121 C for 15 minutes. The D-cycloserine was dissolved in distilled water (0.1 gram per 25 ml), dispensed in screw-cap dilution bottles, and steamed for 20 minutes. The solution was stored at 4 C. A final concentration of 400 μg of the antibiotic per ml of SPS basal medium was obtained by adding 10 ml of D-cycloserine solution to a liter of the plating medium after autoclaving and just prior to plating.

The agar plates were inverted and incubated in a nitrogen atmosphere in an anaerobic incubator (National Appliance Co., Portland, Oregon) at 37 C for 24 hours. The plates were placed in the incubator and a vacuum of 24-27 inches of Hg was produced with a Cenco Hyvac vacuum

pump (Cenco Instruments Corp., Chicago, Ill.). The chamber of the incubator was filled with nitrogen gas, re-evacuated, and again filled with nitrogen.

Analysis of Data. Generation times were calculated by using linear regression and measuring the slope of the growth curves during the exponential growth phase. The growth curves were obtained by plotting the specific plate counts obtained vs time. Generation times obtained in Series I experiments were analyzed for significant differences between growth rates in the presence of the protein sources by analysis of variance. Results from Series II were also analyzed for significant differences among the trials using meat proteins alone and trials using meat and vegetable protein combinations. Results from Series III experiments were analyzed for significant differences among the trials using the meat proteins at the 1% level and the meat proteins plus cottonseed fractions. Duncan's multiple range test was also applied (Duncan, 1955).

RESULTS AND DISCUSSION

Media with Vegetable Protein Sources. Table 8 indicates the generation times of C. perfringens strains ATCC 3624 and NCTC 8238 in the basal growth medium supplemented with trypticase and four vegetable protein additives (Series I experiments). Figures 1 and 2 show typical growth curves for one of the organisms in these media. An analysis of variance of the results is presented in Table 9. There were no apparent significant differences, at the 5% level, in generation times of these two strains of C. perfringens as the result of the addition of the various proteins to the basal medium. However, a significant difference was revealed between the results obtained with strain ATCC 3624 and strain NCTC 8238. The average generation times obtained with strain NCTC 8238 were significantly shorter, at the 0.1% level, than those obtained with strain ATCC 3624 in media containing the vegetable protein variables. In contrast, the average generation times obtained in media containing trypticase were significantly longer for strain NCTC 8238 than for strain ATCC 3624 (0.1% level). This significant interaction between strain and media indicates

Table 8. Generation times of two strains of C. perfringens in media[a] supplemented with four protein additives and trypticase.

Strain	Replicate	Soy Flour	Bontrae	Proflo	Pharmamedia	Trypticase
			(min)			
ATCC 3624	1	20.8	18.1	21.4	21.9	16.4
	2	16.5	17.5	19.4	18.0	16.8
	3	19.5	18.0	21.2	19.6	15.6
	Average	18.9	17.9	20.7	19.8	16.3
NCTC 8238	1	14.5	17.1	14.9	16.0	18.5
	2	13.3	17.4	14.9	12.6	20.1
	3	16.8	14.5	14.9	17.8	19.5
	Average	14.9	16.3	14.9	15.5	19.4

[a]All samples contained the basal medium (see Table 4).

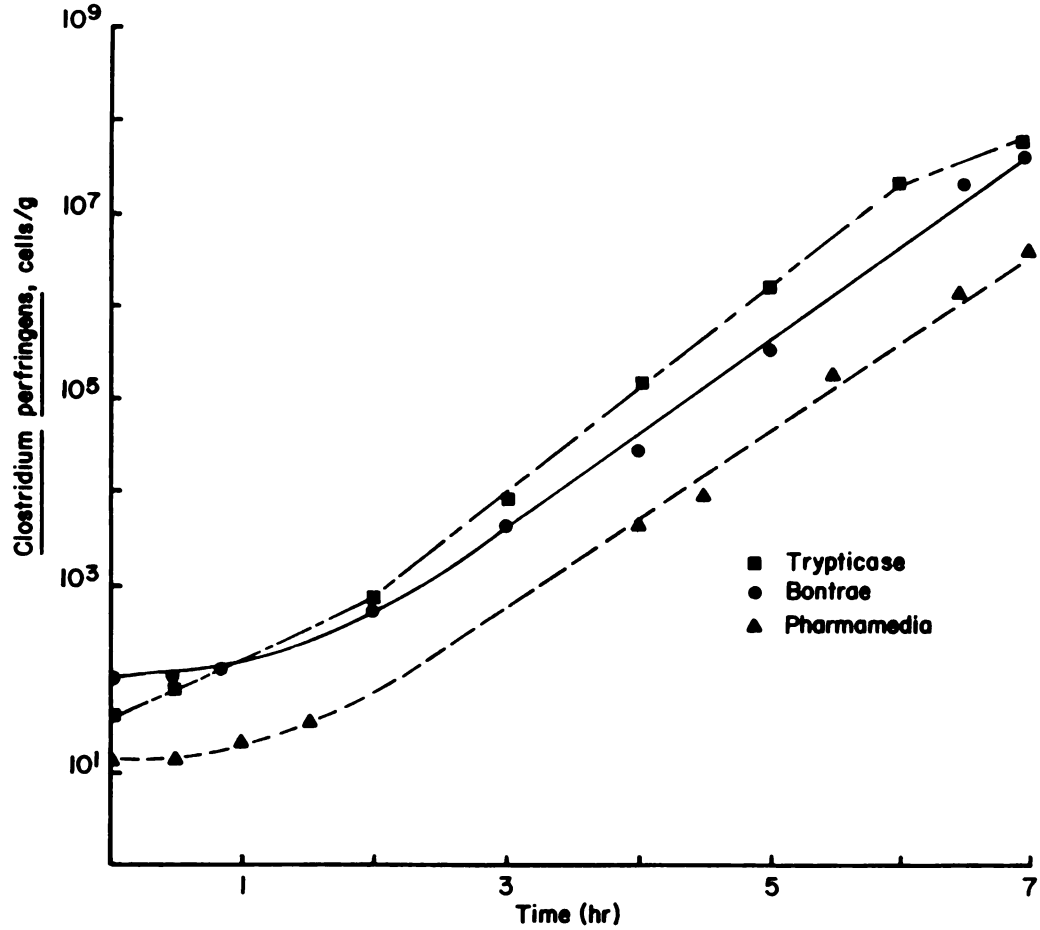

Fig. 1. Typical growth curves of C. perfringens ATCC 3624 in media containing trypticase, Bontrae, and Pharmamedia.

