

**HEAT RESISTANCE AND GROWTH OF *Clostridium perfringens* SPORES AS
AFFECTED BY THE TYPE OF HEATING MEDIUM, AND HEATING AND
COOLING RATES IN GROUND PORK**

A Dissertation

by

MAYRA MÁRQUEZ GONZÁLEZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Food Science and Technology

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ABSTRACT

Heat Resistance and Growth of *Clostridium perfringens* Spores as Affected by the Type of Heating Medium, and Heating and Cooling Rates in Ground Pork. (December 2008)

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The survival and germination of *Clostridium perfringens* spores in different heating media and at different heating rates was studied to determine the fate of *C. perfringens* spores during abusive cooking and cooling of pork products. The heat resistance (HR) of *C. perfringens* spores from three strains that were either previously heat shocked (HS) or non-heat shocked (NHS) was determined individually and as a cocktail in phosphate buffer solution (pH 7.4) (PBS), beef gravy (BG), ground pork (GP) and cured ground pork (CGP) at 75°C. The effect of the heating rate on HR, germination and outgrowth of *C. perfringens* spores in CGP was determined by increasing the temperature from 20 to 75°C at a rate of 4, 8, and 12°C/h prior to heating and holding at 75°C for 48 h. Heating rates at 4°C/h in GP and CGP were repeated with additional cooling from 54.4 to 7.2°C within 20 h (temperature abuse).

Linear survival curves were observed on NHS spores in the four heating media, whereas HS spores showed linear curves when heated in PBS and BG, and biphasic curves when heated in GP and CGP. In general, HS spores were more heat sensitive than

NHS spores. NHS spores heated in GP had greater HR than spores heated in CGP, BG or PBS.

There were no significant differences ($P>0.05$) on the HR of *C. perfringens* spores in CGP heated from 20 to 75°C at 4, 8, or 12°C/h. Heating rates of 8 and 12°C/h showed no difference in germination and outgrowth of inoculated spores, whereas at 4°C/h, growth of *C. perfringens* occurred between 44 and 56°C.

Temperature abuse during cooling of GP resulted in 2.8 log CFU/g increase of *C. perfringens* counts. In CGP, *C. perfringens* counts decreased by 1.1 log CFU/g during cooling from 54.4 to 36.3°C and then increased by 1 log CFU/g until the product reached 7.2°C. However, with an initial inoculum in raw CGP of 5 log CFU *C. perfringens* spores/g, *C. perfringens* counts did not exceed 3.4 log CFU/g during a 20 h abusive cooling. These results suggest there is no risk associated with *C. perfringens* in cured pork products under the conditions tested. Results from the present study indicate that different behavior may be expected with different meat products.

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INTRODUCTION

Clostridium perfringens is a microorganism of major concern in the food industry due to its wide distribution and ability to produce spores. Spores of *C. perfringens* can survive cooking treatments and later grow to large numbers in food products if proper conditions are present (25, 50). A large number of cases of *C. perfringens* foodborne illness occur in the United States every year, most of which are attributed to meat and poultry products (12, 17, 56). Improper handling of cooked products, favoring the germination and growth of surviving spores of *C. perfringens* within the food, has been determined as the most common factor that promoted the outbreaks (3, 12, 13, 16, 36).

Because it is impractical to heat all foods to temperatures high enough to inactivate spores of *C. perfringens* without making food characteristics unacceptable for consumption, control measures rely upon proper handling of cooked products to prevent spore germination and/or multiplication of vegetative cells (25). Current U.S regulation requires that ready-to-eat meat or poultry products meet the stabilization (chilling) standard (91). This stabilization standard requires the prevention of germination and outgrowth of sporeforming bacteria such as *Clostridium botulinum*, and allows no more than a 1 log increase in *C. perfringens* counts within the product during the cooling of cooked meat or poultry products.

This dissertation follows the style of the *Journal of Food Protection*.

Commercial establishments that use customized chilling treatments, which are different from those proposed by the stabilization guidelines (90), should demonstrate the safety of the cooked product. Several challenge studies have been conducted under laboratory conditions to investigate growth of *C. perfringens* during cooling of cooked meat products (48, 80). However, the cooking conditions in these studies were not representative of the cooking process used in commercial establishments. These studies used a heat shock treatment of inoculated meat product at 75°C/20 min instead of the cooking process used during production of cooked meat products.

The objectives of this study were: (i) to determine the effect of the method in which spores are harvested and the nature of the heating medium on the heat resistance of *C. perfringens* spores; (ii) to determine the effect of the heating conditions on the survival and germination of *C. perfringens* spores in cured ground pork; and, (iii) to evaluate the germination and outgrowth of surviving *C. perfringens* spores in ground pork and cured ground pork under temperature abuse heating and cooling treatments.

LITERATURE REVIEW

Clostridium perfringens as a foodborne pathogen

Clostridium perfringens (formerly *Clostridium welchii*) is a Gram positive, non-motile encapsulated rod, capable of producing subterminal ovoid spores (11). Production of spores is an important characteristic in *C. perfringens* classification. Spores have the ability to survive cooking procedures and resume vegetative cell growth when proper conditions are present (50).

C. perfringens strains are classified into five toxicological types (A to E) on the basis of four major toxins produced (alpha (α), beta (β), epsilon (ϵ), and iota (ι)) (11, 39, 50, 76). Most of the strains produce α -toxin (lecithinase, phospholipase C) (50). Only *C. perfringens* type A and C have been associated with human gastroenteritis (11, 32, 36, 50).

C. perfringens food poisoning

Gastroenteritis caused by *C. perfringens* occurs after ingestion of large numbers of vegetative cells (11, 23, 25, 34, 39). Hauschild (34) reported that in order to implicate *C. perfringens* as the agent of a foodborne disease, the viable counts of this organism should be at least 10^5 CFU/g of food.

Symptoms of *C. perfringens* food poisoning include diarrhea, acute abdominal pain, nausea, and occasional vomiting (25, 36, 39). The onset of symptoms have been reported to occur after 6-24 h of ingesting contaminated food (36, 39), and cease

spontaneously after 12-24 h (53). Although *C. perfringens* food poisoning is a relatively mild illness, it may be fatal in elderly or debilitated individuals (25, 53).

Toxin production during sporulation of *C. perfringens* cells in the intestine has been attributed to be the cause of diarrhea (25, 39, 53, 54). McClane (53) reported that *C. perfringens* enterotoxin (CPE) causes morphological damage to the intestine, which results in clinical symptoms (diarrhea and cramps). CPE acts quickly; it only takes 30 min from intestinal binding of CPE to production of onset symptoms. The onset time reported of 6-24 h is the time required for CPE synthesis and release during the growth of vegetative cells (53).

Foods involved with *C. perfringens* foodborne illness usually include meat or poultry that are cooked by boiling, stewing, or lightly roasting, and then held at temperatures above 20°C for an extended period of time or inappropriately refrigerated, such as storing in large amounts for several hours (7, 11, 25, 36). Such conditions have allowed the multiplication of surviving *C. perfringens* to numbers large enough to cause disease. Generally, foods contaminated with such large numbers of vegetative cells of *C. perfringens* do not display signs of spoilage, therefore favoring consumption of contaminated foods by unsuspecting victims (39).

Distribution of *C. perfringens*

C. perfringens Type A is widely distributed in the environment (36). *C. perfringens* has been isolated from human stools, animal feces, sewage, raw meats, and foods (11, 23, 30, 31, 36, 77). It has been isolated from 1 (2.2%) of 45 fecal samples

from healthy individuals and up to 8 (15%) of 53 stool samples from elderly people (healthy and with diarrhea) (36). Strong et al. (77) isolated *C. perfringens* from 3 (2.7%) of 111 commercially prepared frozen foods, 2 (3.8%) of 52 samples of fresh fruit and vegetables, 3 (5.0%) of 60 samples of spices, 3 (1.8%) of 165 of home-prepared foods and 20 (16.4%) of 122 raw meat, poultry, and fish samples. Although *C. perfringens* was not recovered from any of the fish samples analyzed by Strong et al. (77), Hobbs et al. (37) recovered the organism from 18 (9.8%) of 184 samples of vacuum packed fish. Rahmati et al. (59) isolated *C. perfringens* from 17 (4.9%) of 347 fresh and processed seafood samples.

C. perfringens in raw meats

C. perfringens has been isolated frequently from meat samples. Hall and Angelotti (31) reported that *C. perfringens* was present in 93 (58%) of 161 raw unprocessed meat samples analyzed. A higher recovery was reported on veal samples (14 positive samples of 17 samples analyzed) and the lower recovery was reported on pork samples (15 positive samples of 41 analyzed samples). In the same study, these authors reported that the levels of contamination ranged from <10 up to 760 CFU/g. Most of the positive samples contained between 1 and 100 CFU/g. The sample with the highest count was obtained from ground beef.

Hobbs et al. (36) reported the isolation of *C. perfringens* from 11 (20%) of 55 pork samples and 13 (24.1%) of 54 beef samples, whereas none of the 14 lamb samples analyzed tested positive. Strong et al. (77) recovered *C. perfringens* from beef, pork, and

lamb cuts, ground meat, spiced meats, and organ meats. In their study, Strong et al. (77) did not recover *C. perfringens* from veal cuts, fish samples, or chicken samples.

The Food Safety Inspection Service of the United States Department of Agriculture (FSIS) conducted surveys on different meat and poultry products from 1992-1996 to establish a microbiological baseline data on such products. The surveys included 4 191, 2 112 and 1 297 beef, pork and chicken carcasses, respectively, as well as 563, 285 and 296 ground beef, chicken and turkey samples, respectively. According to these surveys, *C. perfringens* was recovered from 2.6 up to 53.3% of the meat and poultry products analyzed. Raw ground meats were more frequently contaminated. *C. perfringens* was recovered from 28.1, 50.6, and 42.9% samples of raw ground turkey, chicken and beef respectively (83-89).

Occurrence of *C. perfringens* on carcass surfaces and whole muscle samples is low. The FSIS isolated *C. perfringens* from 2.6, 8.3, and 10.4% of steers and heifers, cows and bulls, and market hog carcasses, respectively (83, 84, 87). Taormina et al. (79) isolated vegetative cells of *C. perfringens* from 3 (1.6%) of 194 samples of cured whole-muscle meat products, whereas no spores were detected (detection limit was 1 log CFU/g).

C. perfringens in processed meats

Bauer et al. (8) investigated the prevalence of *C. perfringens* during pork processing. These authors did not detect *C. perfringens* on 48 pork carcasses, 6 hearts, 6 spleens, nor from 13 visceral pans examined. *C. perfringens* was recovered from 9

(11.8%) of 76 samples from body cavity fluid and 16 (21.4%) of 76 liver samples, as well as from all of the 13 scalding vat water samples analyzed. These authors also recovered *C. perfringens* in 7 (38.9%) of 18 pork sausage samples obtained from supermarkets. Taormina et al. (79) isolated vegetative *C. perfringens* from 74 (48.7%) of 152 cured ground or emulsified meat precuts samples, whereas spores were detected only in 8 (5.2%) of the 152 samples at levels below 2 log CFU/g. Hall and Angelotti (31) isolated *C. perfringens* from 20 (19.8%) of 101 samples of processed meat and meat dishes. The highest recovery was observed in sausage samples, where *C. perfringens* was isolated from 10 (47.6%) of 21 pork sausage and mettwurst samples.

The higher recovery of *C. perfringens* on processed meats has been attributed to more handling required during the preparation of the product, addition of spices, and use of trimmings, which tend to have a heavier microbial load than other meat cuts due to increased handling during their processing (31, 64).

Although the prevalence of *C. perfringens* in meat and meat products is high, the average number of spores present on such type of products is low. Greenberg et al. (30) reported that the mean of *Clostridium* spp. spores isolated from meat products (beef, pork and chicken samples) was 2.8 spores per gram. The most heavily contaminated sample had 115 spores per gram (30). Taormina et al. (79) isolated *C. perfringens* spores from 8 (5.3%) of 152 samples of cured ground or emulsified meat products, but no spores were isolated from 275 cured and uncured whole muscle samples. Kalinowski et al. (48) reported undetectable levels of *C. perfringens* spores (<3/g) in 195 (99%) of 197 ground meat samples. The only 2 samples from which spores were detected were

ground pork containing 3.3 and 66 spores/g respectively. Turcsán et al. (82) reported counts of *C. perfringens* spores on goose liver samples of 1.0 log CFU/g.

Characteristics of the organism

Growth of C. perfringens

C. perfringens has a temperature growth range between 6 and 52.3°C (7, 25, 31, 70, 74, 96, 97), with the fastest growth occurring between 35 and 48.9°C (7, 9, 11, 25, 74, 96). Temperatures at which growth occurs may vary among different strains and may be affected by the characteristics of the culture medium (7, 96). Barnes et al. (7) reported that vegetative cells of *C. perfringens* F2985/50 were not able to multiply in raw beef blocks (pH of 5.7-5.8) stored at 1, 5, 10, or 15°C, whereas slow growth occurred at 20°C and rapid growth occurred at 37°C. Roberts and Hobbs (62) reported that *C. perfringens* grew well at 15°C in Hartley's digest broth and Tryptose-Peptide-Glucose (TPG) medium containing 0.2% of added yeast extract. However, these authors did not observe any growth of *C. perfringens* incubated at 10°C for more than three weeks. Solberg and Elkind (74) reported that *C. perfringens* grew well in frankfurters stored at 12 and 15°C, but no growth occurred at 10 or 5°C. Juneja et al. (42) reported that no growth occurred in cured pork stored up to 21 days at both 10 and 12°C. In a study by Blankenship et al. (9) *C. perfringens* did not grow in cooked chili held at 26.7°C over 6 h.

In a study conducted with inoculated meat products, Willardsen et al. (96) observed that when the temperature increase rate was 7.5°C/h, *C. perfringens* grew in autoclaved ground beef at 36°C. However, when unautoclaved raw ground beef or raw

beef strips were used, *C. perfringens* did not initiate growth until the temperature reached 41 and 43°C respectively (96).

Barnes et al. (7) found that the minimal growth temperature of a food poisoning strain of *C. perfringens* was related to pH and growth medium. At pH 7.2, less time was required for *C. perfringens* to develop visible turbidity in Robertson's cooked meat broth at 20°C (48 h) than the time required to develop visible turbidity at pH 5.8 (74 h). According to these authors, *C. perfringens* would have a lower minimal growth temperature in beef with a pH higher than 5.7-5.8.

Growth of *C. perfringens* at 50°C and above has been reported (19, 70, 97). Collee et al. (19) observed an unusual growth pattern for *C. perfringens* at 50°C. These authors found that the number of *C. perfringens* cells decreased to <500 cells/ml after inoculation in cooked meat broth within the first 2-4 h at 50°C. The surviving cells followed an increase to a maximum count of 6 log cells/ml within 6 h. This growth pattern was reproduced with both spores and vegetative cells. Collee et al. (19) named this unusual growth the "Phoenix phenomenon". The name "Phoenix phenomenon" was coined possibly after the "Phoenix" myth, which symbolizes immortality through "death and resurrection" (70). The "Phoenix phenomenon" has been reported to occur also at temperatures above 50°C under strict anaerobic conditions and different culture media by Shoemaker and Pierson (70). Mead (55) also observed the "Phoenix phenomenon" in chicken leg medium (pH 6.8) at 50°C.

The generation time of *C. perfringens* is <20 min in cooked meat products at optimum temperatures (20, 97). Generation times vary among strains and the generation

time of the same strain may vary with different types of growth medium. Willardsen et al. (97) reported the generation time of *C. perfringens* NCTC 8238 to be as short as 7.3 min in cooked ground beef at 41°C. In the same study, the authors reported that at 45°C *C. perfringens* showed a shorter lag time in autoclaved ground beef (1.2 h) than in fluid thioglycollate medium (FTG, 1.9 h) and that the growth was more rapid in autoclaved ground beef (generation time=8.5 min) than in FTG (generation time=12.2 min). At a temperature increase rate of 7.5°C/h, mean generation times of *C. perfringens* were 11, 8.1 and 6.2 min in FTG, autoclaved ground beef and raw beef respectively.

Sporulation of C. perfringens

Temperatures at which *C. perfringens* produce spores are more restricted than the temperatures for growth (20). Optimum sporulation occurs at 37° to 40°C (20). The time required to obtain maximum numbers of spores varies among strains. Labbe and Duncan (51) obtained spores in Duncan-Strong (DS) medium incubated at 37°C after 15 h for strain FD1 and an incubation period of 20 h was required for strain NCTC 8798. Ando (2) reported that at 37°C the maximum sporulation of strains NCTC 8238 and S40 occurred usually within 12-16 h.

Most of *C. perfringens* spores require heat activation to germinate (7). However, heterogeneity among *C. perfringens* spores has been reported. Labbe and Duncan (51) reported that *C. perfringens* strain FD1 produced heat sensitive spores that required no prior heat shock for germination, and strain NCTC 8798 produced heat resistant spores that required prior heat shock for optimal germination.

