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## Heat sensitivity of *Clostridium perfringens*

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## **Heat sensitivity of *Clostridium perfringens***

L.M. Wijnands  
A. van der Meij-Florijn  
E.H.M. Delfgou-van Asch  
F.M. van Leusden

Contact:  
L.M. Wijnands  
Laboratory for Zoonoses and Environmental Microbiology  
lucas.wijnands@RIVM.nl

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## Abstract

### Heat sensitivity of *Clostridium perfringens*

The RIVM is investigating various aspects in the development of food borne disease caused by the spore forming bacterium *Clostridium perfringens*. One of the aspects included in the investigations is heat sensitivity of the micro organism, since food that undergoes prolonged heating, cooling and re-heating, such as stews or soups, is an important vector.

The heat sensitivity of spores (a form of the organism that can survive a variety of environmental conditions) and vegetative cells (actively growing cells) at three different temperatures was determined in phosphate buffered saline (PBS) from five strains of bacteria isolated from food. As there have been reports that food components may influence the heat sensitivity of bacteria this property from vegetative cells was also determined in 10-fold diluted pea soup.

In PBS bacterial spores are sensitive to temperatures higher than 95 degrees Celsius and vegetative cells are sensitive to temperatures just over 45 degrees Celsius. In diluted pea soup the vegetative cells show slightly lower heat sensitivity compared with PBS. Sensitivity for the various temperatures is strain dependent.

The results from the heat sensitivity tests were used to calculate Z-values. These Z-values represent the temperature in a process that gives rise to a ten-fold increase/decrease in heat sensitivity. A Z-value can also be used as a degree of the risk that a strain may form in food preparation processes. Strains with a high Z-value are more risky, as a higher change in temperature is necessary to reduce the number of cells of such strains.

The Z-value calculations gave no uniform results: no strain showed high or low values for both spores and vegetative cells. Therefore these values should not be used to formulate preventive measures for individual strains, but to formulate general measures to prevent the onset of disease by *C. perfringens*.

Key words: *Clostridium perfringens*, heat sensitivity, Z-value

## Rapport in het kort

### Hittegevoeligheid van *Clostridium perfringens*

Voedsel verhitten is een belangrijke manier om voedselvergiftigingen door de bacterie *Clostridium perfringens* te voorkomen. De bacterie doet zich in voedsel in twee vormen voor: als spore, een overlevingsvorm die onder andere goed bestand is tegen hogere temperaturen en droogte, en als actief groeiende bacteriecellen (vegetatieve cel). Uit onderzoek van het RIVM blijkt dat sporen van de bacterie bij temperaturen van 95 graden Celsius en hoger snel afsterven. Vegetatieve cellen sterven bij temperaturen van 45 graden Celsius en hoger snel af.

*Clostridium perfringens* is een bacterie die per jaar circa 100.000 tot 150.000 voedselinfecties veroorzaakt. De Voedsel en Waren Autoriteit (VWA) heeft dit onderzoek opgezet om meer zicht te krijgen op het aantal voedselvergiftigingen door dit type bacterie, de oorzaak en het verloop ervan. Met de uitkomsten kan de VWA maatregelen onderbouwen om het aantal voedselvergiftigingen te verminderen. Ook kan de VWA hiermee consumenten eraan herinneren voedsel goed en lang genoeg te verhitten.

De hittegevoeligheid van de bacterie is onderzocht in zowel een kunstmatig medium ('buffer'), dat doorgaans voor laboratoriumwerk wordt gebruikt, als in een hoeveelheid verdund voedsel. Er zijn namelijk aanwijzingen dat het type voedsel invloed heeft op de hittegevoeligheid van de bacterie, vermoedelijk door de wijze waarop de bacterie zich aan voedsel hecht. In het onderzoek is meegenomen dat de temperatuur van het onderzochte voedsel in verhouding staat tot zowel de eetbaarheid ervan als de noodzaak voedselvergiftigingen te voorkomen.

Trefwoorden: *Clostridium perfringens*, hittegevoeligheid, voedselbereiding

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## Summary

*Clostridium perfringens* is a Gram positive spore-forming bacterium that can cause food borne disease. The estimates for incidence of disease in the Netherlands vary from 10,000 – 50,000 to approximately 150,000 cases per annum. These estimates are based primarily on reports of outbreaks to the Food and Consumer Product Safety Authority (VWA) and the Municipal Health Services (GGD).