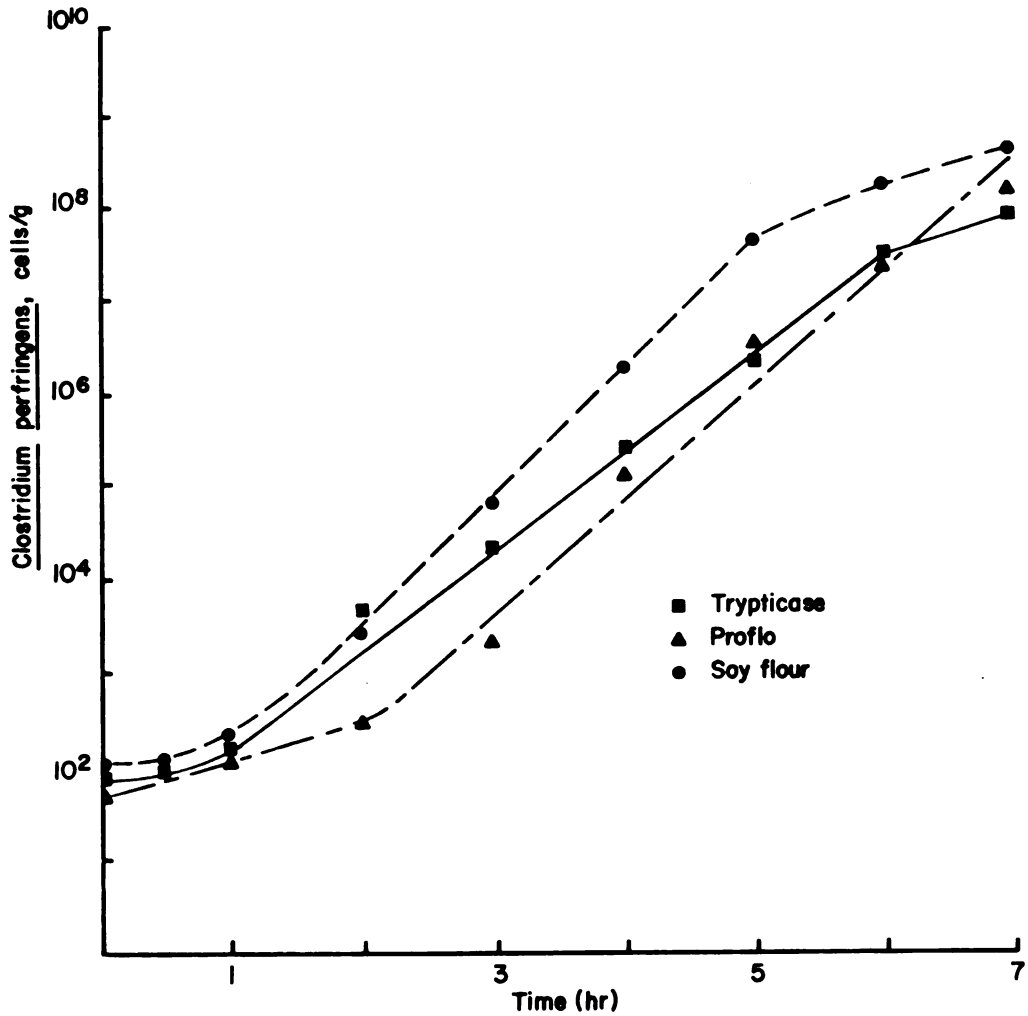

Fig. 2. Typical growth curves of C. perfringens NCTC 8238 in media containing trypticase, soy flour, or Proflo.

Table 9. Analysis of variance of generation times of two strains of C. perfringens in media supplemented with four protein additives and trypticase.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	29	174.4		
Strains	1	48.38	48.38	20.2*
Protein Sources	4	4.32	1.08	0.45
Interaction	4	73.9	18.48	7.73**
Error	20	47.8	2.39	

*Significant at the 0.1% level.
**Significant at the 5% level.

differences between strains in their ability to utilize the substrates. There appeared to be no difference, however, within a strain in the ability to utilize the various vegetable protein sources as compared to the trypticase control. Apparently these vegetable protein sources are all comparatively similar in terms of protein availability to the organisms under the conditions used in this investigation.

Media with Meat and Vegetable Protein Sources.

Table 10 summarizes data on the growth of C. perfringens ATCC 3624 in growth media containing meat and vegetable proteins. Typical growth curves for the organism in these media are shown in Figures 3, 4, and 5. Analyses of variance of these results are presented in

Table 10. Generation times of C. perfringens ATCC 3624 in media containing meat or meat and vegetable protein.

Sample[a]	Generation times, min 1	2	3	Average
Beef control	11.3	10.9		11.1
Beef + Soy Flour	12.0	12.2	11.5	11.9
Beef + Proflo	13.9	13.6	12.9	13.5[b]
Beef + Pharmamedia	17.1	16.8	17.1	17.0[b]
Chicken control	12.1	12.6		12.4
Chicken + Soy Flour	12.1	12.7	12.8	12.5
Chicken + Proflo	17.0	16.2	16.5	16.6[c]
Chicken + Pharmamedia	14.9	14.0	14.9	14.6[c]
Turkey control	12.0	12.0		12.0
Turkey + Soy Flour	11.3	12.9	12.6	12.3
Turkey + Proflo	14.5	14.2	13.6	14.1[d]
Turkey + Pharmamedia	14.4	15.0	15.6	15.0[d]

[a]All samples contained the basal growth medium (see Table 4).

[b]Significantly different from beef control at 0.1% level.

[c]Significantly different from chicken control at 0.1% level.

[d]Significantly different from turkey control at 0.1% level.