Barnes et al. (7) reported that only about 3% of the spores of *C. perfringens* F2985/50 were able to germinate in raw beef without prior heat shock, and almost all spores germinated after heating the meat. Roberts and Hobbs (62) reported that *C. perfringens* was not able to germinate within 14 days at 5°C in four different systems which have been recommended for germination of clostridial spores, or on TPG medium at 5°C.

Survival of C. perfringens

C. perfringens spores are very resistant to freezing and storage at -5 or -20°C, whereas vegetative cells are destroyed during freezing and by storage at freezing temperatures (7). Canada et al. (15) reported that vegetative cells of *C. perfringens* were not able to survive 48 h at -17.7°C in phosphate buffer (pH 7.2) and only 0.4% and <0.1% of the original population survived in 0.1% peptone water and 2% sucrose solution respectively at the same storage conditions. In the same study, 26.6, 44.1 and 58.3% of original *C. perfringens* spores were able to survive after 48 h at -17.7°C in phosphate buffer, peptone water and sucrose solution respectively.

C. perfringens vegetative cells have been reported to die during refrigerated storage (15, 48, 74). Canada et al. (15) found that only 33.4% of the original population of vegetative cells survived 48 h at 7.1°C whereas up to 100% of the original population of spores was able to survive the same storage conditions in phosphate buffer, peptone water or sucrose solution.

Kalinowski et al. (48) reported that *C. perfringens* levels decreased by 2.5 log CFU/g in cured turkey stored at 0.6 and 4.4°C for 7 days and up to 2.7 log CFU/g at 10°C for 7 days. Solberg and Elkind (74) reported that *C. perfringens* spore counts remained constant for more than 169 days stored in distilled water at 5°C, whereas vegetative cell counts were reduced up to 3 log cycles within the first 1 or 2 weeks of storage and then remained constant several months.

C. perfringens spores are able to survive cooking treatments, whereas vegetative cells are destroyed (14, 15, 71, 81). Canada et al. (15) reported that vegetative cells of *C. perfringens* were not able to survive heating at 80°C, nor 100°C for 10 min in phosphate buffer, peptone water, or sucrose solution. Up to 60.7% of the original population of spores was able to survive heating at 80°C for 10 min and only 8% of the original spore population survived heating at 100°C for 10 min in peptone water.

Byrne et al. (14) reported that D-values for vegetative cells of *C. perfringens* in pork luncheon roll ranged from 1 min at 60°C to 33.2 min at 50°C. These authors estimated that cooking pork luncheon roll at 70°C for 1.3 min would achieve a 6 log reduction of vegetative *C. perfringens*. Tuomi et al. (81) reported that vegetative *C. perfringens* were not able to survive during heating of beef gravy to 74°C. These authors also reported that vegetative *C. perfringens* did not survive after adding the fresh cultures in beef gravy previously heated to 70°C.

The survival of *C. perfringens* vegetative cells at temperatures commonly used to hold foods has been reported. Strong and Ripp (78) reported that after roasting inoculated turkey rolls at 74 or 85°C, *C. perfringens* was able to survive up to 6 h when

holding at 68°C. *C. perfringens* survival at 68°C was also observed in inoculated ground beef casseroles baked to 82°C and 71°C. Solberg and Elkind (74) found that both spores and vegetative cells survived the heating procedures used in the processing of frankfurters (heating to an internal temperature of 68-69°C in 30-48 min).

Growth of *C. perfringens* during cooking and cooling of meat products has been reported. Willardsen et al. (97) reported that growth of vegetative cells of *C. perfringens* in ground beef heated at different rates started between 32 and 39°C, and inactivation of cells began at 51-55°C for all heating rates. In an additional study conducted with rolled raw beef slices at increasing temperature rates of 6.0°C/h, 7.5°C/h and 8.5°C/h, *C. perfringens* growth ceased at 53, 54, and 55 °C respectively (96).

C. perfringens, as well as other *Clostridium* spp., is inhibited by sodium nitrite when nitrite is heated in the medium where the cells are suspended (57, 58, 63). Perigo et al. (58) reported that sodium nitrite heated in laboratory media was inhibitory against *Clostridium sporogenes*. Perigo and Roberts (57) later reported the same inhibitory effect of heated sodium nitrite against *C. botulinum* and *C. perfringens*. According to these authors, the inhibitory effect of heated sodium nitrite is due to the production of an unknown inhibitory substance during heating. Ashworth and Spencer (4) reproduced the Perigo effect on minced pork, and attributed the safety and stability of canned and cured meats to the Perigo effect.

Sodium nitrite at levels of 200 ppm or below does not inhibit *C. perfringens* growth without prior heating (29, 60). Riha and Solberg (60) reported that higher concentrations of sodium nitrite were required to inhibit growth of *C. perfringens*

without prior heating (800-1500 ppm) compared to autoclaved medium (pH 7.2) containing sodium nitrite at 25-50 ppm. Gough and Alford (29) found that *C. perfringens* spores were able to survive up to 48 days in curing brine solutions (containing 200 ppm of sodium nitrite) stored at 3°C.

Challenge studies

By preventing growth of *C. perfringens* during the cooling of cooked meat products, the requirement of outgrowth inhibition of other pathogenic sporeforming organisms such as *C. botulinum* and *Bacillus cereus*, can be satisfactorily met (24, 45, 46).

The FSIS requires that commercial establishments producing any ready-to-eat meat or poultry product meet approved chilling rates (90, 91). FSIS stabilization guidelines state that cooling of cooked uncured meat and poultry products from 54.4°C to 26.7°C should take place within 1.5 h and cooling from 26.7°C to 4.4°C should take no more than 5 h. Cured meat and poultry products should be cooled from 54.4°C to 26.7°C within 5 h, and from 26.7 to 7.2°C within 10 h.

Deviations of proposed chilling guidelines or customized chilling rates should be validated to guarantee the safety of such products. The establishment should demonstrate that no more than 1 log increase of *C. perfringens* occurred in the finished product during the customized or alternative stabilization treatment.

Numerous challenge studies for growth of *C. perfringens* during cooling of cooked meat products have been reported in beef (40, 45, 65, 66, 69, 71, 79-81, 98), pork (40, 79, 80, 93, 98) and poultry (40, 48, 75, 79, 92) systems.

Juneja et al. (45) reported that *C. perfringens* was able to grow by 4-5 log CFU/g in cooked ground beef when the cooling time from 54.4 to 7.2°C at exponential rate was greater than 18 h. In the same study, the authors reported that when the cooling process was completed within 15 h or less, *C. perfringens* counts did not increase by ≥ 1 log CFU/g (45).

Predictive bacterial growth models have been developed to describe the germination and outgrowth of *C. perfringens* during cooling by using isothermal and non-isothermal growth data (42, 47, 66, 73). However, growth models are not always appropriate to predict *C. perfringens* growth during cooling (72).

In all of the previous experiments, the increase of *C. perfringens* counts during chilling was dependent on the inoculum concentration, cooking process, cooling rate, and concentration of antimicrobials (salt, nitrite, organic acids salts, and spices). However, these studies have not considered the overall impact of the thermal process used in commercial establishments.

Shigehisa et al. (69), studied the survival and outgrowth of spores and vegetative cells of *C. perfringens* in roast beef cooked from 20 to 60°C at different heating rates (7 to 25 °C/h) and chilled from 60 to 15°C at linear chilling rates of 5 to 25°C/h. The natural chilling of foods does not occur in a linear fashion, the chilling has a exponential

behavior and the length of the chilling treatment depends upon the size and shape of food and the temperature of the cooling chamber (22).

While in several challenge studies the growth of *C. perfringens* during cooling temperatures has been addressed by comparing counts at the beginning and the end of the cooling process, no data have been reported on the behavior of *C. perfringens* spores along the cooking and cooling process of cured meats. An evaluation of the behavior of *C. perfringens* spores exposed to different cooking treatments, as well as during cooling of cured meat products would be helpful to evaluate the potential of such spores to survive the cooking process and grow during abusive cooling.

MATERIALS AND METHODS

Bacterial cultures

Three strains of *C. perfringens* (ATCC[®] 12916 [NCTC 8238], ATCC[®] 12917 [NCTC 8239], and ATCC[®] 14809 [NCTC 10239] obtained from the American Type Culture Collection (ATCC[®], Manassas, VA) were used in this study. Lyophilized cultures were revived by initially adding 0.3 ml of cooked meat medium (CMM, Difco[™] BD Diagnostic Systems, Sparks, MD) to each freeze-dried culture and mixing. The resuspended mixture was then transferred into 10 ml CMM. The hydrated cultures were incubated at 37°C for 24 h in an anaerobic jar (GasPak[™] BD Diagnostic Systems). An aliquot of each of the incubated CMM was streaked on blood agar (Trypticase[™] Soy Agar w/5% sheep blood BD Diagnostic Systems) and incubated at 37°C for 24 h in an anaerobic jar. Isolated colonies of each strain were transferred into cryocare vials (Key Scientific Products, Round Rock, TX) and stored at -80°C. Stock working cultures were prepared monthly by transferring one bead from each frozen vial to CMM and incubating at 37°C for 24 h under anaerobic conditions as described above.

Spore suspension preparation

Spore suspensions were separately prepared from each CMM stock working culture according to the method reported by Juneja et al. (41). A 0.1 ml aliquot of the stock culture was transferred into 10 ml of freshly steamed fluid thioglycollate medium (FTG, Difco[™]). Stock cultures were heat shocked by immersing inoculated FTG tubes

in a circulating ethylene glycol bath set at 75°C (Polyscience[®], Model 7305, Niles, IL) for 20 min, removed, chilled immediately in an ice water bath, and then incubated at 37°C (ATCC[®] 12917 and ATCC[®] 14809) and 42°C (ATCC[®] 12916) for 18h. A 1.0 ml aliquot of each of the incubated FTG tubes was transferred into 10 ml of fresh steamed FTG and incubated at their respective temperatures for 4 h. One-ml aliquots from each of the 4 h incubated tubes were then transferred into 10 ml of freshly steamed FTG medium and incubated for 4 h. The entire contents of each incubated FTG media were transferred into 100 mL of freshly prepared modified Duncan Strong sporulation medium (mDS). The original formulation of Duncan Strong sporulation medium (26) was modified to enhance sporulation by substituting starch with raffinose (52), supplemented with caffeine (41), and adjusting the pH after autoclaving to 7.8 ± 0.1 (33).

Formulation of mDS consisted of the following ingredients per liter: proteose peptone (Difco[™]) 15 g, Bacto[™] yeast extract (BD Diagnostic Systems) 4 g, sodium thioglycollate (Difco Laboratories, Detroit, MI) 1 g, sodium phosphate dibasic heptahydrate (Fisher Scientific, Fair Lawn, NJ) 10 g, raffinose (United States Biochemical Corporation, Cleveland, OH) 4 g, and caffeine (Sigma-Aldrich, Inc., St Louis, MO) 100 mg. All ingredients were dissolved in distilled water and autoclaved at 121°C for 15 min. The pH of the autoclaved medium was adjusted to 7.8 ± 0.1 by adding filter-sterilized 0.66 M sodium carbonate (Sigma-Aldrich) solution.

Inoculated mDS medium was incubated at 37°C (ATCC[®] 12917 and ATCC[®] 14809) and 42°C (ATCC[®] 12916) for 16 h. Cultures were checked for sporulation on a heat fixed smear prepared with 0.01 ml of the incubated mDS medium, spread on a 1

cm² surface, and stained with malachite green (Allied Chemical Corporation, New York, NY) and safranin (BD Diagnostic Systems) solutions using the Schaeffer and Fulton method (68). An estimation of the number of spores per ml was determined by direct microscopic count of the stained smear (27). Incubated mDS containing $\geq 10^6$ spores/ml were transferred into sterile 50 ml polypropylene centrifuge tubes (Falcon[®], BD Labware, Franklin Lakes, NJ), centrifuged, and washed twice with distilled water at 4°C for 15 min using a Jouan centrifuge B4i (Jouan, Inc., Winchester, VA) at 1509 g. The recovered pellets were resuspended with 10 ml of sterile distilled water and spore suspensions were kept at 4°C until use within one month of being prepared.

Enumeration of heat-resistant spores

Tryptose-sulfite-cycloserine agar without egg yolk (TSC) was freshly prepared on each experiment day. Dehydrated Shahidi Ferguson Perfringens (SFP) agar base (Difco[™]) was used as TSC base, prepared according to manufacturer instructions, and autoclaved at 121°C for 15 min. The autoclaved SFP base was supplemented with 20 ml (per 250 ml base) of filtered solution of D-cycloserine (Sigma-Aldrich, 1 g in 200 ml of distilled water). TSC agar was kept at 45°C until use.

Heat-resistant spore counts of each strain were determined for each batch of spores prepared. A 1 ml portion of the spore suspension was diluted with 9 ml of 0.1% peptone water and heated in a circulating ethylene glycol bath set at 75°C (Polyscience[®]) for 20 min to kill vegetative cells and activate heat-resistant spores. Heat-shocked spore suspensions were removed, chilled immediately in an ice water bath, and additional

serial dilutions were made in 0.1% peptone water. Appropriate dilutions were plated on TSC agar using the dual-layer pour plating method (92). Heat-resistant spore counts were made after 24 h incubation of plates at 37°C under anaerobic conditions.

Preparation of *C. perfringens* cocktail

A cocktail of spores from the three *C. perfringens* strains was prepared immediately before use by mixing 2 ml of each spore suspension in sterile polystyrene tubes (15 ml capacity, Falcon[®], BD Labware).

Thermal resistance of *C. perfringens* spores in phosphate buffered saline

Phosphate buffered saline (PBS) solution of pH 7.4 (Calbiochem, EMD Biosciences, Inc., La Jolla, CA) was prepared on each day of the experiment, dispensed in glass screw-cap test tubes in 10 ml portions and autoclaved at 121°C for 15 min. The heat resistance of each *C. perfringens* strain and the cocktail of the three strains were determined. A 0.1 ml aliquot of each spore suspension was diluted in 10 ml of PBS so that the final concentration of *C. perfringens* spores was ca. 5 log/ml. Inoculated PBS tubes were sealed with laboratory film (Parafilm[®], Menasha, WI) and immersed in a circulating ethylene glycol bath set at 75°C (Polyscience[®]). The tubes were allowed to equilibrate and to activate the spores for 20 min in the bath. One tube of each strain was removed after 20 min and immediately chilled in an ice bath to determine the initial inoculum of spores. At successive time intervals (every 8 h for a total of 40 h) one tube from each strain was removed and immediately chilled to determine the survivor number

for each *C. perfringens* strain. Decimal dilutions of each sample were prepared with 0.1% peptone water, plated on TSC agar and incubated under anaerobic conditions as described before.

The experiment was repeated on three different days. The temperature of the samples was monitored by 2 type K thermocouples inserted at the center of 2 uninoculated tubes with PBS. The temperature readings were recorded every 2 min using a 4 channel data logging thermometer (Model 80024, Sper Scientific, Ltd., Scottsdale, AZ). Temperature data were obtained by using TestLink SE 309 software.

Thermal resistance of *C. perfringens* spores in beef gravy

Beef gravy was prepared on each day of experiment according to the formulation reported by Juneja et al. (43). Formulation of beef gravy consisted of the following ingredients per 100 ml: proteose peptone (Difco™) 1.5 g, Bacto™ beef extract (BD Diagnostic Systems) 5 g, Bacto™ yeast extract (BD Diagnostic Systems) 0.5 g, and soluble starch (Matheson Company, Inc., Norwood, OH) 1.7 g. All ingredients were dissolved in distilled water by boiling, dispensed into 10 ml portions in screw-cap glass tubes and autoclaved at 121°C for 15 min. The pH of autoclaved beef gravy was 6.8.

To test the heat resistance of each *C. perfringens* strain, 0.1 ml aliquots of the spore suspension were diluted in 10 ml of beef gravy so that the final concentration of *C. perfringens* spores was ca. 5 log/ml. Inoculated beef gravy tubes were sealed with laboratory film (Parafilm®) and immersed in an ethylene glycol bath set at 75°C (Polyscience®). The tubes were allowed to equilibrate and to activate the spores for 20

min in the bath. One tube of each strain was removed after 20 min and immediately chilled in an ice bath to determine the initial inoculum of spores. At successive time intervals (every 8 h for a total of 48 h) one tube from each strain was removed and immediately chilled to determine survivor number of *C. perfringens*. Decimal dilutions of each sample were prepared with 0.1% peptone water, plated on TSC agar and incubated under anaerobic conditions as described before.

The experiment was repeated on three different days. The temperature of the samples was monitored by 2 type K thermocouples inserted at the center of 2 uninoculated beef gravy tubes. The temperature readings were recorded as described previously.