From outbreak reports can be derived that temperature abuse during the processing of food is a common cause. Insufficient cooling capacity of refrigerators or too low temperatures during reheating of prepared food is often the cause of growth of *C. perfringens* to high numbers causing diarrhoea after the ingestion of the contaminated food.

In the Netherlands *C. perfringens* is regularly detected in food commodities, but more exact details on the strains, such as the ability to produce the enterotoxin Cpe or the heat sensitivity of spores and vegetative cells, are lacking.

Therefore, from a number of *C. perfringens* strains, isolated from food commodities in the Netherlands and collected by the VWA, the heat sensitivity of spores and vegetative cells was determined in phosphate buffered saline (PBS). From vegetative cells the heat sensitivity was also determined in diluted pea soup to investigate possible protective influence of food constituents on the vegetative cells. The heat sensitivity data were also used to calculate Z-values as a measure for the adaptation of heat processes to increase or decrease the surviving number of spores/vegetative cells.

The Z-values of the spores of the various strains do not differ dramatically, but those of vegetative cells, in PBS as well as in diluted pea soup, show more variation. Also, the results are not uniform in the sense that one strain shows the lowest heat sensitivity in spore form and in vegetative cell form, and in PBS and in diluted pea soup.

These investigations are part of a larger study set up to identify combinations of strains and processes that increase the risk for food borne disease and to (re-)formulate measures that will lead to reduced incidence of food borne disease by *C. perfringens*.

# 1 Introduction

*Clostridium perfringens* type A is a rod shaped spore-forming Gram positive bacterium capable of causing food borne disease. The symptoms of the disease are mainly watery diarrhoea and general malaise. The actual disease symptoms are caused by an enterotoxin, Cpe, which is produced during sporulation of *C. perfringens* in the small intestine. Incubation time of the disease is about 6 – 24 hours, the duration of the symptoms is about 24 hours, and the disease is self-limiting. As the disease is not notifiable in the Netherlands, it is hard to estimate the number of disease cases. The main instrument for such estimation is the report of disease cases to the Food and Consumer Product Safety Authority (VWA) or the Municipal Health Service (GGD). However, only a very small number of *C. perfringens* induced disease cases are reported. Most important reason for this low amount of reports are the relatively mild and short lasting symptoms, not urging people to seek medical assistance. The National Health Council (GezondheidsRaad) estimates 10,000 to 50,000 cases per annum (Health Council of the Netherlands 2000). More recently the number of disease cases due to *C. perfringens* was estimated to be approximately 150,000 (Van Kreijl, Knaap et al. 2006).

The basis for our investigations regarding *C. perfringens* induced diarrhoea is the following flow scheme, which can also be derived from outbreak reports (Regan, Syed et al. 1995; Keita-Perse, Pradier et al. 1999; Holtby, Tebbutt et al. 2008). Food products contaminated with *C. perfringens*, vegetative cells and/or spores, are cooked during which process vegetative cells are killed. Spores survive the cooking process. Subsequently the cooked food is cooled inefficiently/inadequately, during which process spores germinate and grow out to higher numbers. At a later stage the prepared food is reheated for consumption, often too slow and to a too low temperature, inducing multiplication of vegetative cells to high numbers. These are ingested with the heated food, and, as they easily survive the gastric passage, reach the small intestine unharmed, where they sporulate and produce enterotoxin (Cpe). This enterotoxin is responsible for the onset of diarrhoea.

The main topics of investigation in this project concern 1) the characterization of strains present in food and 2) the capacity of food strains to sporulate and produce enterotoxin in a gastrointestinal model system and the role of food products during this process. In risk assessment terms item 1) can be characterized as part of exposure assessment, and item 2) as part of hazard characterization.

The characterization includes determination of the incidence of potentially enterotoxin producing strains in food products by means of Polymerase Chain Reaction (PCR) and determination of the heat sensitivity of strains occurring in food products.

In the model system *C. perfringens* vegetative cells, embedded in a food matrix, are submitted to simulated gastric conditions and subsequently transferred to simulated intestinal conditions. The process is monitored for sporulation and enterotoxin production. The most important parameters for research are starting concentration of the vegetative cells and type of food matrix.