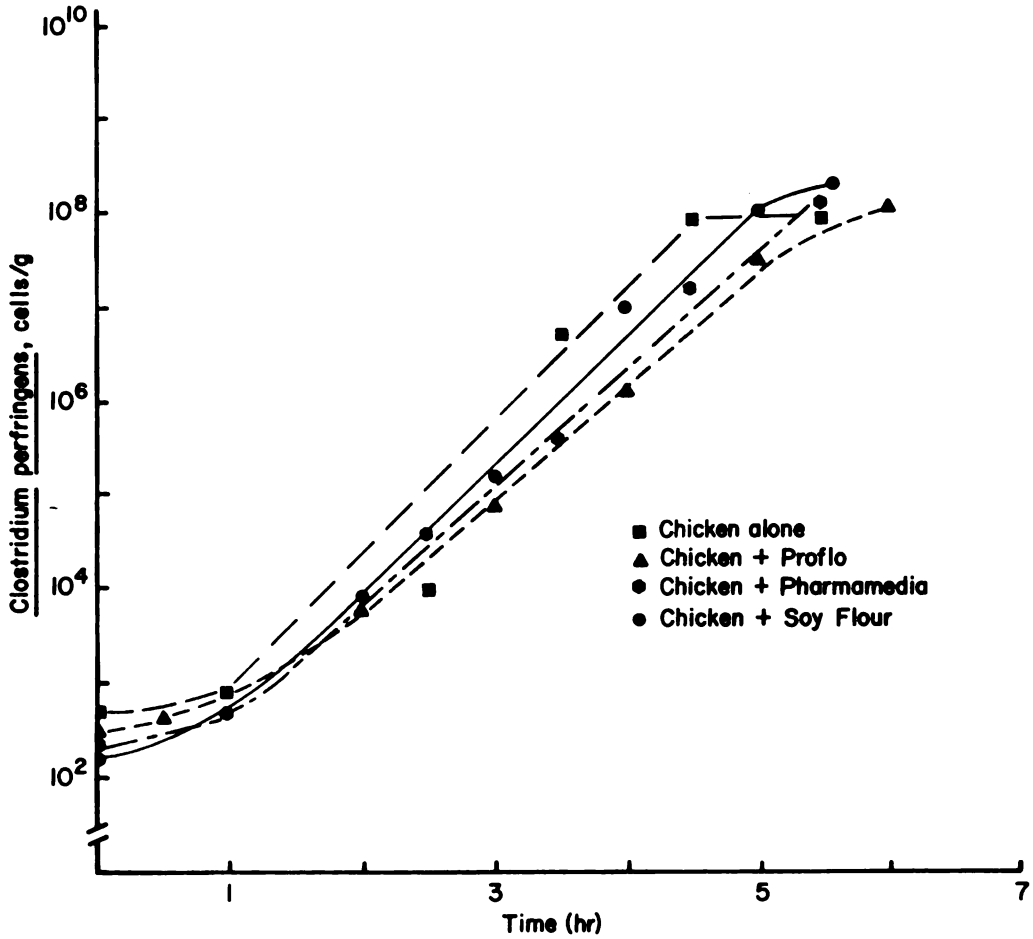

Fig. 3. Typical growth curves of C. perfringens ATCC 3624 in media containing chicken or chicken and vegetable protein.

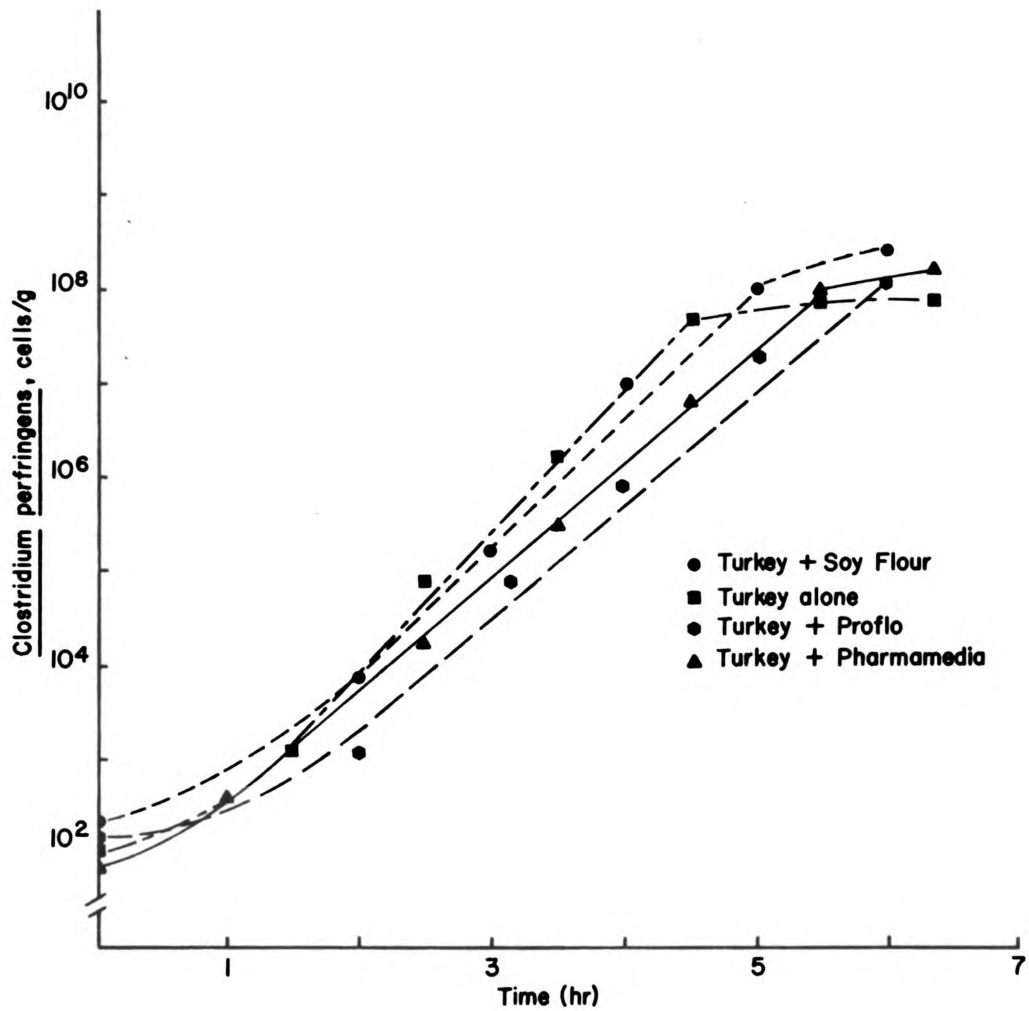

Fig. 4. Typical growth curves of C. perfringens ATCC 3624 in media containing turkey or turkey and vegetable protein.