Thermal resistance of *C. perfringens* spores in ground pork

A raw bone-in ham was obtained from the E. M. "Manny" Rosenthal Meat Science and Technology Center (Texas A&M University, College Station, TX). The ham was transported in an insulated container to the laboratory located in an adjacent building and trimmed free of skin, fat, bone and connective tissue. A meat grinder (Model 4612, Hobart Corporation, Froy, OH) was used to grind the ham. Prior to using, the components of the grinder (feed tray, stuffing tube, auger, grinder plate, and blade) were individually wrapped in foil and autoclaved at 121°C for 15 min. The ham was ground using a ¼-inch plate, distributed in 300 g portions into sterile bags (100 oz capacity, 4.0 mil/0.102 thick, Fisher Scientific, Pittsburgh, PA), compressed on a flat

surface to achieve a thin layer (5 mm thick), packages were heat sealed, and stored at -80°C until subsequent use.

Frozen packages were thawed by submerging a 300 g package in a plastic tub with water at 25°C for 30 min before use. The thawed packaged was dried using paper towels and sanitized with 70% isopropyl alcohol presaturated wipes (SATWipes[®], Contec, Inc., Spartanburg, SC). The bag with the thawed sample was aseptically opened and four 50 g portions were transferred into autoclaved plastic beakers (600 ml capacity, Nalgene[®], Fisher Scientific). Each 50 g portion was inoculated with 1.0 ml of the spore suspension of each strain and the cocktail so the final concentration of *C. perfringens* spores was ca. 5 log/g, and hand mixed for 2 min using a sterile spatula (1 min clockwise and 1 min counterclockwise). Inoculated ground pork was distributed in 3 g portions into sterile screw-cap polystyrene tubes (15 ml capacity, Falcon[®], BD Labware), capped, and sealed with laboratory film (Parafilm[®]). Inoculated ground pork tubes were immersed in a circulating ethylene glycol bath set at 75°C (Polyscience[®]). The tubes were allowed to equilibrate and to activate the spores for 20 min in the bath. One tube of each strain was removed after 20 min and immediately chilled in an ice bath to determine the initial spore inoculum concentration. At successive time intervals (every 8 h for a total of 48 h) one tube from each strain was removed and immediately chilled to determine survivor number of *C. perfringens*.

The content of each tube was removed using sterile forceps and placed in filtered stomacher bags (Seward Ltd, West Sussex, UK), weighed and diluted with 9 times the weight of the sample with 0.1% peptone water. The samples were pummeled for 2 min

in a Stomacher Lab Blender 400 (Model BA6021, A. J. Seward, London, UK), plated on TSC agar, and incubated under anaerobic conditions as described before.

The experiment was repeated on three different days. The temperature of the samples was monitored by two type K thermocouples inserted at the center of two uninoculated tubes with ground pork. The temperature readings were recorded as described before.

To verify the absence of native *C. perfringens* cells and spores on the ground pork used on each day of experiment, a 10 g portion of uninoculated ground pork sample was diluted with 90 ml of 0.1% peptone water, plated on TSC, and incubated as described before. The pH of uninoculated ground pork was determined on each sampling day in a 1:1 mixture of ground pork:deionized water prior to heating with a ThermoOrion pH meter (model 250A, Orion Research, Inc., Boston, MA). The water activity (a_w) was determined on a representative sample of uninoculated ground pork with an Aqualab a_w meter (model series 3, Decagon Devices, Inc., Pullman, WA).

Thermal resistance of *C. perfringens* spores in cured ground pork

A raw cured bone-in ham was obtained from the E. M. "Manny" Rosenthal Meat Science and Technology Center (Texas A&M University). The ham was cured with a 20% pump of a brine solution (10% salt, 10% sucrose, 2.5% sodium tripolyphosphate, 0.27% sodium erythorbate, 1.6% Prague powder). The cured ham was transported in an insulated container to the laboratory located in an adjacent building and trimmed free of skin, fat, bone and connective tissue. A meat grinder (Hobart Corporation) was used to

grind the ham. Prior to using, the components of the grinder (feed tray, stuffing tube, auger, grinder plate, and blade) were individually wrapped in foil and autoclaved at 121°C for 15 min. The cured ham was ground using a ¼-inch plate, distributed in 300 g portions in sterile bags (Fisher Scientific), compressed on a flat surface to achieve a thin layer (5 mm thick), packages were heat sealed and stored at -80°C until subsequent use.

Frozen packages were thawed by submerging a 300 g package in a plastic tub with water at 25°C for 30 min before use. The thawed packaged was dried using paper towels and sanitized with 70% isopropyl alcohol presaturated wipes (Contec, Inc.) The bag with the thawed sample was aseptically opened and four 50 g portions were transferred into autoclaved plastic beakers (Fisher Scientific). Each 50 g portion was inoculated with 1.0 ml of the spore suspension from each strain and the cocktail with the final concentration of *C. perfringens* spores at ca. 5 log/g, and hand mixed for 2 min using a sterile spatula (1 min clockwise and 1 min counterclockwise). Inoculated cured ground pork was distributed in 3 g portions into sterile screw-cap polystyrene tubes (Falcon[®]), capped, and sealed with laboratory film (Parafilm[®]). Inoculated cured ground pork tubes were immersed in a circulating ethylene glycol bath set at 75°C (Polyscience[®]). The tubes were allowed to equilibrate and to activate the spores for 20 min in the bath. One tube of each strain was removed after 20 min and immediately chilled in an ice bath to determine the initial inoculum level of spores. At successive time intervals (every 8 h for a total of 48 h) one tube from each strain was removed and immediately chilled to determine survivor number of *C. perfringens*.

The content of each tube was removed using sterile forceps and placed on filtered stomacher bags (Seward Ltd), weighed and diluted with 9 times the weight of the sample with 0.1% peptone water. The samples were pummeled for 2 min in a Stomacher (Seward), plated on TSC agar and incubated under anaerobic conditions as described before. The experiment was repeated on three different days.

The temperature of the samples was monitored by 2 type K thermocouples inserted at the center of 2 uninoculated tubes with cured ground pork. The temperature readings were recorded as described before.

To verify the absence of native *C. perfringens* cells and spores on the cured ground pork used on each day of experiment, a 10 g portion of uninoculated cured ground pork sample was diluted with 90 ml of 0.1% peptone water, plated on TSC and incubated as described before. The pH of uninoculated cured ground pork was determined each sampling day in a 1:1 mixture of cured ground pork:deionized water prior to heating with a ThermoOrion pH meter (Orion Research, Inc.). The a_w was determined on a representative sample of uninoculated cured ground pork with an Aqualab a_w meter (Decagon Devices, Inc.). A representative sample of cured ground pork was analyzed for nitrite content as described in method 973.31 of Association of Official Analytical Chemists (5).

Thermal resistance of *C. perfringens* following heat shock

A 5 ml aliquot of each spore suspension (ATCC[®] 12916, 12917, and 14809) was heat shocked in a circulating ethylene glycol bath set at 75°C for 20 min. After heat

shock, the tubes containing the spore suspension were removed from the bath and immediately chilled in an ice bath. Equal amounts of each heat shocked spore suspension were used to prepare a cocktail of heat shocked *C. perfringens* spores.

Heat shocked spore suspensions of individual strains and the cocktail were used to inoculate PBS as described before. One tube of each inoculated strain and the cocktail were used to determine the initial inoculum of spores without any further heat treatment. Another set of inoculated PBS tubes was immersed in a circulating ethylene glycol bath set at 75°C (Polyscience®). One tube of each strain and the cocktail were sampled at successive time intervals (every 8 h for a total of 48 h) and immediately chilled to determine survivor number of *C. perfringens* as described before.

The thermal resistance of heat shocked *C. perfringens* spores was also determined on beef gravy, ground pork, and cured ground pork. Each heating media was inoculated with heat shocked spores as described before and immersed in an ethylene glycol bath set at 75°C. Beef gravy and ground pork samples were removed from the 75°C bath every 8 h for a total of 48 h, and cured ground pork samples were removed every 1.5 h for a total of 9 h. At each sampling time, samples were immediately chilled to determine survivor number of *C. perfringens* as described before.

The experiment on each heating media was repeated on three different days and the temperature of the samples was monitored as described before.

Calculation of D₇₅-values

D₇₅-values were determined by plotting the log counts of survivor *C. perfringens* as a function of time (hours). For linear curves, the reciprocal of the slope obtained by linear regression analysis (67) was used to determine D₇₅-value. For non-linear curves, two D₇₅-values were obtained using non-linear regression analysis (67).

Effect of the heating conditions on the survival and germination of *C. perfringens* spores in cured ground pork

A 150 g portion of raw cured ground pork, obtained as described before, was inoculated with 3 ml of cocktail of spores from the 3 *C. perfringens* strains. The final concentration of *C. perfringens* spores was ca. 5 log/g. Inoculated cured ground pork was distributed in 3 g portions into sterile screw-cap polystyrene tubes (Falcon[®]), capped, and sealed with laboratory film (Parafilm[®]). Inoculated cured ground pork tubes were immersed in a programmable circulating water bath set at 20°C (VWR, Model 1187 P, Polyscience[®], Niles, IL). The inoculated cured pork tubes were allowed to equilibrate for 15 min, and two tubes were removed from the bath, and immediately chilled in an ice bath to determine the initial spore content. After equilibration of the inoculated cured ground pork at 20 °C, the temperature of the water bath was increased in a linear fashion at a rate of 4°C/h, 8°C and 12°C/h until the temperature of the water bath reached 75°C. At successive temperature intervals (every 4°C) two tubes were removed from the water bath and immediately chilled in an ice bath to enumerate total *C. perfringens* counts and heat resistant spores of *C. perfringens*.

Total *C. perfringens* counts were determined from the content of the two tubes placed in a filtered stomacher bag, diluted with 0.1 % peptone water and plated on TSC agar as described before. Enumeration of heat resistant spores was determined by heating 10 ml aliquots of the diluted sample in a circulating ethylene glycol bath set at 75°C for 20 min, followed by immediately chilling in an ice bath. The heated samples were plated on TSC agar and incubated under anaerobic conditions as described before. D_{75} -values were determined for each heating rate. After the temperature of the inoculated cured pork in the tubes reached 75°C, the tubes were transferred to an ethylene glycol bath set at 75°C, held at 75°C and sampled every 8 h for a total of 48 h to determine D_{75} -values as described before.

The experiment was repeated on three different days. The temperature of the samples was monitored by 2 type K thermocouples inserted at the center of 2 uninoculated tubes with cured ground pork. The temperature readings were recorded as described previously. The absence of native *C. perfringens* cells and spores, and the pH of the cured ground pork used on each day of experiment were determined as described previously.

Germination and outgrowth of surviving *C. perfringens* spores following a slow cooking process and chilled under extended cooling treatment

Thawed 300 g portions of raw ground pork and raw cured ground pork obtained as described previously were inoculated with 5 ml of *C. perfringens* spore cocktail to a final concentration of 5 log of spores per g. Inoculated ground pork (cured and non-

cured) was distributed into 3 g portions into sterile screw-cap polystyrene tubes (Falcon[®]), capped, and sealed with laboratory film (Parafilm[®]).

Inoculated pork tubes were immersed in a programmable circulating water bath set at 20°C (VWR) and allowed to stabilize, followed by heating from 20 to 71°C at a temperature increase rate of 4°C/h. After cooking to 71°C, the temperature of the bath was programmed to cool exponentially from 71°C to 54.4 °C in 3 h and from 54.4 to 7.2°C in 20 h. The times and temperatures computed were obtained from the equation derived by Sabah et al. (65):

$$T = T_{\text{initial}} \exp(k_{\text{cool}}t)$$

where T is the final temperature (°C), T_{initial} is the start temperature, k_{cool} is the cooling rate, and t is the time (h) to reach the desired temperature. In this study, two k_{cool} values were obtained with the use of 71°C as T_{initial} , 54.4°C as T , and 3 h as t for the first k_{cool} value and the use of 54.4°C as T_{initial} , 7.2°C as T , and 20 h as t for the second k_{cool} value. The times and temperature (°C) computed for the extended cooling treatment are shown in Fig.1.

Three inoculated tubes of each type of ground pork (cured and non-cured) were removed from the water bath after equilibration at 20°C to determine inoculum level. After the cooking process, three tubes were taken at every hour during the cooling from 54.4 to 7.2°C. Total counts of *C. perfringens* were determined from the content of the three tubes placed in a filtered stomacher bag, diluted with 0.1 % peptone water and plated on TSC agar as described before. Enumeration of heat resistant spores was also

determined by plating on TSC appropriate dilutions of heated diluted sample (at 75°C for 20 min) as described before.

The experiment was repeated on two different days. The temperature of the samples was monitored by 2 type K thermocouples inserted at the center of 2 uninoculated tubes with cured and non-cured ground pork. The temperature readings were recorded as described before. The absence of native *C. perfringens* cells and spores, and the pH of the cured and non-cured ground pork used on each day of experiment were determined as described before.

Data analysis

Analysis of variance (67) was performed on the data obtained from the thermal resistance and heating rate experiments to compare the effects of heating medium and heating rate on the survival of *C. perfringens* spores. LSM test was used to separate the means with significance level of $\alpha = 0.05$. Mean generation times were determined with the data obtained from the temperature abuse cooling treatment when *C. perfringens* grew rapidly using the equation derived by Willardsen et al. (97).

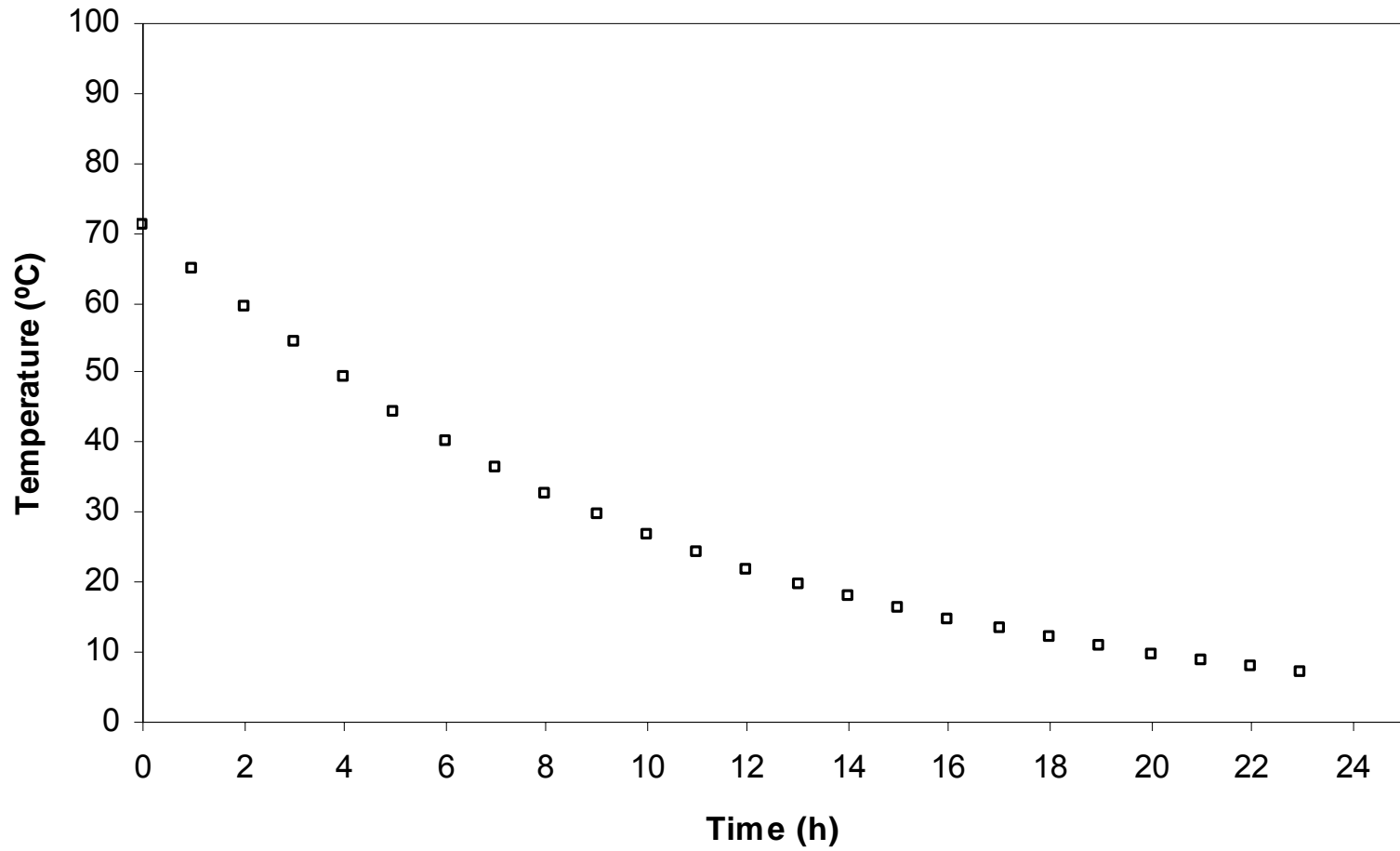


FIGURE 1. *Programmed temperature profile for cooked cured and non-cured ground pork during cooling from 71 to 54.4°C in 3 h and from 54.4°C to 7.2°C in 20 h.*

RESULTS

Characteristics of ground pork and cured ground pork

The counts of native *C. perfringens* vegetative cells and spores were below the detection limit (1 CFU/g) in both raw ground pork and cured ground pork. A_w values of raw ground pork and cured ground pork were 0.993 and 0.973 respectively. pH values of ground pork and cured ground pork ranged between 6.27 to 6.47 and 6.11 to 6.52 respectively. Incoming nitrite content in cured ground pork was 170 ppm.