The result of these investigations should be the identification of most risky product-preparation combinations, which may be of use in setting standards and phrasing hygiene measures.



In proper cooking processes vegetative cells of pathogenic bacteria are killed, thus unable to cause disease. However, food production processes are constantly changing. Minimal or reduced processing has increased, but with that the risk of survival of vegetative cells of bacteria has increased. Also the survival of spores of pathogenic bacteria is favoured by these changed preparation processes. During later stages in the food preparation these spores may germinate, grow and contribute to the onset of disease. Adaptation of food preparation protocols, without reducing the nutritional and sensory properties of the food, may overcome these problems. For that it is necessary to have data on the reduction of spores and vegetative cells through heat, so-called D-values, decimal reduction values, indicating the time at a certain temperature necessary to reduce the number of colony forming units with one log-unit. Such data are not abundant, especially, on vegetative cells. Some of the available data have been summarized below. Sarker et al. (2000) determined D-values from vegetative cells and spores of food poisoning isolates and strains carrying plasmid *cpe*-gene (Sarker, Shivers et al. 2000). At 100 °C spores from food poisoning isolates had D-values ranging from 30 to 124 minutes. At 55 °C the D-values of vegetative cells from food poisoning isolates ranged from 11 to 16 minutes. The D-values of spores and vegetative cells from plasmid *cpe*-gene carrying strains ranged from 0.5 to 1.9 minutes at 100 °C and from 5 to 9 minutes at 55 °C respectively. More recently, Le Marc et al. (2008) assessed heat resistance of spores from various *C. perfringens* strains, and found them to range from 1 to more than 300 minutes at 95 °C (Le Marc, Plowman et al. 2008). From D-values a Z-value may be calculated, being the change in temperature necessary to change the D-value with one log-unit. Z-values can be of help to adapt preparation processes. Here too there is a difference between food poisoning strains and non-food poisoning strains. The Z-values from the former type ranges from 1.5 to 9 °C, and from the latter they range from 10 to 11.5 °C (Roberts 1968).

In this report results of investigations concern the identification of the most risky *C. perfringens* strains as part of the characterization of strains present in food. This was done by determining D-values of spores and vegetative cells of *C. perfringens* strains isolated from food commodities suspected for having caused food borne disease in the Netherlands. The strains used for these investigations were able to sporulate in the presence of bile. Moreover, D-values were determined in buffer as well as in a (diluted) food commodity prone for the occurrence of *C. perfringens*, since recent research has indicated that the presence of food constituents in the experimental matrix may increase the heat sensitivity. Calculation of Z-values from the D-values was carried out to identify the most risky strains as previously described (Adams and Moss 2004). A Z-value is the temperature change necessary to change the D-value tenfold. Thus it can be used to show the impact of a change in temperature in a preparation process on the ability of cells to survive such process change. The higher the Z-value, and therefore the higher the change in temperature, the more effort has to be done to decrease the survivability of cells, and thus the riskier the strain.

## 2 Methods

### Strains

For these investigations *C. perfringens* strains able to sporulate in Duncan and Strong medium (Duncan and Strong 1968) supplemented with bile ( $20 \pm 2$  mg/50 ml) were used. All strains derive from food commodities suspected for being involved in food borne disease. Identity of the strains was confirmed by colony morphology and haemolysis on blood agar plates, and by using nitrate-motility and lactose-gelatinase medium.

The strains and their origin are listed in Table 1.

**Table 1. Strains**

RIVM-no.	Provided by	Original number	Sample ID*	Food commodity
Cp 3	VWA <sup>#</sup> (Zuid)	C 0011	38088483	Meat in curry sauce
Cp 5	VWA (Zuid)	C 0031	37412686	Potato dish
Cp 7	VWA (Zuid)	C 0057	38520458	Fried rice
Cp 8	VWA (Zuid)	C 0058	38793012	Poultry dish
Cp 9	VWA (Zuid)	C 0059	38793004	Pasta dish