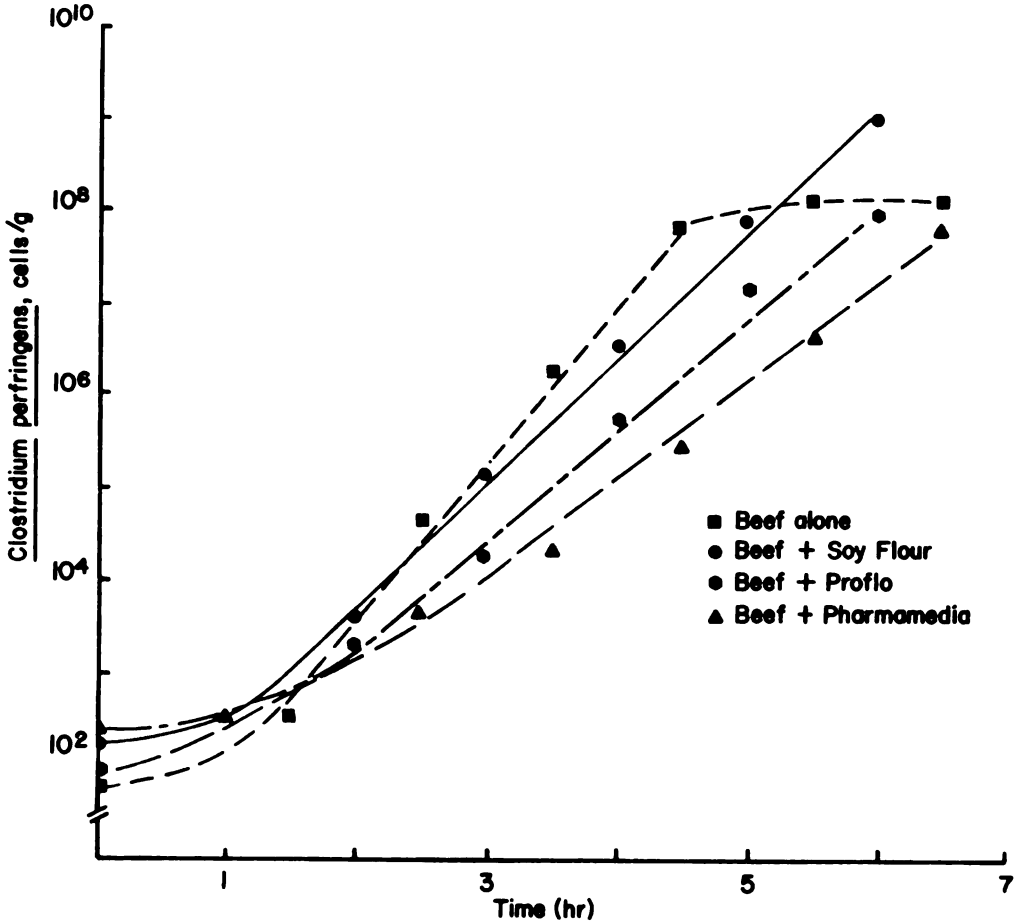

Fig. 5. Typical growth curves of C. perfringens ATCC 3624 in media containing beef or beef and vegetable protein.

Table 11 for the trials with turkey, in Table 12 for the trials with beef, and in Table 13 for the trials with chicken. These analyses showed apparent significant differences, at the 0.1% level, among the average generation times obtained in the trials using the various vegetable protein sources in combination with meat. Duncan's multiple range test was applied to the data to compare differences among the average generation times obtained in this set of trials (Series II). Table 14 shows the multiple range test for the data of Tables 11 through 13. Comparisons showed that the average generation time obtained with media containing turkey plus soy flour was not significantly different from the average generation time obtained with the turkey control (meat alone). Average generation times obtained with the cottonseed products, Proflo and Pharmamedia, plus turkey were significantly longer than those obtained with the turkey control. Generation times obtained with turkey and Pharmamedia were not significantly different from those obtained with turkey and Proflo.

In the case of beef, comparisons showed that generation times obtained with media containing beef plus soy flour were not significantly different from the average generation time obtained with the beef control (meat alone). Average generation times obtained with beef plus the cottonseed products were significantly longer

Table 11. Analysis of variance of generation times of C. perfringens ATCC 3624 in media containing turkey or turkey and vegetable protein.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	10	19.34		
Proteins	3	16.74	5.58	14.99*
Error	7	2.61	0.372	

*Significant at the 0.1% level.

Table 12. Analysis of variance of generation times of C. perfringens ATCC 3624 in media containing beef or beef and vegetable protein.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	10	56.95		
Proteins	3	55.97	18.66	134.42*
Error	7	0.97	0.14	

*Significant at the 0.1% level.

Table 13. Analysis of variance of generation times of C. perfringens ATCC 3624 in media containing chicken or chicken and vegetable protein.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	10	33.60		
Proteins	3	32.44	10.81	65.50*
Error	7	1.16	0.17	

*Significant at the 0.1% level.

than those obtained with the beef control. Generation times obtained with beef plus Proflo were significantly shorter than those obtained with beef plus Pharmamedia.

The multiple range test also showed that, in the case of chicken, the average generation time obtained with media containing chicken plus soy flour was not significantly different from the average generation time obtained with the chicken control (meat alone). In these trials the generation times obtained with chicken plus Pharmamedia were significantly shorter than those with chicken plus Proflo.

Generation times observed with meat plus soy flour in this investigation are consistent with findings published by Busta and Schroder (1970). Natural meat systems appear to be excellent growth media for C. perfringens and the addition of soybean proteins in the form of soy flour

Table 14. Multiple range test for data of Tables 11, 12, and 13: Generation times of C. perfringens ATCC 3624 in media containing meat or meat and vegetable protein.

	Shortest significant ranges 1% level			Comparisons			
	n=2	n=3	n=4				
[1]Q_p	4.949	5.145	5.260	Chicken	Soy[3]	Pharm[4]	Pro[5]
[2]R_p	1.218	1.266	1.294	12.4	12.5	14.6	16.6
Q_p	4.949	5.145	5.260	Turkey	Soy	Pro	Pharm
R_p	1.826	1.899	1.941	12.0	12.3	14.1	15.0
Q_p	4.949	5.145	5.260	Beef	Soy	Pro	Pharm
R_p	1.119	1.163	1.189	11.1	11.9	13.5	17.0

[1]Q_p = Tabular values for 7 degrees of freedom (Table from Duncan, 1955; Harter, 1960; Harter, 1961).

[2]R_p = Shortest significant ranges.

[3]Soy = soy flour and meat.

[4]Pro = Proflo and meat.

[5]Pharm = Pharmamedia and meat.

does not appear to affect the potential for rapid growth of the organism.

The addition of cottonseed proteins in the form of cottonseed flour did have an inhibitory effect on the growth rate of C. perfringens strain ATCC 3624. In each set of trials the generation time of the organism was significantly longer, at the 1% level, in the media containing meat plus cottonseed flour than in the media containing protein from meat alone and meat plus soy flour. Manufacturer's processing of the cottonseed flour may have caused a change in solubility or some modification of the cottonseed protein which may affect the growth of C. perfringens. Excessive heat during processing of the cottonseed flour can affect the protein solubility and amino acid availability of the final product. With cottonseed, only the barest minimum of dry heat in excess of that needed for removing the solvent can be tolerated without causing browning and coagulation of water-soluble proteins (Martinez et al., 1970). Another possibility is that the presence of one or more inhibitors affected the growth of C. perfringens. Manufacturer's specifications state that the typical gossypol content of Proflo and Pharmamedia is 0.029%. Even this small amount, however, may have been responsible for all or part of the inhibition observed. Gossypol is highly reactive chemically and has undesirable physiological effects. Gossypol reacts with

other seed constituents during processing of cottonseed and is responsible, in part, for reduction of the nutritive quality of the protein of cottonseed meal and for other adverse physiological effects in nonruminants (Berardi and Goldblatt, 1969).

According to manufacturer's literature, Pharmamedia contains approximately 5.5% glucose while Proflo contains approximately 6.1%. A study of the effects of food components on C. perfringens conducted by Schroder and Busta (1973) indicated that the addition of D-glucose to sodium caseinate or to isolated soy protein greatly extended the lag phase of growth. Other sugars tested did not have this inhibitory effect. These findings offer another possible explanation of the inhibitory effect of cottonseed flour as observed in these experiments.