Thermal resistance

There were significant differences in the heat resistance among different strains of *C. perfringens* as well as the types of heating media. The results of thermal resistance conducted with non-heat shocked spores showed linear survivors curves for each individual strain as well as the cocktail of the three strains in PBS (Fig. 2), beef gravy (Fig. 3), ground pork (Fig. 4) and cured ground pork (Fig. 5) exposed to heating at 75°C. The D_{75} -values of the *C. perfringens* strains individually tested ranged from 22.67 to 41.03 h, 29.03 to 70.80 h, 36.93 to 65.43 h, and 22.7 to 26.33 h in PBS, beef gravy, ground pork and cured ground pork respectively (Table 1). D_{75} -values of the cocktail were 31.57 h, 52.23 h, 38.43 h and 28.13 h in PBS, beef gravy, ground pork and cured ground pork respectively (Table 1). *C. perfringens* ATCC 12916 showed the highest D_{75} -value ($P<0.05$) when the heating medium was PBS, whereas ATCC 14809 showed the highest D_{75} -value ($P<0.05$) when the heating medium was beef gravy or ground pork

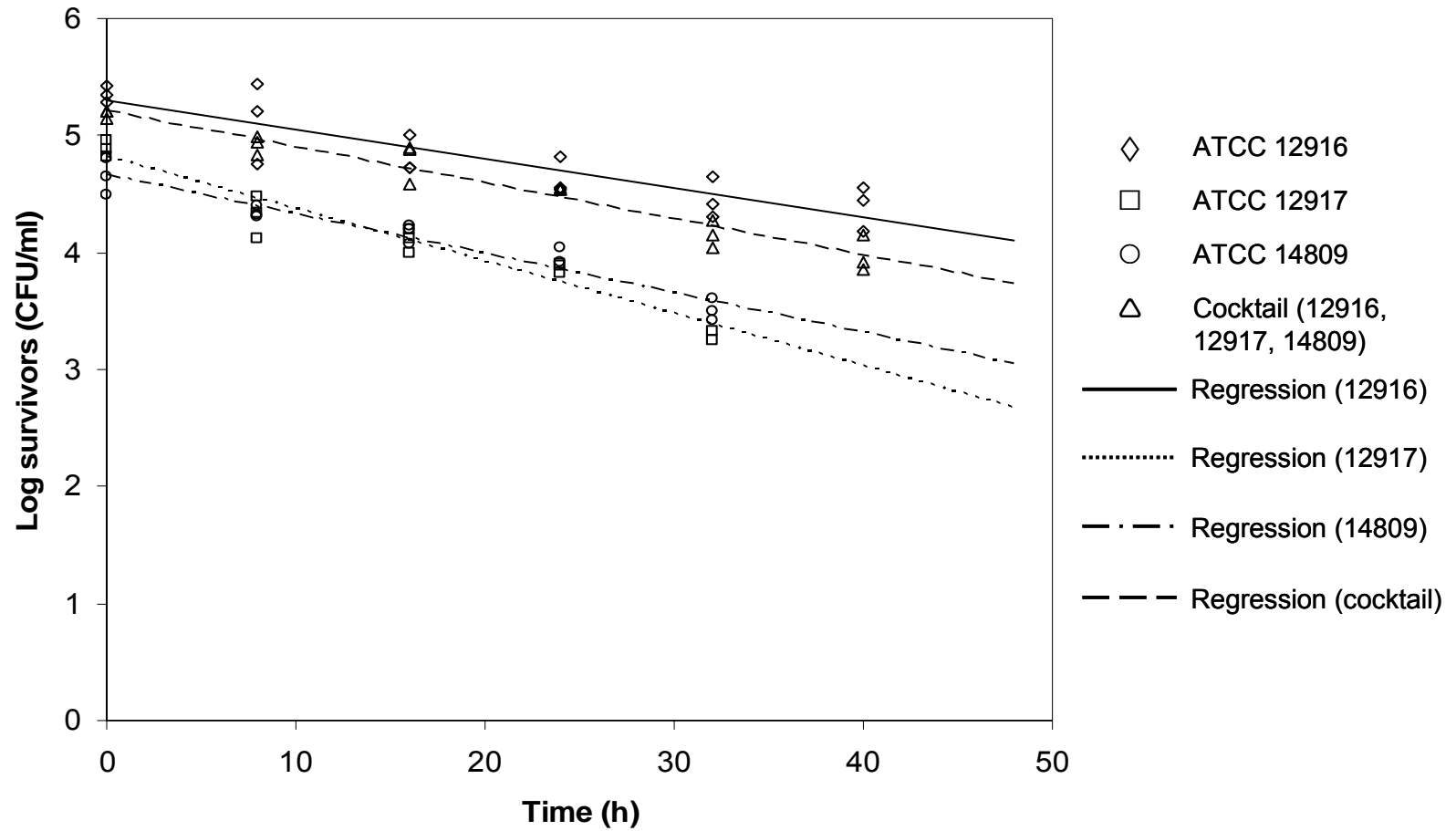


FIGURE 2. Survivor data and regression lines for non-heat shocked *Clostridium perfringens* spores heated at 75°C in phosphate buffered saline solution (pH 7.4) for 40 h.

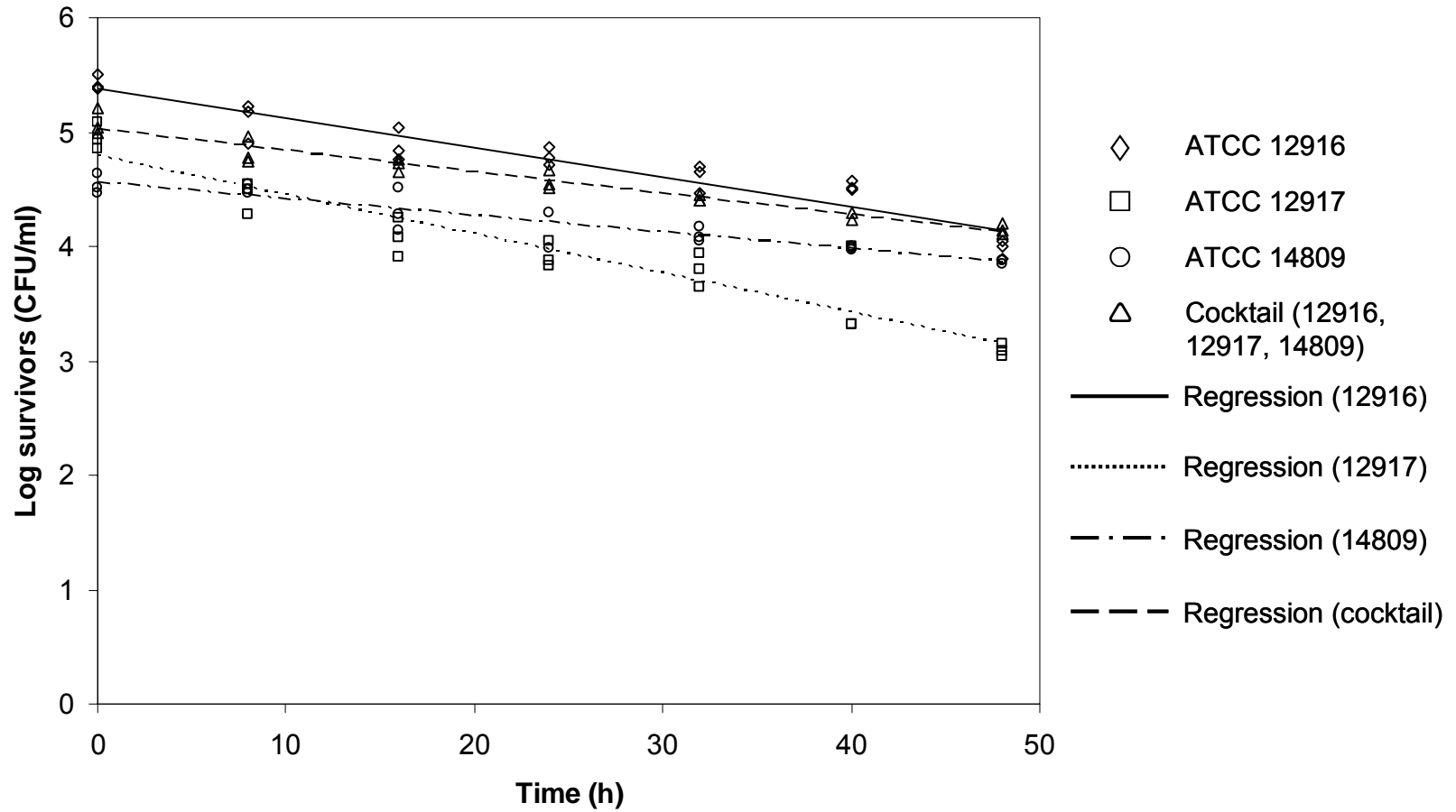


FIGURE 3. Survivor data and regression lines for non-heat shocked *Clostridium perfringens* spores heated at 75°C in beef gravy for 48 h.

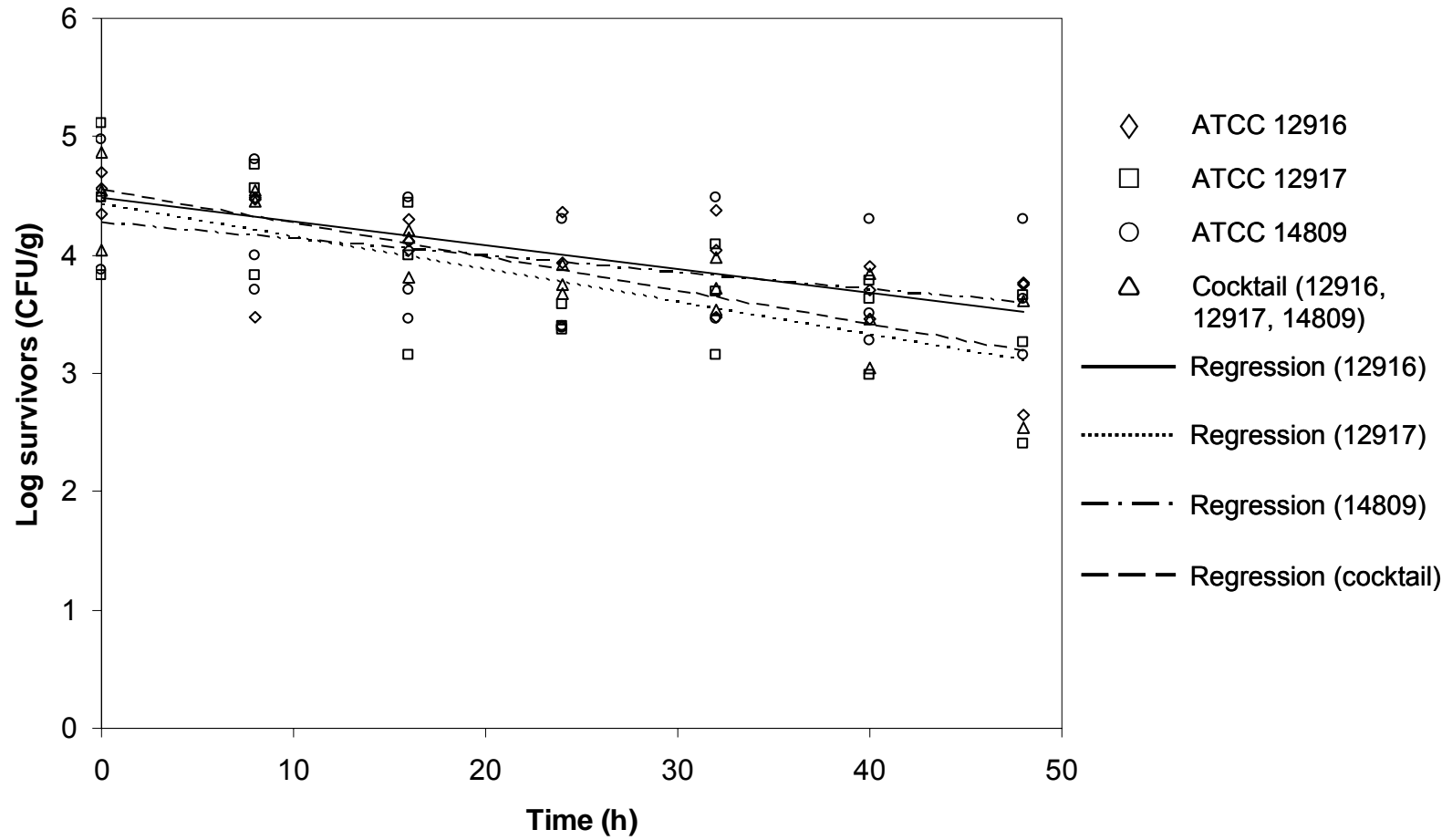


FIGURE 4. Survivor data and regression lines for non-heat shocked *Clostridium perfringens* spores heated at 75°C in ground pork for 48 h.

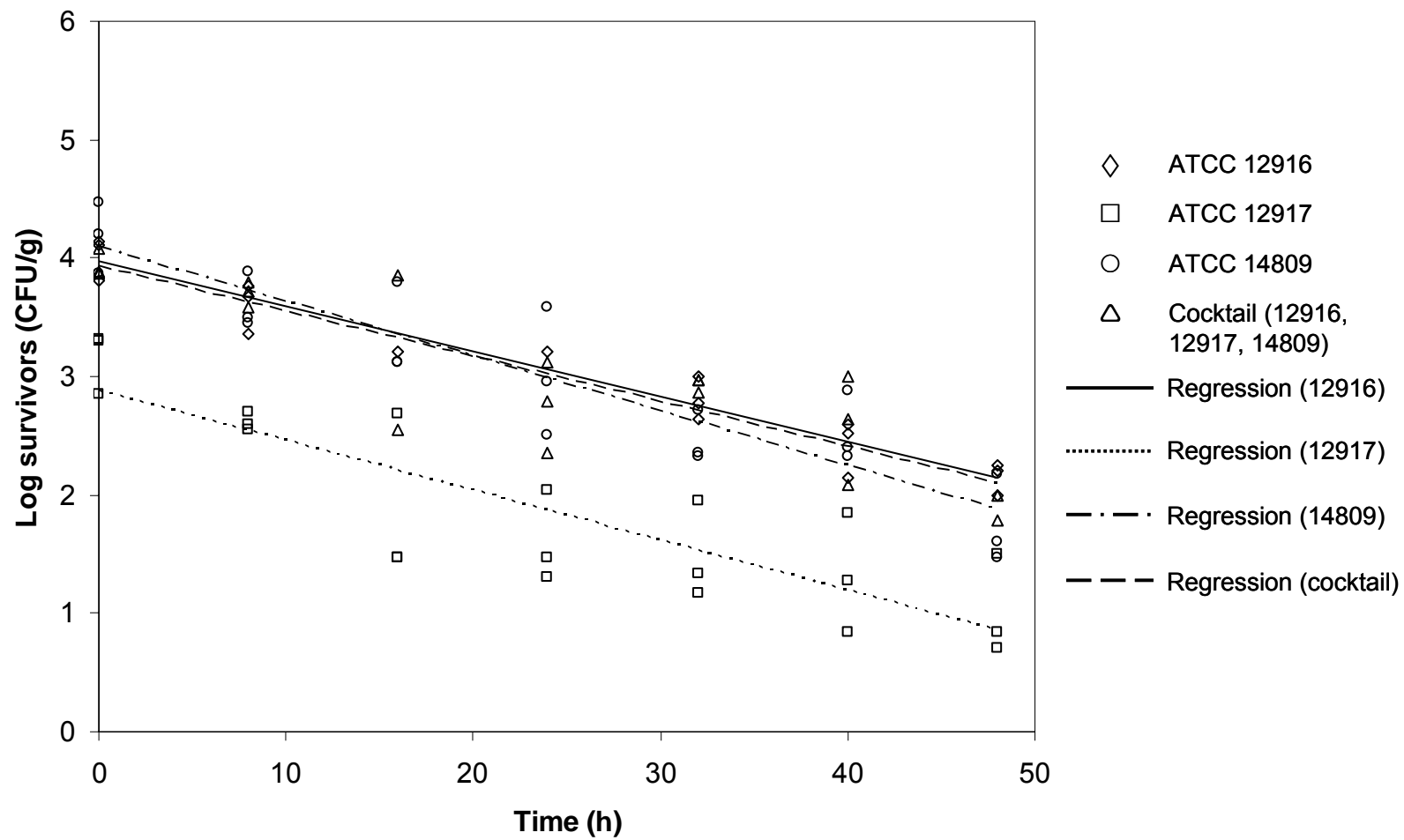


FIGURE 5. Survivor data and regression lines for non-heat shocked *Clostridium perfringens* spores heated at 75°C in cured ground pork for 48 h.