\*= number from ISI-database of the Food and Consumer Product Safety Authority

# = Food and Consumer Product Safety Authority

### Determination of D-values from spores

Survival of spores and vegetative cells was determined essentially according to a previously described method (Dufrenne, Tatini et al. 1994). Five screw capped glass tubes (16 x 100 mm) with Teflon inlays containing 8 ml phosphate buffered saline pH 7.0 (PBS) were preheated in a oil bath at 90 °C. When the contents of the tube had reached the temperature of the oil bath, the pressure in the tube was released by using a hypodermic needle, and subsequently a 100 µl portion of a spore suspension was injected with a syringe through the Teflon inlay. This tube remained in the oil bath for 120 minutes. After 30 minutes the procedure was repeated with the next tube, which remained in the oil bath for 90 minutes. This was repeated at 60 and 30 minutes before ending the experiment, after a total incubation time of 120 minutes. The rack with tubes was taken out of the oil bath and placed in melting ice. When the tubes had cooled down the last of the five tubes was injected as described before for Time = 0. The procedure was carried out in duplicate.

The numbers of surviving spores for each the two series were determined by spread plating decimal dilutions on Trypton Soy Agar and incubating the plates anaerobically at 37 °C during 20 – 22 hours.

D-values were determined using the following formula (Adams and Moss 2004):

$$D = (t_2 - t_1) / (\log N_1 - \log N_2), \text{ where } N_1 \text{ and } N_2 \text{ are the numbers of cells at time points } t_1 \text{ and } t_2 \text{ respectively.}$$

The linear portion of the survivor curve on a semi log scale was used.

The procedure was repeated at 95 °C and 100 °C with incubation times of 60, 45, 30, 15 and 0 minutes and 10, 7, 4, 2 and 0 minutes, respectively. For these temperatures D-values were determined as well.

#### Determination of D-values from vegetative cells in PBS

This procedure resembled the method for spores as described above except for the incubation times and incubation temperatures. These were 45 °C, 50 °C and 55 °C for 60, 30 and 15 minutes respectively. Also the incubations were carried out in a water bath instead of an oil bath.

#### Determination of D-values from vegetative cells in 10% pea soup in PBS

This procedure resembled the method for vegetative cells in PBS as described above except for the incubation times and incubation temperatures. These were 50 °C, 55 °C and 60 °C for 60, 20 and 10 minutes respectively. These incubations were carried out in capped test tubes instead of Teflon sealed screw cap vials.

An overview of all incubation temperatures and incubation times is shown in Table 2.

**Table 2 General incubation temperatures and - times**

<b>Spores Temperature / Time (°C) / (minutes)</b>	<b>Veg. cells (PBS) Temperature / Time (°C) / (minutes)</b>	<b>Veg. cells (10% pea soup) Temperature / Time (°C) / (minutes)</b>
90 / 120	45 / 60	50 / 60
95 / 60	50 / 30	55 / 20
100 / 10	55 / 15	60 / 10

#### Extra incubation temperatures

With strain Cp 8 three intermediate temperatures were tested: two, 97.5 °C and 92.5 °C, with spores in PBS, and one, 47.5 °C, with vegetative cells in PBS.

#### Calculation of Z-values

After plotting the log transformations of the D-values against the temperature, the Z-value for a strain can be determined as follows (Adams and Moss 2004):

$$Z = (T_2 - T_1) / (\log D_1 - \log D_2), \text{ where } D_1 \text{ and } D_2 \text{ are the D-values at temperatures } T_1 \text{ and } T_2 \text{ respectively.}$$

### **3 Results**

The results of the D-value determinations and calculations for the Z-values for spores in phosphate buffered saline are shown in Tables 3. Compared to the other four strains the D-value at 90 °C for the spores of strain Cp 3 is low. This low D-value has its direct effect on the Z-value of strain Cp 3, which is higher than that of the other strains.

The D-value at 90 °C of spores from strain Cp 9 is relatively high in comparison to the other strains, resulting in a relatively low Z-value.

Overall there are large differences in D-values at 90 °C, whereas these differences no longer exist at 95 °C and 100 °C (see Figure 1).

In Table 4 the results of the D-value determinations and Z-value calculations for vegetative cells in phosphate buffered saline are shown. The values for Cp 9 at 45 °C and 50 °C are remarkably high in comparison to the values of the other strains at those temperatures which are more or less in the same order (see Figure 2). The Z-values of the vegetative cells in PBS show more variety than those of the spores: a range from 5.70 to 14.31 for vegetative cells versus a range from 4.38 to 5.44 for spores.