Comparison of the generation times obtained with media containing meat plus Proflo and meat plus Pharmamedia revealed that in the case of turkey, the generation times obtained with Proflo additive were not significantly different from those obtained with Pharmamedia additive. In the case of beef, the trials using Proflo additive resulted in generation times that were significantly shorter than those obtained with Pharmamedia additive. With chicken, however, the trials using Pharmamedia resulted in significantly shorter, at the 1% level, generation times than did the trials using Proflo. These

results suggest that there is an interaction between the rate of growth of the organism and the composition of the protein sources. For example, different amino acid compositions of the different meat and vegetable protein sources may result in varying levels of the nutrients necessary for the growth of C. perfringens. Thus, beef plus Pharmamedia may have a different level of some nutrient or factor necessary for the growth of C. perfringens than the combination beef plus Proflo. The results obtained in the trials using turkey suggest that turkey contains some factor that is lacking (or present in a different concentration) in beef and chicken. Alternatively, turkey may lack, or contain a lesser concentration of, some factor that is contained in both Proflo and Pharmamedia, since the generation times obtained with turkey plus these cottonseed products were not significantly different from each other. In the trials with chicken, since the generation times with Proflo additive were longer than with Pharmamedia, chicken may lack, or contain a lesser concentration of, some factor that is in Pharmamedia and absent, or at a lower concentration, in Proflo.

A study conducted by Clifford et al. (1974) revealed that at a 1.0% level of substitution, the inclusion of xylose or ribose in the plating medium inhibited the growth of C. perfringens spores while the

inclusion of glucose enhanced growth (the greatest increase being with 0.01 and 0.1% concentrations). Xylose is present in Proflo and Pharmamedia at levels of approximately 1.4% and 1.3%, respectively, according to manufacturer's literature. The combination of mannose, ribose, and rhamnose is present at a concentration of 2.3% in both Proflo and Pharmamedia. While Clifford et al. (1974) did not identify the point of inhibition (i.e., spore activation, germination, or outgrowth) the presence of these carbohydrate components in the cottonseed products suggests another possible explanation for the inhibitory effects of cottonseed flour observed in these experiments.

Media with Meat and Cottonseed Flour Protein and Carbohydrate Fraction. Table 15 lists the generation times of C. perfringens ATCC 3624 in growth media containing meat or meat and cottonseed flour protein and carbohydrate fractions. Figures 6, 7, and 8 show typical growth curves of the organism in these media. Analysis of variance of the data in Table 15 is summarized in Table 16. Significant differences were revealed and a multiple range test was applied to the data to compare the differences (Table 17). The analyses revealed no apparent significant difference, at the 5% level, between generation times obtained with media containing meat (beef, chicken, or turkey at the 1% substitution level) and meat with Pharmamedia protein

Table 15. Generation times of C. perfringens ATCC 3624 in media containing meat or meat and cottonseed flour protein or carbohydrate fractions.

Sample[a]	Generation times, min 1	2	Average
Beef control (1%)	14.9	14.8	14.9
Beef + Pharmamedia protein fraction	15.0	14.3	14.7
Beef + Pharmamedia carbohydrate fraction	16.9	15.1	16.0
Beef + Proflo protein fraction	15.3	14.9	15.1
Beef + Proflo carbohydrate fraction	15.5	16.0	15.8
Chicken control (1%)	15.3	13.2	14.3
Chicken + Pharmamedia protein fraction	16.2	14.0	15.1
Chicken + Pharmamedia carbohydrate fraction	16.6	15.9	16.3
Chicken + Proflo protein fraction	15.2	16.9	16.1
Chicken + Proflo carbohydrate fraction	15.4	15.7	15.6
Turkey control (1%)	14.6	15.4	15.0
Turkey + Pharmamedia protein fraction	15.2	14.7	15.0
Turkey + Pharmamedia carbohydrate fraction	16.9	16.2	16.6
Turkey + Proflo protein fraction	16.7	16.9	16.8
Turkey + Proflo carbohydrate fraction	16.5	16.3	16.4

[a]All samples contained the basal growth medium (see Table 4).

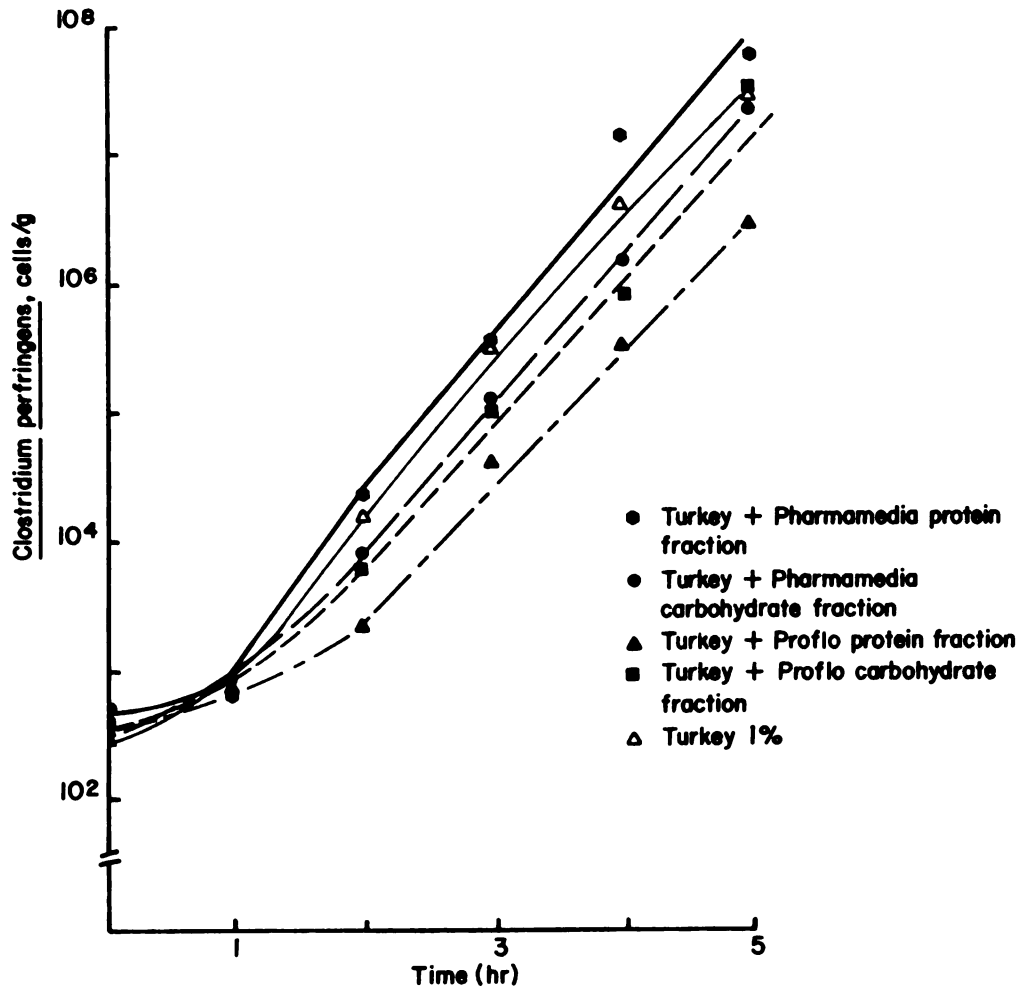

Fig. 6. Typical growth curves of C. perfringens ATCC 3624 in media containing turkey or turkey and cottonseed flour protein or carbohydrate fractions.