TABLE 1. D_{75} -values (expressed in h) of *Clostridium perfringens* non-heat shocked spores as affected by heating medium and strain.

Strain	D_{75} -value ^a in:			
	Phosphate buffer solution (pH 7.4)	Beef gravy	Ground pork	Cured ground pork
ATCC 12916	41.03 ± 1.87 c ^b y ^c	38.63 ± 0.65 B y	51.20 ± 4.81 B z	26.33 ± 0.58 AB x
ATCC 12917	22.67 ± 2.93 A x	29.03 ± 1.30 A y	36.93 ± 1.56 A z	23.93 ± 2.66 AB x
ATCC 14809	29.50 ± 2.26 B x	70.80 ± 9.00 D y	65.43 ± 6.13 C y	22.70 ± 5.89 A x
COCKTAIL ^d	31.57 ± 2.31 B x	52.23 ± 3.93 C z	38.43 ± 6.07 A y	28.13 ± 4.14 B x

^a D_{75} -value = Decimal reduction time: time in hours to inactivate 90% of the population at 75°C. Values are means of three independent repetitions ± standard deviation.

^bMean values in the same column that are not followed by the same letter (ABC) are significantly different ($P < 0.05$).

^cMean values in the same row that are not followed by the same letter (xyz) are significantly different ($P < 0.05$).

^dCocktail= mixture of three *C. perfringens* strains (ATCC 12916, 12917 and 14809).

(Table 1). In general, higher D_{75} -values were observed in ground pork, whereas the lower thermal resistance was observed in cured ground pork.

The results of thermal resistance conducted with heat shocked spores showed linear survivor curves on each individual strain as well as on the cocktail of the three strains in PBS (Fig. 6), and beef gravy (Fig. 7). Non-linear regression curves were observed on heat shocked spores of individual strains and the cocktail when heated in ground pork (Fig. 8) and cured ground pork (Fig. 9). The D_{75} -values of the heat shocked *C. perfringens* spores individually tested ranged from 14.49 to 47.62 h, and 19.23 to 43.47 h in PBS and beef gravy respectively (Table 2). D_{75} -values of the cocktail were 23.81 h and 27.02 h in PBS and beef gravy respectively (Table 2). *C. perfringens* ATCC 12916 showed the highest D_{75} -value in both heating media (PBS and beef gravy). In ground pork, heat shocked spores of *C. perfringens* showed a rapid death within the first 6 h of heating (D_{75} -values ranged between 4.35 and 5.88 h), and then the thermal resistance increased up to 34.5 to 47.62 h (Table 2).

Effect of cooking rate

The behavior of *C. perfringens* total counts and heat resistant counts at different heating rates are shown in Figures 10-12. Initial total counts of *C. perfringens* were lower than the counts of heat resistant spores. In general, there were not significant differences on the total counts and heat resistant counts of *C. perfringens* inoculated in cured ground beef and heated at 8 or 12°C/h to a final temperature of 72°C (Tables 3 and 4). When the temperature of the cured pork reached 75°C, the total counts of *C.*

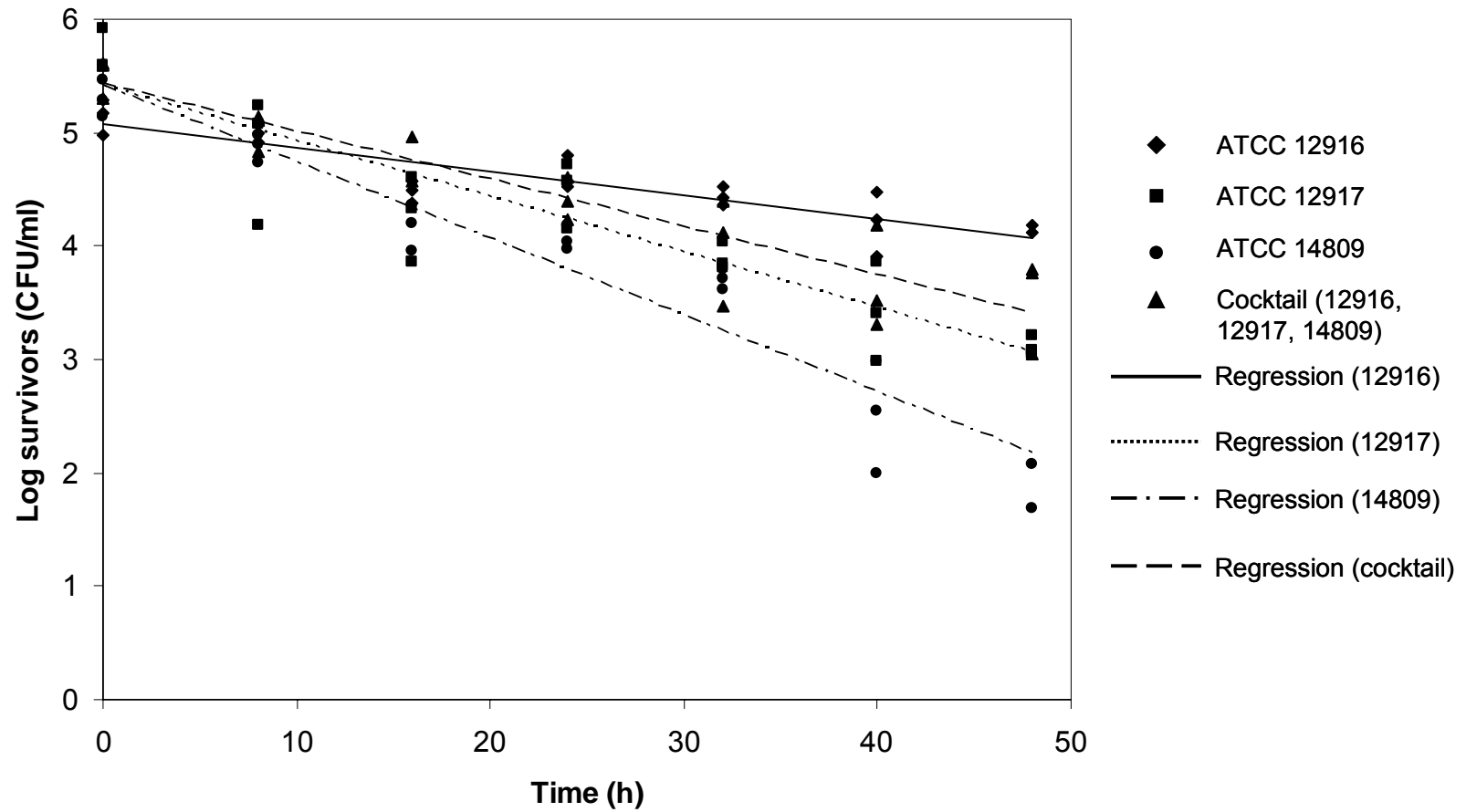


FIGURE 6. Survivor data and regression lines for heat shocked *Clostridium perfringens* spores heated at 75°C in phosphate buffered saline solution (pH 7.4) for 48 h.

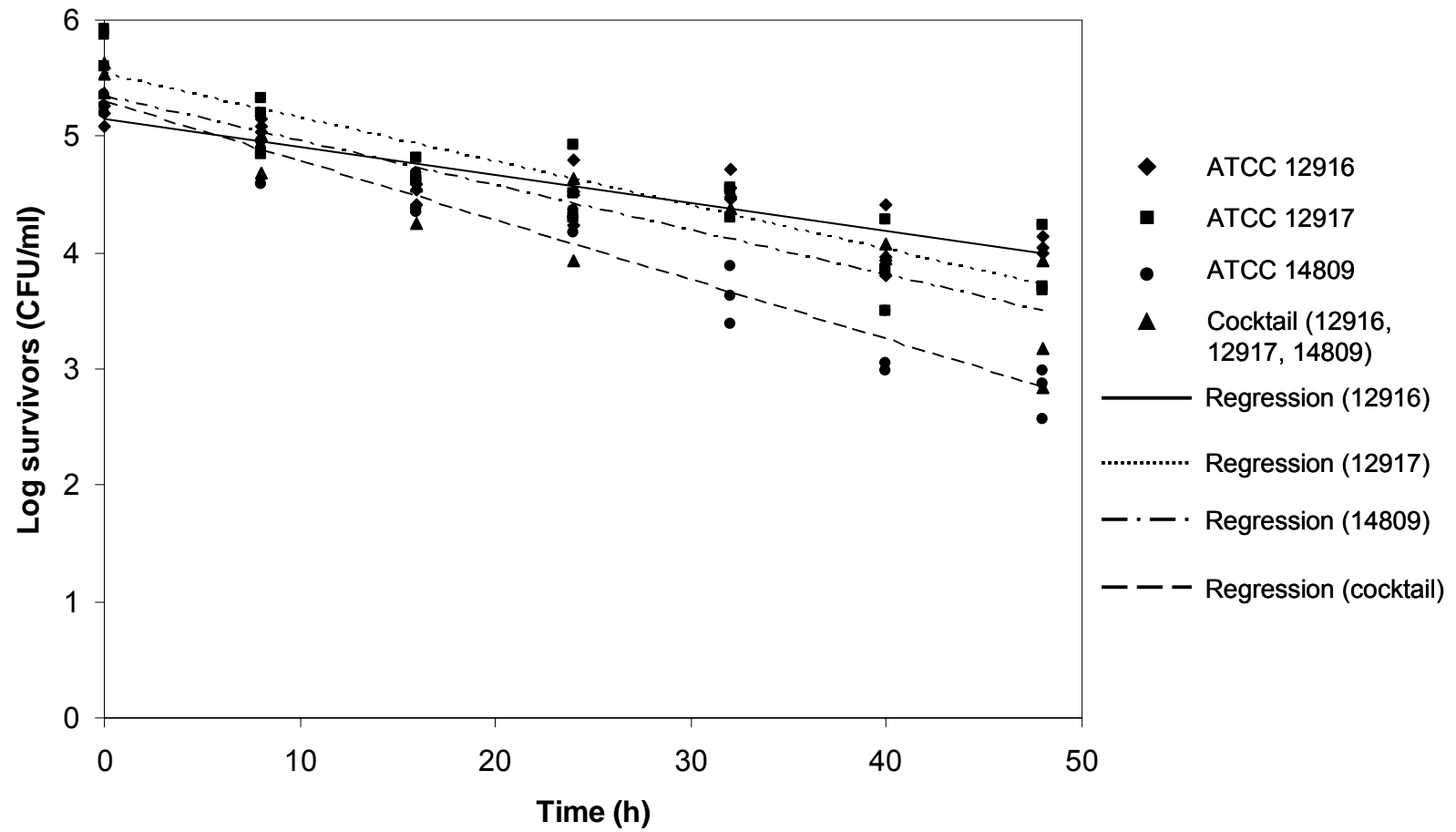


FIGURE 7. Survivor data and regression lines for heat shocked *Clostridium perfringens* spores heated at 75°C in beef gravy for 48 h.

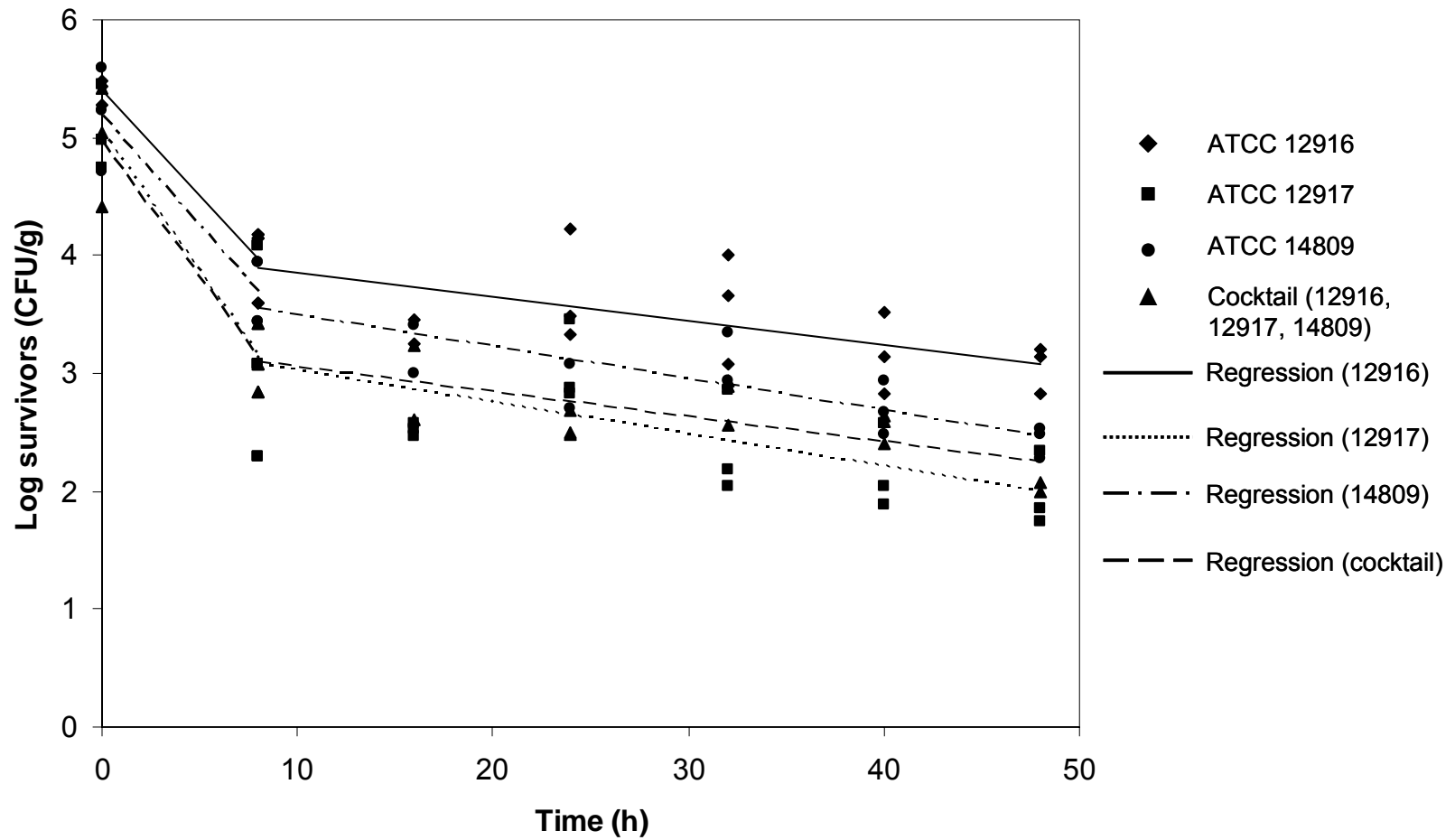


FIGURE 8. Survivor data and regression lines for heat shocked *Clostridium perfringens* spores heated at 75°C in ground pork for 48 h.