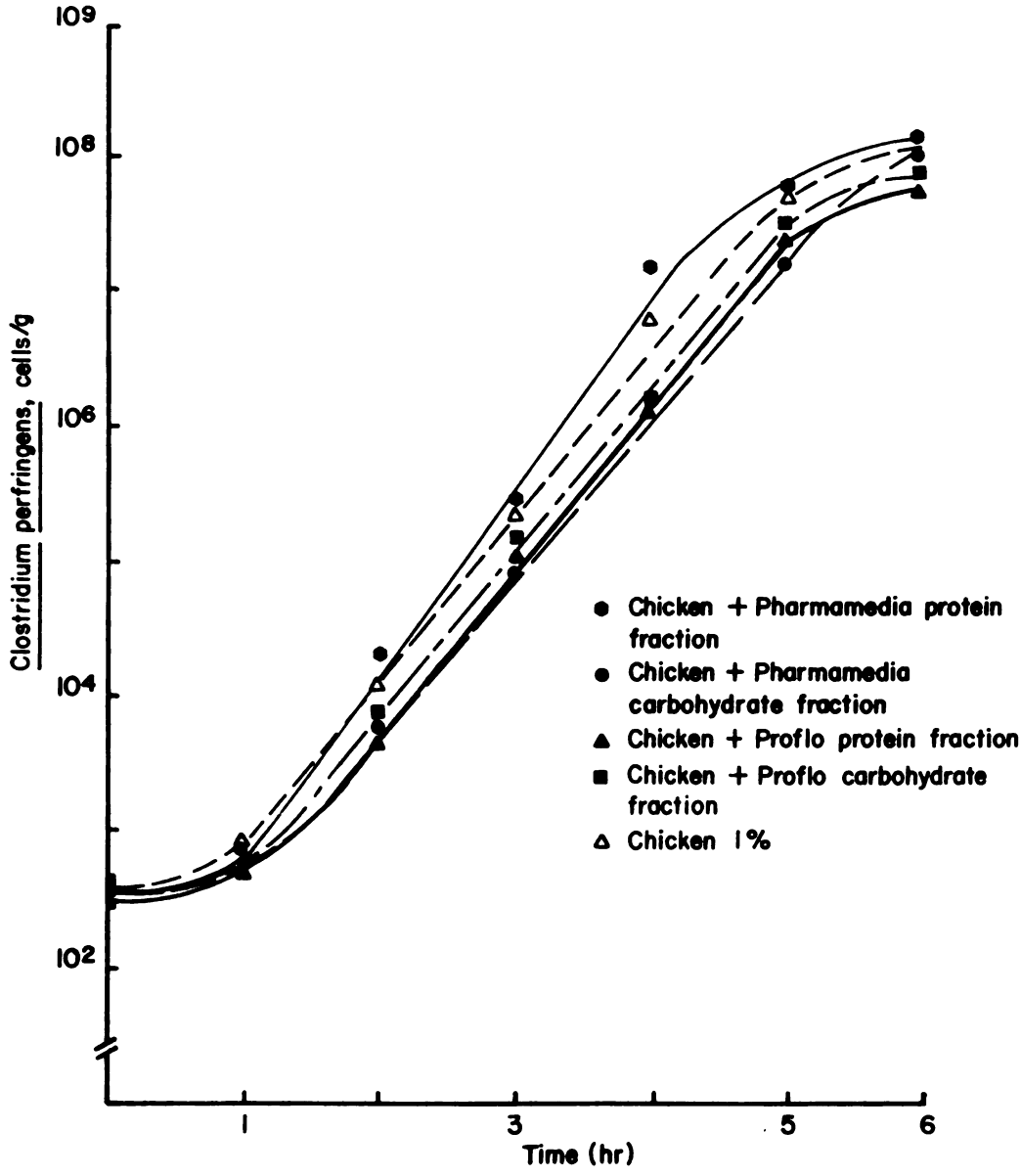

Fig. 7. Typical growth curves of C. perfringens ATCC 3624 in media containing chicken or chicken and cottonseed flour protein or carbohydrate fractions.

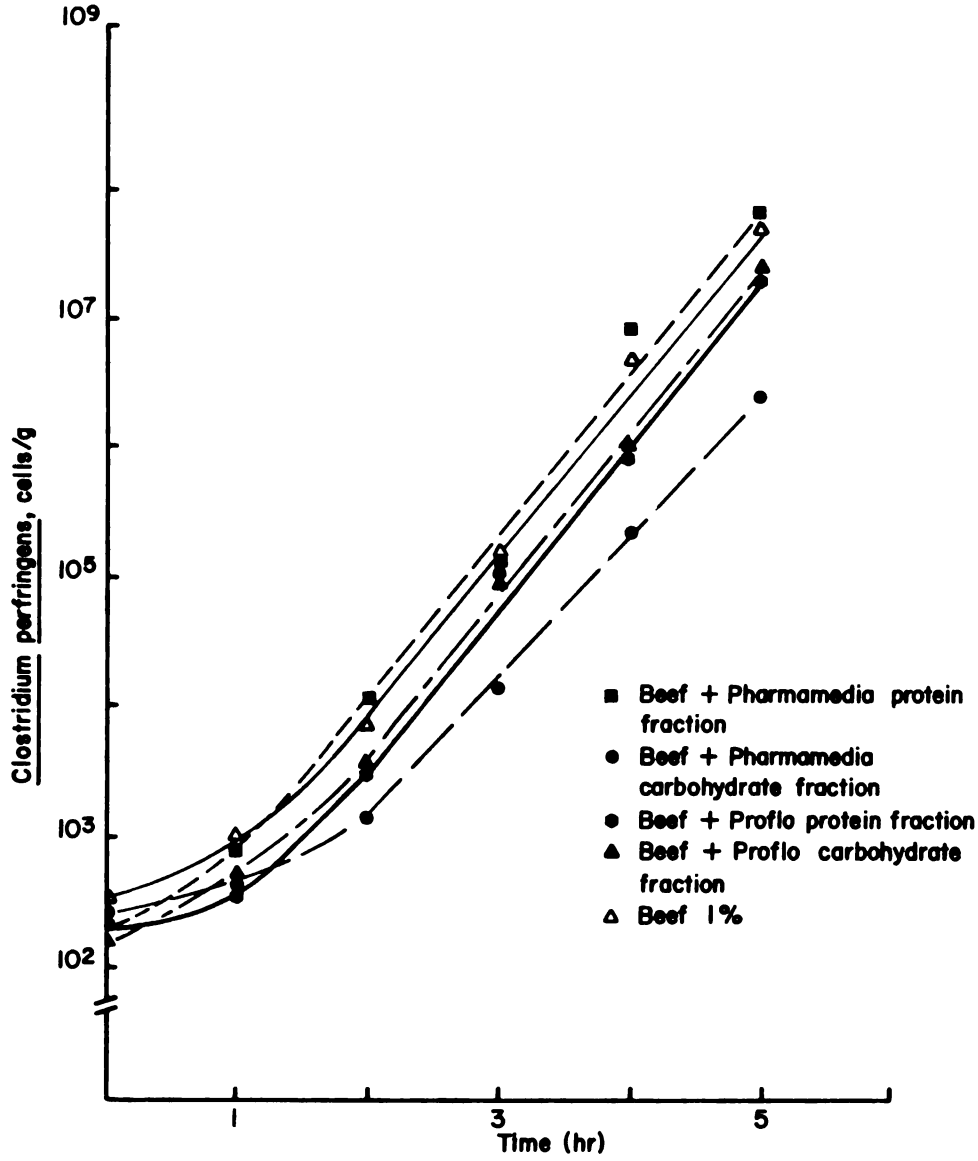

Fig. 8. Typical growth curves of C. perfringens ATCC 3624 in media containing beef or beef and cottonseed flour protein or carbohydrate fractions.

Table 16. Analysis of variance of generation times of C. perfringens ATCC 3624 in media containing meat or meat and cottonseed flour protein or carbohydrate fractions.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	29	25.82	0.89	
Meat	2	2.43	1.213	3.441*
Additives	4	11.91	2.98	8.449**
Interaction (M x A)	8	2.31	0.289	0.819
Error	26	9.17	0.353	

*Significant at the 5% level.

**Significant at the 1% level.

fraction. No apparent significant difference was seen, at the 5% level, among the generation times that were observed with media containing meat and Proflo carbohydrate fraction, meat and Proflo protein fraction, and meat and Pharmamedia carbohydrate fraction. Although the generation times observed with media containing meat only were not significantly different from generation times observed with media containing meat and Pharmamedia protein fraction, the generation times obtained with these two types of media were significantly different, at the 1% level, from the generation times obtained with media containing meat and Pharmamedia carbohydrate fraction, Proflo carbohydrate fraction, or Proflo protein fraction. These results

Table 17. Multiple range test for data of Table 15: generation times of C. perfringens ATCC 3624 in media containing meat or meat and cottonseed flour protein or carbohydrate fractions.

	Shortest significant ranges 1% level				Comparisons[1]				
	n=2	n=3	n=4	n=5					
[2]Q_p	3.889	4.056	4.168	4.250	Alone	Ph-p	Pr-c	Pr-p	Ph-c
[3]R_p	0.943	0.983	1.010	1.030	14.73	14.93	15.93	16.0	16.30
Q_p	3.889	4.056	4.168		Beef*		Chicken		Turkey*
R_p	0.730	0.762	0.783		15.3		15.4		15.9

[1]Key: Alone = meat (beef, chicken, or turkey) as sole protein source at 1% protein level.
Ph-p = meat + Pharmamedia protein fraction
Ph-c = meat + Pharmamedia carbohydrate fraction
Pr-p = meat + Proflo protein fraction
Pr-c = meat + Proflo carbohydrate fraction

[2]Q_p = Tabular values for 30 degrees of freedom (Table from Duncan, 1955; Harter, 1960; Harter, 1961).

[3]R_p = Shortest significant range.

*At 5% level of significance, beef and turkey were significantly different from each other, but not from chicken.

suggest that while the addition of Pharmamedia protein fraction had no significant effect on the growth of C. perfringens ATCC 3624 in media containing beef, chicken, or turkey, the addition of Proflo or Pharmamedia carbohydrate fractions or Proflo protein fraction exerted an inhibitory effect on the growth of the organism. The fact that the inhibitory effect was observed in media containing both Proflo and Pharmamedia carbohydrate fractions suggests that one or more inhibitory factors may be present in the carbohydrate fraction of the cottonseed products used in this investigation.

A significant difference was also revealed, at the 5% level, among generation times observed with media containing beef, chicken, or turkey at the 1% level of substitution. Generation times observed with media containing beef were significantly shorter than generation times observed with turkey. No significant difference was seen between generation times observed with chicken and those observed with beef or between generation times observed with chicken and those observed with turkey. These results suggest that there may be different amounts of nutrients or growth factors in these types of meat that account for the slightly different rates of growth of C. perfringens in the media.

Since the inhibitory effect of D-glucose on the growth of C. perfringens had been previously reported

(Schroder and Busta, 1973), an attempt was made to eliminate this component from the carbohydrate fraction of Pharmamedia by treatment with glucose oxidase. The basal growth medium (Table 4) was prepared using turkey and Pharmamedia carbohydrate fraction that had been treated with glucose oxidase. An experiment was conducted (using appropriate controls) to determine the generation time of C. perfringens ATCC 3624 in this medium. Results of this experiment are listed in Appendix Table I. Typical growth curves of the organism are shown in Appendix Figure II. These data, however, are inconclusive due, in part, to the small number of trials conducted. The use of media containing carbohydrate fraction from which the glucose had been enzymatically removed did not result in significantly shorter generation times. The inhibitory effect of the carbohydrate fraction does not appear to be due to its glucose content.

SUMMARY AND CONCLUSIONS

The effects of various vegetable protein sources on the growth of Clostridium perfringens were studied. Results show that the addition of vegetable protein sources as soy flour, Bontrae, Proflo, and Pharmamedia to growth media for C. perfringens had neither a stimulatory nor an inhibitory effect on the rate of growth of the organism as compared to the addition of trypticase. A difference between strains was shown since heat sensitive strain ATCC 3624 had significantly longer generation times in media containing vegetable protein sources than did heat resistant strain NCTC 8238. Thus, a difference between strains in ability to utilize the substrate was shown, although the vegetable protein sources used were apparently similar in terms of protein availability to the organism.