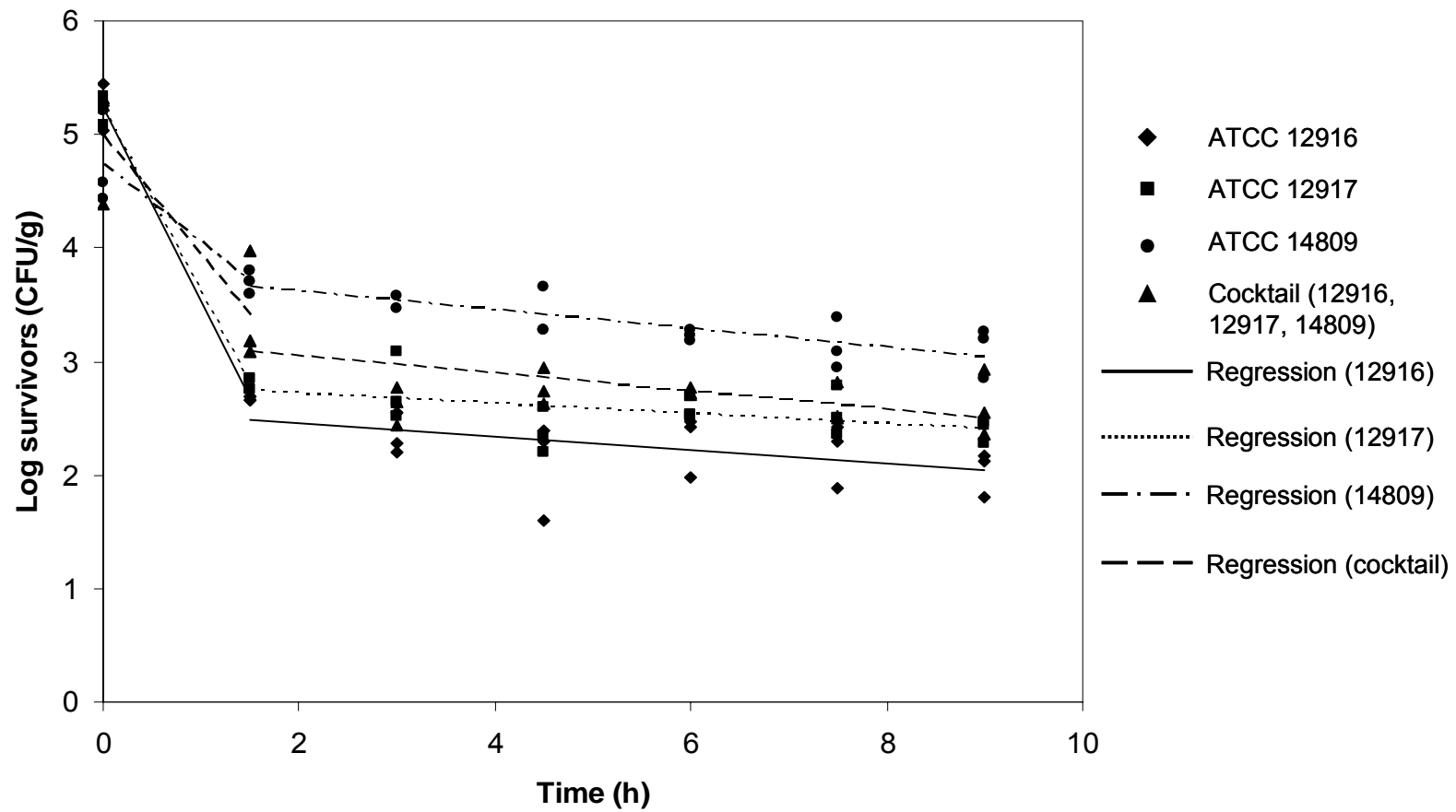


FIGURE 9. Survivor data and regression lines for heat shocked *Clostridium perfringens* spores heated at 75°C in cured ground pork for 9 h.

TABLE 2. D_{75} -values (expressed in h) and survivor data of *Clostridium perfringens* heat shocked spores as affected by heating medium and strain.

Strain	Heating medium	Slope 1	Slope 2	D_1^a	D_2^b
ATCC 12916	PBS	-0.021	- ^c	47.62	-
	Beef gravy	-0.023	-	43.47	-
	Ground pork	-0.170	-0.022	5.88	45.45
	Cured ground pork	-1.680	-0.068	0.59	14.70
ATCC 12917	PBS	-0.049	-	20.37	-
	Beef gravy	-0.037	-	27.02	-
	Ground pork	-0.230	-0.029	4.35	34.50
	Cured ground pork	-1.630	-0.040	0.61	25.00
ATCC 14809	PBS	-0.069	-	14.49	-
	Beef gravy	-0.052	-	19.23	-
	Ground pork	-0.189	-0.027	5.29	37.04
	Cured ground pork	-0.390	-0.073	2.56	13.70
COCKTAIL ^d	PBS	-0.042	-	23.81	-
	Beef gravy	-0.037	-	27.02	-
	Ground pork	-0.229	-0.021	4.37	47.62
	Cured ground pork	-1.040	-0.062	0.96	16.13

^a D_1 = D_{75} -value: time in hours to inactivate 90% of the population at 75°C. In concave survivor curves, the decimal reduction time prior to the intersection point.

^b D_2 = D_{75} -value: time in hours to inactivate 90% of the population at 75°C. In concave survivor curves, the decimal reduction time following the intersection point.

^c"-"=curve is linear with only one slope.

^dCocktail= mixture of three *C. perfringens* strains (ATCC 12916, 12917 and 14809).

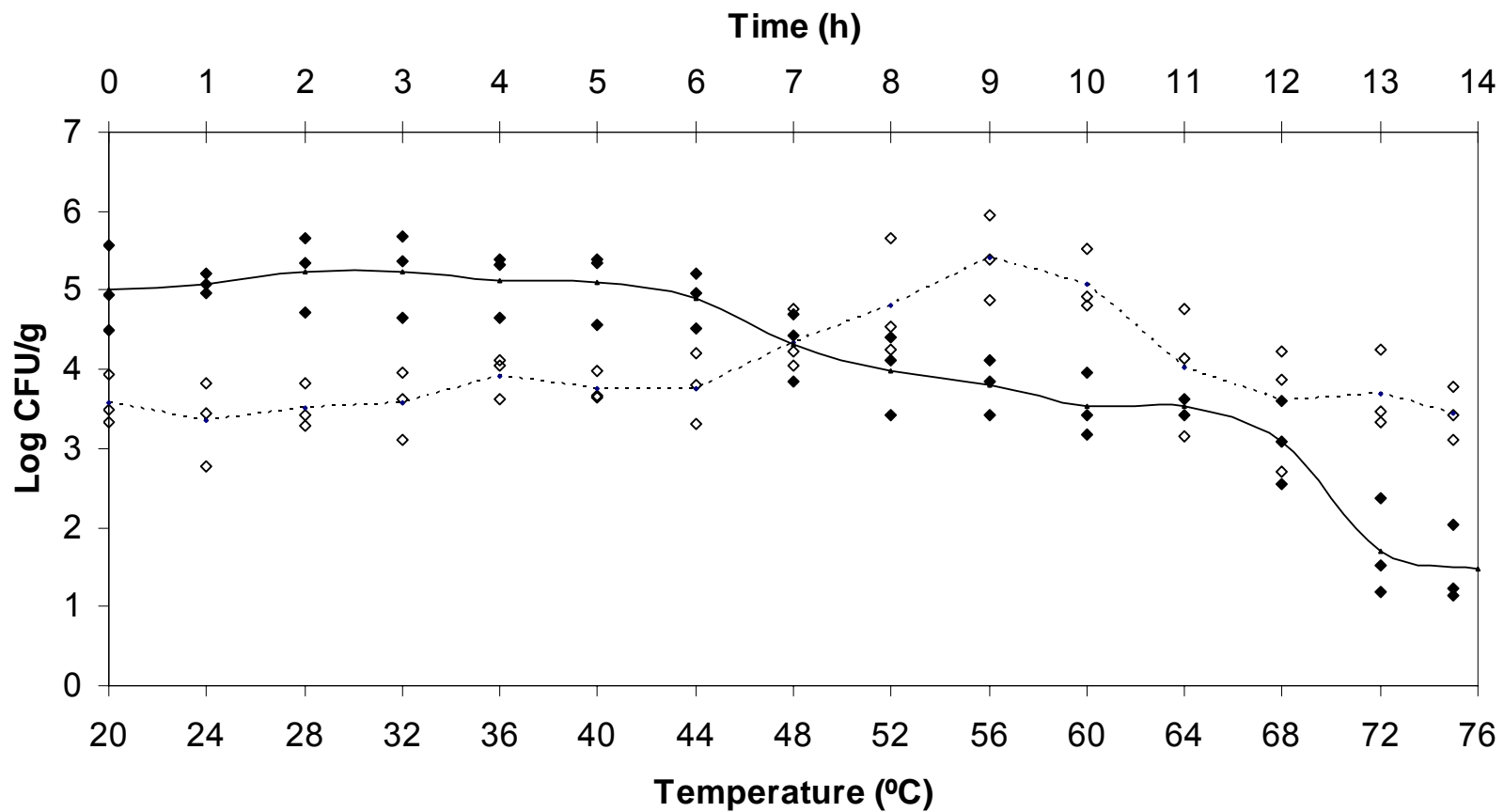


FIGURE 10. Survivor data for total counts (\diamond) and heat resistant spore counts (\blacklozenge) of *Clostridium perfringens* in cured ground pork heated from 20 to 75°C at a rate of 4°C/h. Lines represent the average of three repetitions of total counts (dotted line) and heat resistant spore counts (continuous line) of a three strain cocktail.

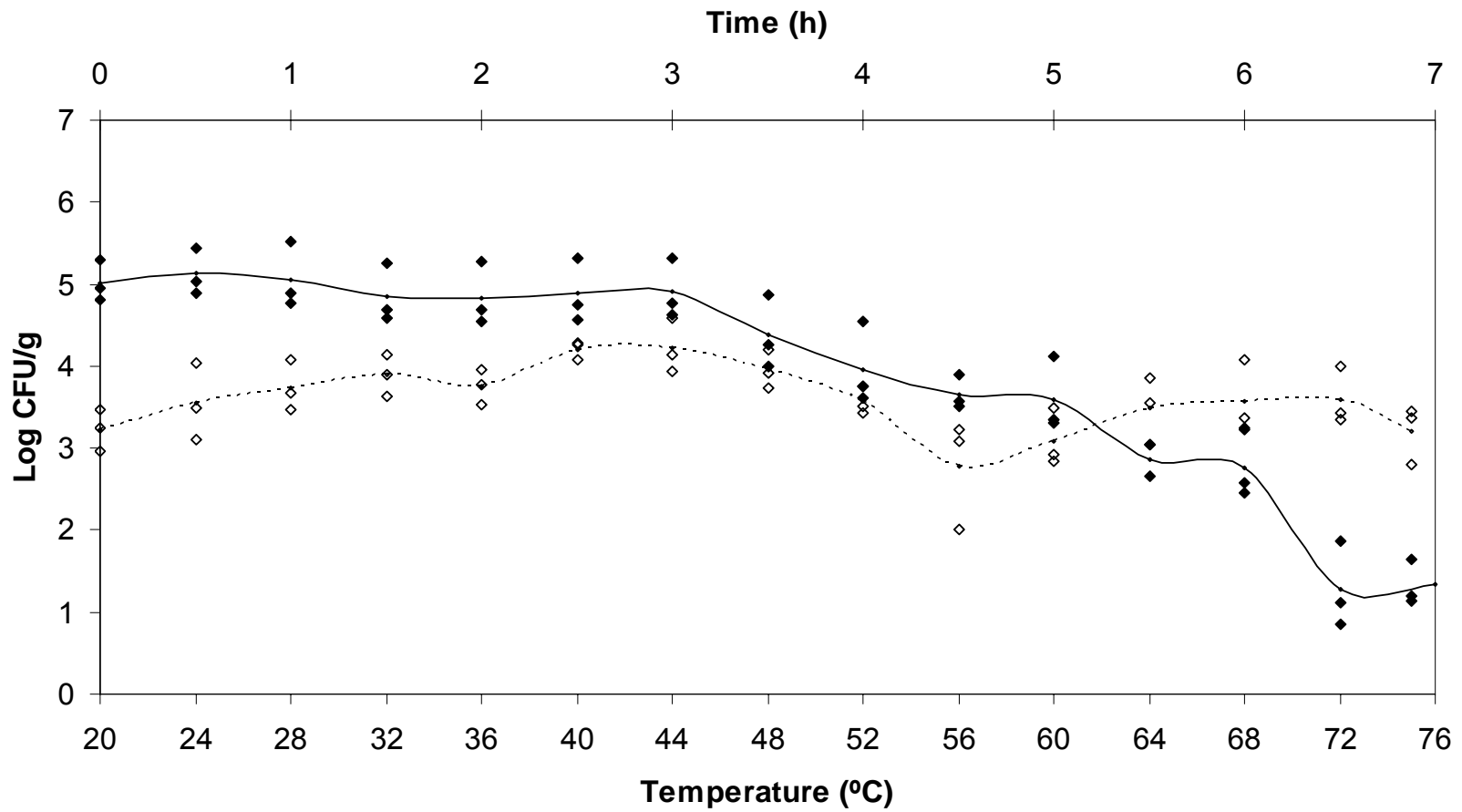


FIGURE 11. Survivor data for total counts (\diamond) and heat resistant spore counts (\blacklozenge) of *Clostridium perfringens* in cured ground pork heated from 20 to 75°C at a rate of 8°C/h. Lines represent the average of three repetitions of total counts (dotted line) and heat resistant spore counts (continuous line) of a three strain cocktail.

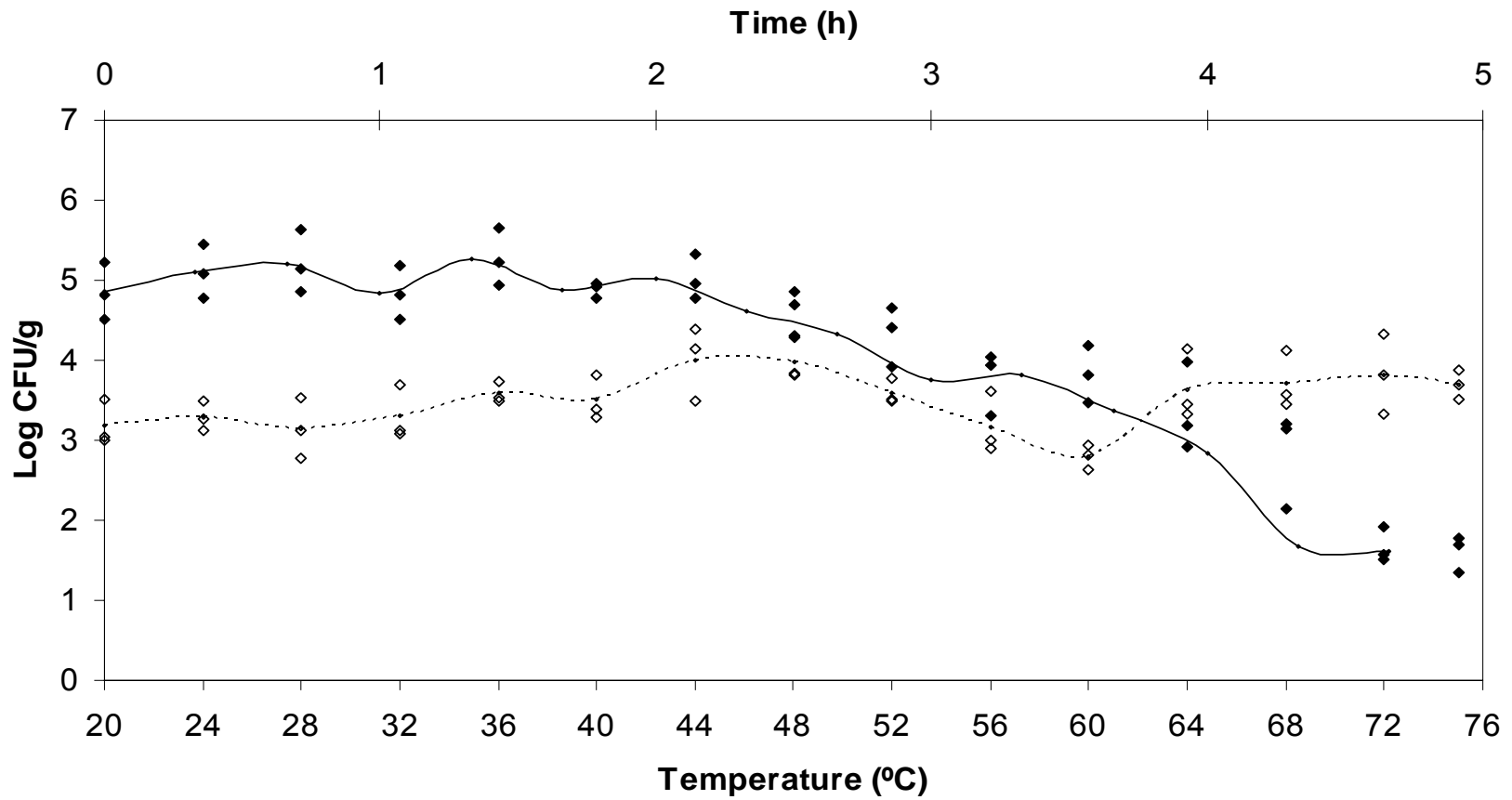


FIGURE 12. Survivor data for total counts (\diamond) and heat resistant spore counts (\blacklozenge) of *Clostridium perfringens* in cured ground pork heated from 20 to 75°C at a rate of 12°C/h. Lines represent the average of three repetitions of total counts (dotted line) and heat resistant spore counts (continuous line) of a three strain cocktail.

TABLE 3. Comparison of total counts of *Clostridium perfringens* (in log CFU/g) during cooking of cured ground pork from 20 to 75°C as affected by sampling temperature and heating rate.

Sampling temperature	Log CFU/g ^a at heating rate of:			
	75°C/20 min	12°C/h	8°C/h	4°C/h
20°C	3.5 ± 0.49 A ^b x ^c	3.2 ± 0.28 AB x	3.3 ± 0.25 ABCD x	3.6 ± 0.31 AB x
24°C	ND ^d	3.3 ± 0.20 ABC x	3.5 ± 0.45 BCDE x	3.3 ± 0.50 A x
28°C	3.8 ± 0.68 A x	3.1 ± 0.35 AB x	3.8 ± 0.31 BCDE x	3.5 ± 0.26 AB x
32°C	ND	3.3 ± 0.35 ABC x	3.9 ± 0.25 CDE x	3.6 ± 0.45 AB x
36°C	3.5 ± 0.53 A x	3.6 ± 0.11 BCD x	3.7 ± 0.21 BCDE x	3.9 ± 0.26 AB x
40°C	ND	3.5 ± 0.26 BCD y	4.2 ± 0.11 E y	3.8 ± 0.17 AB x
44°C	4.0 ± 0.67 A x	4.0 ± 0.46 D x	4.2 ± 0.36 E x	3.8 ± 0.45 AB x
48°C	ND	4.0 ± 0.29 D x	3.9 ± 0.25 DE x	4.3 ± 0.42 BCD x
52°C	3.7 ± 0.67 A x	3.6 ± 0.17 BCD x	3.6 ± 0.21 BCDE x	4.8 ± 0.76 CDE y
56°C	ND	3.2 ± 0.38 AB x	2.8 ± 0.67 A x	5.4 ± 0.55 E y
60°C	3.5 ± 0.47 A y	2.8 ± 0.15 A x	3.1 ± 0.38 AB xy	5.1 ± 0.38 DE z
64°C	ND	3.6 ± 0.44 BCD x	3.5 ± 0.45 BCD x	4.0 ± 0.85 ABC x
68°C	3.8 ± 0.57 A x	3.7 ± 0.36 BCD x	3.6 ± 0.44 BCDE x	3.6 ± 0.79 AB x
72°C	ND	3.8 ± 0.50 CD x	3.6 ± 0.38 BCDE x	3.7 ± 0.53 AB x
75°C	4.2 ± 0.52 A x	3.7 ± 0.20 BCD xy	3.2 ± 0.35 ABC y	3.4 ± 0.35 AB y

^aLog CFU/g represent mean values ± standard deviation of three independent experiments.

^bMean values in the same column that are not followed by the same letter (ABC) are significantly different ($P < 0.05$).

^cMean values in the same row that are not followed by the same letter (xyz) are significantly different ($P < 0.05$).

^dND= Not determined.

TABLE 4. Comparison of heat resistant spore counts of *Clostridium perfringens* (in log CFU/g) during cooking of cured ground pork from 20 to 75°C as affected by sampling temperature and heating rate.

Sampling temperature	Log CFU/g ^a at heating rate of:			
	75°C/20 min	12°C/h	8°C/h	4°C/h
20°C	5.0 ± 0.40 A ^b x ^c	4.8 ± 0.35 AB x	5.0 ± 0.25 A x	5.0 ± 0.56 AB x
24°C	ND ^d	5.1 ± 0.30 A x	5.1 ± 0.26 A x	5.1 ± 0.10 AB x
28°C	5.1 ± 0.38 A x	5.2 ± 0.36 A x	5.1 ± 0.38 A x	5.2 ± 0.50 A x
32°C	ND	4.8 ± 0.35 AB x	4.9 ± 0.38 A x	5.2 ± 0.57 A x
36°C	5.1 ± 0.42 A x	5.3 ± 0.40 A x	4.8 ± 0.42 A x	5.1 ± 0.38 AB x
40°C	ND	4.9 ± 0.10 AB x	4.9 ± 0.36 A x	5.1 ± 0.44 AB x
44°C	5.3 ± 0.58 A x	5.0 ± 0.25 A x	4.9 ± 0.36 A x	4.9 ± 0.26 AB x
48°C	ND	4.6 ± 0.31 AB x	4.4 ± 0.46 AB x	4.3 ± 0.46 BC x
52°C	4.8 ± 0.44 A x	4.3 ± 0.40 BC x	3.9 ± 0.49 BC x	4.0 ± 0.51 C x
56°C	ND	3.7 ± 0.38 CD x	3.7 ± 0.21 C x	3.8 ± 0.36 CD x
60°C	4.5 ± 0.64 A y	3.8 ± 0.35 CD x	3.6 ± 0.46 C x	3.5 ± 0.42 CD x
64°C	ND	3.4 ± 0.57 DE x	2.9 ± 0.21 D x	3.5 ± 0.14 CD x
68°C	3.6 ± 0.55 B x	2.8 ± 0.61 E x	2.8 ± 0.38 D x	3.1 ± 0.50 D x
72°C	ND	1.7 ± 0.21 F x	1.3 ± 0.57 E x	1.7 ± 0.62 E x
75°C	2.6 ± 0.49 C y	1.6 ± 0.26 F x	1.3 ± 0.32 E x	1.4 ± 0.49 E x

^aLog CFU/g represent mean values ± standard deviation of three independent experiments.

^bMean values in the same column that are not followed by the same letter (ABC) are significantly different ($P < 0.05$).

^cMean values in the same row that are not followed by the same letter (xyz) are significantly different ($P < 0.05$).

^dND= Not determined.

perfringens heated at a rate of 12°C/h were significantly higher than the counts of the inoculated product heated at 8°C/h (Table 3). Heat resistant spore counts did not differ significantly during heating either at 8 or 12°C/h (Table 4).

At a slow heating rate (4°C/h) heat resistant spore counts did not differ from the counts obtained by heating at 8 or 12°C/h. Conversely, total counts of *C. perfringens* increased by 1.2 log CFU/g when the temperature of the product reached 52°C (Table 4). Activation of spores occurred at 52°C (Table 4) when the inoculated meat was heated at 12 and 8°C/h. The counts of heat resistant spores obtained at 52°C were lower than the counts obtained at 20°C ($P < 0.05$). When a slow cooking rate was applied (4°C/h), activation of spores occurred at 48°C rather than at 52°C as observed with the other heating rates (Table 4).

In all cooking treatments, the maximum amount of activated spores was obtained when the temperature of the product reached 72°C. These counts did not differ with the counts obtained at 75°C ($P > 0.05$) (Table 4).

The D_{75} -values obtained at the different cooking rates applied were 39.7 ± 13.4 , 32.13 ± 6.10 and 35.7 ± 11.42 h for 12°C/h, 8°C/h and 4°C/h respectively (Fig. 13). There were no significant differences ($P > 0.05$) among the D_{75} -values at the different cooking rates, neither with the D_{75} -value obtained in the previous experiment, where the samples were allowed to equilibrate and activate spores at 75°C for 20 min ($P > 0.05$).

The germination and outgrowth of *C. perfringens* spores heated at 75°C for 20 min was also determined (Tables 3 and 4). By using this treatment to activate spores, total counts of *C. perfringens* did not differ significantly ($P > 0.05$) than the counts

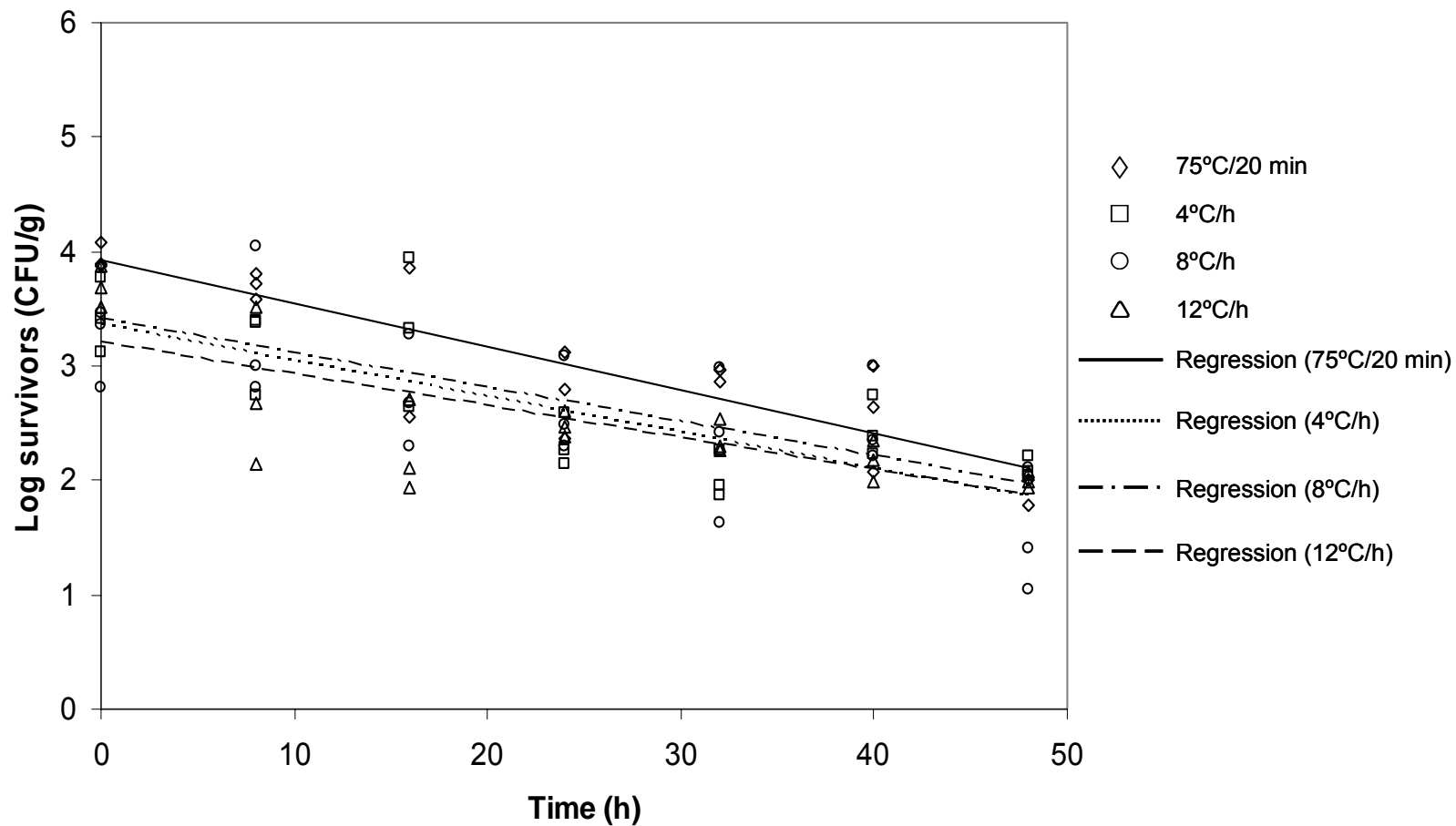


FIGURE 13. Survivor data and regression lines for non-heat shocked *Clostridium perfringens* spores cocktail (ATCC 12916, 12917 and 14809) held at 75°C for 48 h in cured ground pork after heating at 75°C/20 min, and from 20 to 75°C at heating rates of 4, 8, and 12°C/h.

obtained when the product was heated at a rate of 12°C/h (Table 3). Significant differences ($P<0.05$) were observed when spores activated at 75°C/20 min were compared with the counts obtained when the product was heated at 8 and 4°C/h (Table 3). Counts of heat resistant spores heated at 75°C/h were significantly higher ($P<0.05$) than the counts of heat resistant spores obtained when the product was heated at 4, 8 or 12°C/h (Table 4).

Effect of extended cooling rate

The behavior of *C. perfringens* during cooling of ground pork from 54.4°C to 7.2°C in 20 h is shown in Fig. 14. Total count of *C. perfringens* at 54.4°C was 4.0 CFU/g. No growth was observed during the initial cooling treatment until the temperature of the product reached 49.2°C (1 h approximately). *C. perfringens* exhibited slow growth at temperatures between 49.2 and 44.4°C (Fig. 14). Maximum growth rate was observed between 44.4°C and 32.8°C. At 32.8°C the total count of *C. perfringens* reached 7.3 log CFU/g. The mean generation time between the temperature range of 44.4 and 32.8 °C was 19.5 min. Below 32.8°C growth of *C. perfringens* was practically null. At 7.2°C, total count of *C. perfringens* was 7.8 log CFU/g. Fig. 14 also shows the heat resistant spore counts in ground pork. At the beginning of the cooling treatment (54.4°C) the mean count was 3.6 log CFU/g. This number decreased to 0.8 log CFU/g until the temperature of the product reached 36.3 °C (approximately 4 h of cooling), followed by a gradual increase to 3.7 log CFU/g within the next 6 h, time at which the

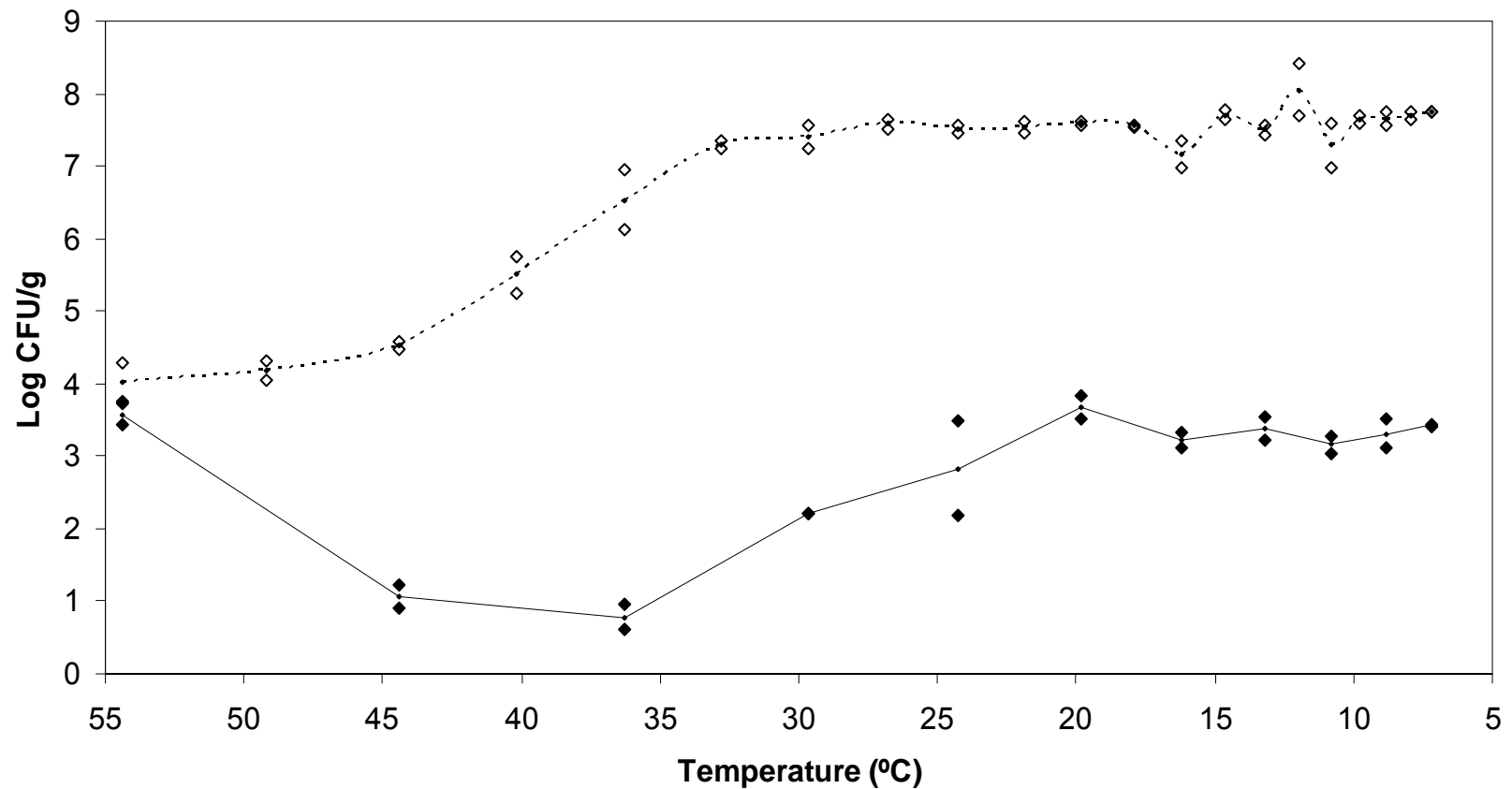


FIGURE 14. Survivor data for total counts (\diamond) and heat resistant spore counts (\blacklozenge) of *Clostridium perfringens* in cooked ground pork chilled from 54.4 to 7.2°C in 20 h. The data show the growth of a three strain cocktail. Lines represent average of two repetitions of total counts (dotted line) and heat resistant spore counts (continuous line).

temperature of the product reached 19.8 °C. The counts then remained constant until the end of the cooling treatment.