Clostridium perfringens strain ATCC 3624 was grown in media containing meat and vegetable protein sources. Results show that the addition of cottonseed protein in the form of Proflo and Pharmamedia has an inhibitory effect on the growth rate of the organism when compared to the growth rate in media containing beef, chicken, or turkey

as the sole protein source or media containing meat and soy flour. The growth rate of the organism in media containing soy flour and meat was similar to the growth rate in media containing meat as the sole source of protein. Since generation times of the organism in media containing chicken and Pharmamedia were significantly shorter than in media containing chicken and Proflo, an interaction between protein sources in a given type of media was indicated. Generation times in media containing beef and Proflo were significantly shorter than in media containing beef and Pharmamedia. Generation times of the organism in media containing turkey and Proflo were not significantly different from generation times in media containing turkey and Pharmamedia. Some factor or factors may be present in Pharmamedia and Proflo that exert an inhibitory effect on the growth of C. perfringens. The interaction observed between meat and cottonseed protein sources may be due to differing concentrations of one or more factors in the protein sources that are utilized by the organism for growth.

Clostridium perfringens ATCC 3624 was grown in media containing meat and meat with cottonseed flour protein and carbohydrate fractions. Results show that the addition of Proflo or Pharmamedia carbohydrate fractions to media containing meat exerted an inhibitory effect on the growth of the organism. One or more inhibitory factors

may be present in the carbohydrate fraction of the cottonseed products.

A significant difference was also seen among growth rates observed with media containing various meat protein sources. Generation times observed with media containing beef were significantly shorter than with media containing turkey. No significant difference was seen between generation times observed with chicken and those observed with beef or between generation times observed with chicken and those observed with turkey. Meat is generally an excellent growth medium for C. perfringens and different levels of nutrients or growth factors among these types of meat may account for the slightly different rates of growth observed.

Clostridium perfringens ATCC 3624 was grown in media containing turkey and Pharmamedia carbohydrate fraction which had been treated with glucose oxidase. The results were inconclusive. The use of media containing carbohydrate fraction from which the glucose had been enzymatically removed did not result in significantly shorter generation times. Glucose does not appear to be responsible for the inhibitory effect of the carbohydrate fraction of Pharmamedia.

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APPENDICES

APPENDIX I

APPENDIX I

Generation times of C. perfringens ATCC 3624 in media containing turkey, Pharmamedia carbohydrate fraction, and glucose oxidase.

Sample[1]	Generation times, min 1	2	Average
Turkey[2]	14.5	15.6	15.1
Turkey + Pharmamedia carbohydrate fraction[2]	14.7	16.6	15.7
Turkey + Pharmamedia carbohydrate fraction + glucose oxidase	14.8	14.5	14.2

[1]All samples contained the basal medium (see Table 4).

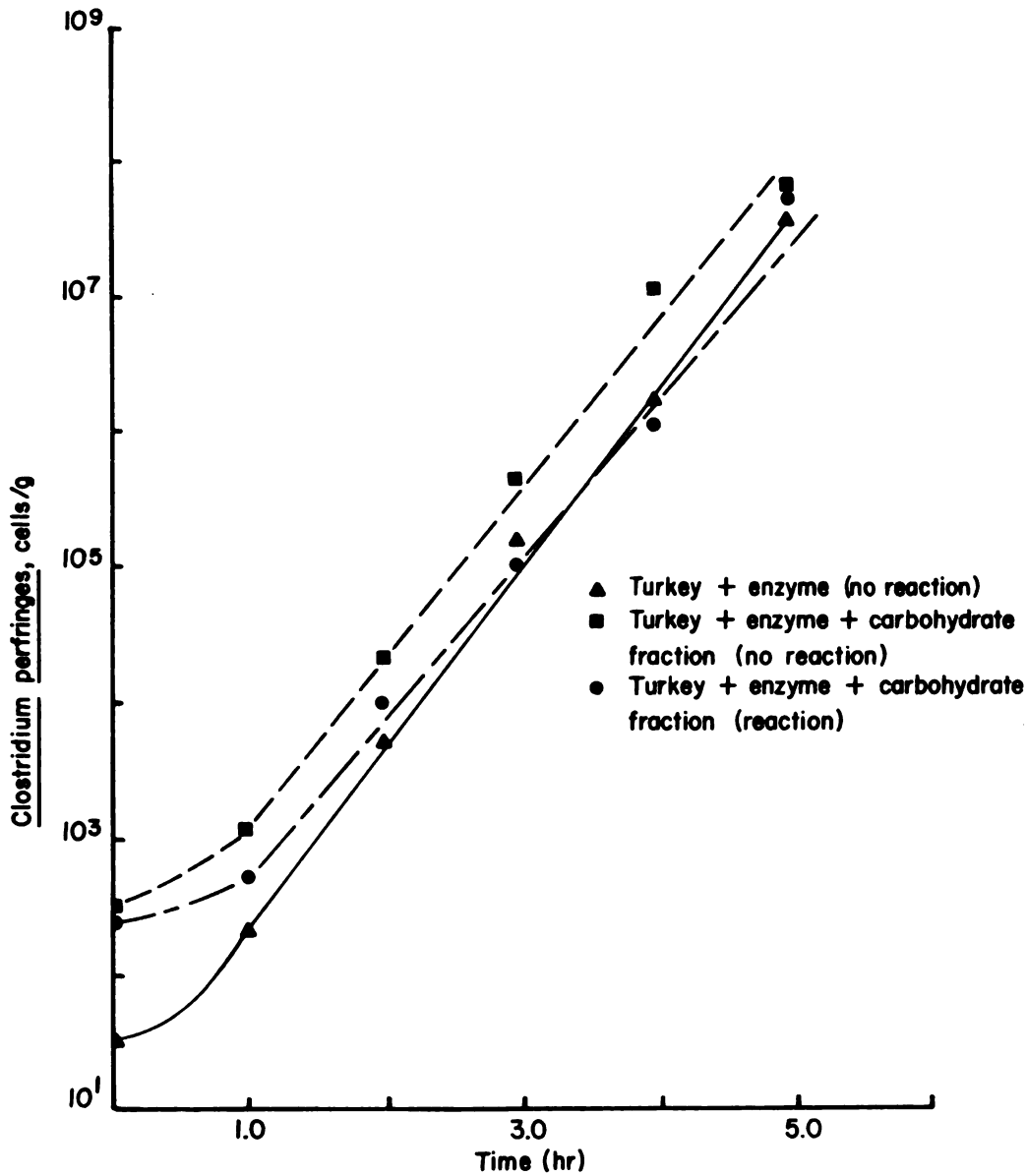
[2]Glucose oxidase added, but reaction stopped immediately by autoclaving the medium.

Analysis of variance of generation times of C. perfringens ATCC 3624 in media containing turkey, Pharmamedia carbohydrate fraction, and glucose oxidase.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F value
Total	5	4.97	0.994	
Treatment	2	2.45	1.225	1.458
Error	3	2.52	0.84	

APPENDIX II

APPENDIX II


10^9
10^7
10^5
10^3
10^1
Clostridium perfringes, cells/g
1.0
3.0
5.0
Time (hr)
▲ Turkey + enzyme (no reaction)
■ Turkey + enzyme + carbohydrate fraction (no reaction)
● Turkey + enzyme + carbohydrate fraction (reaction)