In cured ground pork the total count of *C. perfringens* at 54.4°C was 3.0 CFU/g (Fig. 15). Total count of *C. perfringens* decreased to 1.9 log CFU/g during the initial cooling step until the product temperature reached 36.3°C (4 h approximately). At this moment the total *C. perfringens* count started to increase at a slow rate. When the product temperature reached 24.2°C (4 h approximately), the total *C. perfringens* count was 2.7 log CFU/g and this count oscillated between 2.3 and 2.8 until the end of the cooling treatment (7.2°C). Despite the fact no increase was observed during the cooling conditions tested (20 h to chill from 54.4 to 7.2°C) between the initial counts at 54.4°C and the final counts at 7.2°C, a 1 log increase was observed in the total counts of *C. perfringens* between the temperature range of 36.3 and 7.2°C. The mean generation time between 36.6 and 24.2°C, temperature range where slight growth occurred, was 90.9 min.

The count of heat resistant spores during cooling of cured ground pork was 2.0 log CFU/g at the beginning of the cooling treatment (54.4°C) and decreased to 0.3 log CFU/g when the temperature of the product was 36.3°C (approximately 4 h of cooling) (Fig. 15). No further increase in heat resistant spore counts was observed during the following 16 h, time at which the temperature of the product reached 7.2°C. Counts of heat resistant spores ranged between 0.2 and 0.3 log CFU/g until the end of the cooling treatment.

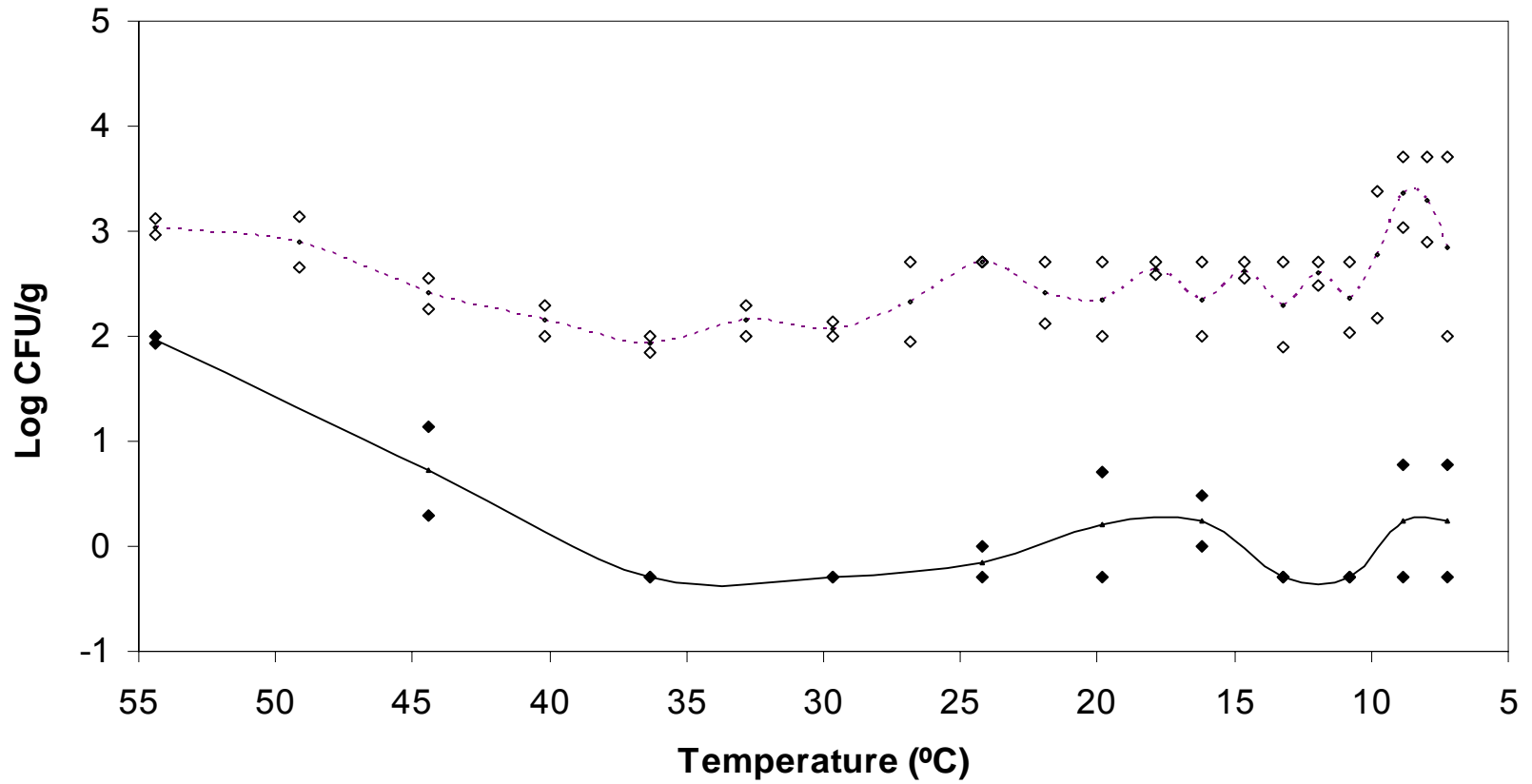


FIGURE 15. Survivor data for total counts (\diamond) and heat resistant spore counts (\blacklozenge) of *Clostridium perfringens* in cooked cured ground pork chilled from 54.4 to 7.2°C in 20 h. The data show the growth of a three strain cocktail. Lines represent average of two repetitions of total counts (dotted line) and heat resistant spore counts (continuous line).

DISCUSSION

C. perfringens has different sporulation capacity among strains (2, 7, 21, 26, 38, 41, 51). This has been reported to be affected by the composition of the medium, temperature of incubation, age of the cells and, the time of incubation (7, 21, 38, 41). These parameters may also affect the heat resistance of the spores obtained under different conditions. Barnes et al. (7) reported that spores obtained from Ellner's medium stored at room temperature became less heat resistant after storing *C. perfringens* cultures for more than 3 days. These authors also reported that spores of *C. perfringens* F2985/50 survived heating at 100°C for 4 h when the spores were produced in Robertson's meat medium, compared with a 3 h survival of spores produced in Ellner's medium. Barnes attributed the higher resistance of *C. perfringens* spores in Robertson's meat medium to the higher amount of spores produced in this medium.

The results of this study revealed that ATCC 12916 strain was not able to produce enough spores at 37°C to achieve an inoculum of 5 log per g or ml of medium. It has been reported that *C. perfringens* produces spores more rapidly at 43°C (28, 35). This particular strain has a shorter generation time at 41°C compared with 37°C in meat products. *C. perfringens* ATCC 12916 was grown at 42°C during spore preparation for this study in order to achieve a high number of spores in the same incubation period (16 h) than the spores obtained with the other two strains (ATCC 12917 and 14809).

Differences in thermal resistance among strains of *C. perfringens* and the effect of heating medium on the thermal resistance have been reported previously (1, 10, 19,

61, 94, 95). Adams (1) reported that *C. perfringens* ATCC 3624 suspended in water showed a larger D value when heated at 90°C than when heated in phosphate buffer. Collee et al. (19) reported that spores of *C. perfringens* strain F2985/50 survived steaming at 100°C for 4 h when suspended in cooked meat broth, while the identical inoculum suspended in distilled water did not survive the minimum exposure time of 50 min.

The results obtained in the present study, concluded that D₇₅ values of non-heat shocked *C. perfringens* spores were higher in beef gravy than PBS. Bradshaw et al. (10) also reported higher heat resistance of *C. perfringens* spores in beef gravy than in phosphate buffer. According to Bradshaw et al. (10) the increased resistance observed in beef gravy may be attributed to a protective effect of the beef gravy constituents to the spore outgrowth mechanism from heat inactivation or the presence of a substance that stimulates germination of heat injured spores. The repair of heat injured *C. perfringens* spores has also been reported to occur in meat broth and milk (6). Higher thermal resistance obtained in ground pork in the present study suggests that repair of damaged spores also occur in this food.

The results of thermal resistance of this study were consistent with Bradshaw et al. (10) findings. These authors reported that D-values of spore suspensions of *C. perfringens* in 0.067 M (pH 7.0) phosphate buffer were 18.57-22.4 min and 3.15-5.18 min at 98.9°C and 104°C respectively. Higher D-values were observed in spores heated in commercial beef gravy, 26.0-31.4 min and 6.6-8.0 min at 98.9 and 104.4°C respectively. In the same study, the authors also reported that the increased thermal

resistance of spores found in commercial beef gravy, was not exhibited when the samples were heated at higher temperatures. D-values obtained in spore suspensions heated in phosphate buffer at 110 and 115°C (0.8-1.29 min) were higher than the D-values obtained with beef gravy (0.1-0.95 min).

The lower survivor counts in cured ground pork may be due to the effect of curing salts, which make spores more susceptible to heat treatment (2, 4, 18). Ando et al. (2) reported that increasing the concentration of sodium nitrite from 0.1 to 0.8 mol/L in 50 mmol/l phosphate buffer (pH 6.0) stimulated germination at 40°C. Ashworth and Spencer (4) demonstrated that the Perigo effect occurs in pork. These authors reported that addition of sodium nitrite to minced pork meat before heating enhanced inhibition of *C. perfringens*.

The higher thermal resistance of *C. perfringens* ATCC 12916 may be attributed in part to the temperature at which spores were produced (42°C). Solberg and Elkind (74) reported that *C. perfringens* cells grown at 37°C showed higher heat resistance than cells grown at 23°C.

The effect of increased thermotolerance on heat shocked spores was observed only on heat shocked spores of *C. perfringens* ATCC 12916 heated in PBS ($P < 0.05$). No increased thermotolerance occurred in the strains heated in beef gravy, ground pork and cured ground pork. These findings differ from the results reported by Juneja et al. (44) in beef gravy. These authors reported an increased thermotolerance of heat shocked spores when heated in beef gravy at 100°C. Differences in temperatures used to measure heat

resistance in this study (75°C vs. 100°C) and the method used to heat the samples may account for the differences.

The lower heat resistance of heat shocked *C. perfringens* spores on cured ground pork agrees with previous reports. Chumney et al. (18) reported that injured spores (heated at 90°C/6 h) are more sensitive to NaCl and sodium nitrite compared with non-heat injured spores. These authors suggest that cellular damage occurred during the heat treatment which makes the spores more sensitive to these agents.

Initial total counts of *C. perfringens* were lower than the counts of heat resistant spores. These results agree with Barnes et al. (7) and Roberts (61) results, which reported that only about 0.1-3% of spores were able to germinate without prior heat shock.

The rapid germination and outgrowth of *C. perfringens* spores in cured ground pork heated at a rate of 4°C/h may be attributed to an early activation of the spores and the slow come up during the cooking process, which favored the germination and outgrowth of the spores. These results indicate the potential for growth of naturally occurring spores during slow come up time during processing of cooked cure products.

The results presented in this study were found to be consistent with the data reported by Hall and Angelotti (31). These authors reported no growth of *C. perfringens* occurred at temperatures within 4.4 to 15.6°C. However, within the optimal range of temperature large numbers of cells may be produced within 3 h as reported previously by Duncan (25).

The results of the present study differ from the results reported by Barnes et al. (7). These authors reported that after heating, germination of spores occurred and lost their heat resistance during cooling; which was not recovered under the storage conditions tested. The results obtained during the abusive chilling indicated that even during cooling of cooked ground pork, no growth of vegetative cells was seen at temperatures below 32.8°C, spore counts continued to increase until the temperature of the product reached 19.8°C.

Hall and Angelotti (31) reported that strain 8239 inoculated on beef cubes with natural gravy, was able to increase 100 fold in <4h at 46°C but rapid death occurred at 49°C, whereas slow decrease in counts occurred between 4.4-15.6°C. These authors also reported that growth was preceded by a 2-4 h lag phase at 35°C but no lag phase occurred at 46°C.

Shigehisa et al. (69) studied the behavior of *C. perfringens* spores and vegetative cells in roast beef heated at different cooking rates. These authors reported that at slow cooking rates (7°C/h) *C. perfringens* spores germinated at an earlier temperature (ca. 30°C) than spores heated at 13 to 25°C (spores germinated at temperatures higher than 35°C). The findings of the present study are consistent with Shigehisa results. Spores of *C. perfringens* heated in cured ground pork at 4°C germinated earlier than spores heated at 8 and 12°C/h.

Growth occurred during abusive cooling of cured ground pork was slower than in ground pork. The presence of curing salts inhibited the growth of *C. perfringens* at the temperatures at which growth occurred rapidly in ground pork (44.4 to 36.3°C) and

delayed growth at temperatures between 36.3 to 24.2°C. The inhibitory effect of curing salt has been previously reported (2, 29, 42, 48, 51, 98). However, at the cooling conditions tested (20 h to chill from 54.4 to 7.2°C), 1 log increase was observed in the counts of *C. perfringens*.

Zaika (98) reported that inhibition of germination and outgrowth of *C. perfringens* occurred in cured hams inoculated post commercial processing and supplemented with 3.1 % NaCl when cooled exponentially from 54.4°C to 7.2°C within 15, 18 and 21 h.

At constantly raising temperatures, where approximately linear rises are observed, Willardsen et al. (97) reported that rapid growth of vegetative cells of *C. perfringens* occurred in ground beef between 35 and 52°C. The data obtained in the present study indicated that during cooling, where exponential temperature drop takes place, growth occurred in ground pork in a narrow temperature range (32.8° to 44.4°C).

This study investigated germination and outgrowth of *C. perfringens* spores at dynamic temperatures: during cooking at different linear rates and cooling under simulated temperature abuse conditions. In previous studies the growth of *C. perfringens* during cooling under temperature abuse conditions, has been examined (48, 98). However, in these studies the heating of inoculated meat products does not represent the common processes used at commercial establishments.

In a study conducted by Taormina et al. (79), cooking procedures held at commercial establishments were simulated and an extended cooling treatment (20 h) was applied following the cooking process. These authors reported that counts of *C.*

perfringens at 54.4°C (3.0 log CFU/g) were reduced to 2.6 log CFU/g at the middle chilling temperature (26.7°C) and increased to 3.2 CFU/g at the lower chilling temperature (7.2°C). Reduction of total counts and spore counts of *C. perfringens* were also observed in the present study at the middle chilling temperature (36.3°C). The hourly sampling intervals used in the present study indicated the lowest counts were obtained at 36.3°C in cured ground pork, and a 1 log increase in the counts of *C. perfringens* was observed at the end of the cooling process.

Although sporulation of *C. perfringens* in foods has been reported to occur at a temperature range of 25 to 37°C (7, 20, 21, 49), no data have been reported previously in regards to sporulation of *C. perfringens* during cooling of cooked products. The lower number of spores produced in cured ground pork may be attributed to the presence of curing salts as well as the lower water activity of the cured product (20, 98).

CONCLUSION

Thermal resistance of *C. perfringens* spores was affected by the composition of the medium in which spores were heated and differences were also observed among bacterial strains. The thermal resistance at 75°C was not affected by the heating rate when spores of *C. perfringens* were heated in the same medium (cured ground pork). However, the germination and outgrowth of *C. perfringens* during cooking from 20 to 75°C was affected by the heating rate. At the rate of 4°C/h (temperature abuse) growth of *C. perfringens* occurred, which suggests that other sporeforming pathogens may grow as well. *C. perfringens* spores were able to survive and germinate during abusive cooking and cooling of cured ground pork.

This study denoted that germination and outgrowth of *C. perfringens* spores may occur in the interior of contaminated cured pork products under abusive cooling procedures (from 54.4 to 7.2°C in 20 h). Although a 1 log growth was shown to occur during the cooling of cooked cured pork, no increase was detected when comparing the counts at 54.4 and 7.2°C. The number of *C. perfringens* cells did not reach the high levels required to be present in the food to cause disease. With an initial inoculum in raw cured ground pork of 5 log CFU *C. perfringens* spores/g, *C. perfringens* counts did not exceed 3.4 log CFU/g during a 20 h abusive cooling.

Caution should be taken when conducting challenge studies for validation of cooling procedures. The cooking procedures should represent the product and processes used in commercial establishments and these studies should demonstrate that no growth

occurs during the chilling process. It is also important the use of proper strain or a mixed cocktail of strains for the product under study, because the behavior may vary among bacterial strains and among products (phosphate buffer, beef gravy, ground pork and cured ground pork).

Similar behavior of *C. perfringens* spores could be expected in contaminated interiors of whole muscle cured bone-in hams with a similar temperature profile. Therefore, in conducting challenge studies it is paramount to carry out under appropriate dynamic temperatures that represent the processes as it is applied in commercial establishments as well as to investigate growth along the total cooling process. This will guarantee the ability to detect whether a 1 log increase has occurred during the stabilization of the cooked product, as well as validate compliance with current regulations.

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