Food and food constituents may have a protective influence on bacterial cells where the sensitivity for low pH and heat is concerned. Therefore, D-values of vegetative cells were also determined in 10% pea soup. Undiluted pea soup was not used for these investigations because of the high viscosity of the soup and the inherent difficulties with pipetting and diluting. The results of these determinations and the subsequently calculated Z-values are shown in Table 5. Comparison of the results in 10% pea soup with the results in PBS shows a tendency the D-values from strains Cp 3, 5, 7 and 8 in pea soup to be slightly higher than in PBS (see Figure 4). The protective influence of 10% pea soup does not seem to go for strain Cp 9, where the D-value at 50 °C in 10% pea soup is slightly lower than in PBS.

The heat sensitivity of vegetative cells from strain Cp 8 was also tested in 3% pea soup. The resulting D-values and the calculated Z-value did not differ significantly from the D-values in PBS (data not shown).

## 4 Discussion

Determination of D-values and calculation of Z-values provides valuable information of the ability of spores and vegetative cells to survive heating processes. Although only a limited number of strains have been investigated here, the variation among the strains appears to be high. When considering spores, strain Cp 3 is the least heat resistant and strain Cp 9 the most heat resistant. When considering vegetative cells, strain Cp 5 is the least heat resistant and, again strain Cp 9 the most heat resistant.

When considering changes of preparation processes in order to eliminate spores or vegetative cells more safely, the Z-values should be taken into account. The more gradual the change in D-value with rising temperature, the higher the change in temperature necessary to change the D-value with a log unit (i.e. Z-value), and thus to reach a faster reduction in spores. This goes equally for vegetative cells. Remarkably, a high Z-value for spores does not imply a high Z-value for vegetative cells. From the five strains, Cp 3 has the highest Z-value for spores, but Cp 5 has the highest for vegetative cells (compare Tables 1 and 2). Also, a high Z-value in PBS does not imply a high value in 10% pea soup (compare Tables 2 and 3). The risk of a *C. perfringens* strain therefore depends on the experimental medium and on the type of cells. In our investigations Cp 3 must be considered the most risky strain with respect to spores in PBS, Cp 5 with respect to vegetative cells in PSB, and Cp 8 with respect to vegetative cells in 10% pea soup.

The D-value at 90 °C of spores from strain Cp 3 is remarkably low compared to the other strains. Possibly this strain can be classified among the heat sensitive *C. perfringens* strains. This could imply that Cp 3 is a strain without a genomic *cpe*-gene, capable of causing food borne disease (Cornillot, Saint-Joanis et al. 1995). At a later stage in this project this information will become available, and will then be taken into consideration for the final report of this project.

D-values from vegetative cells were also determined in 10% pea soup. In this medium the D-values for most strains at most temperatures are higher than in phosphate buffered saline. This may indicate that food components have a protective influence on the bacterial cells, a phenomenon that has been seen before. For example, *Campylobacter jejuni* is eliminated from broth within several seconds after heating, while attached to chicken meat it takes about ten minutes to reach an equal level of elimination.

Generally, D-values are determined from spores only because of their low heat sensitivity. In our opinion D-value determination of vegetative cells is at least as important as those of spores. After all, vegetative cells of *C. perfringens* may occur in food commodities after germination of spores. They may survive during improper cooling and subsequent improper reheating of food. Their numbers may even increase at these stages. Moreover, previously we have shown that vegetative cells are most important for the onset of diarrhoeal disease by *C. perfringens*, as they easily survive the gastric passage. Upon arrival in the small intestine they will sporulate and produce enterotoxin leading to the disease symptoms (Wijnands, Meij-Florijn et al. 2008). In literature the reports of D-values of vegetative cells are scarce. The D-values found by Sarker et al. (2000) differ from the values found in

this study, mainly where the genome borne *cpe*-gene strains, i.e. the heat resistant strains (Cornillot, Saint-Joanis et al. 1995), are concerned. The D-values for strain Cp 3, a heat sensitive strain without a *cpe*-gene<sup>1</sup>, are more comparable to the data found by Cornillot et al. (1995). Of course the most obvious reason is the difference in strains used for both studies.

The differences in D-values and Z-values, as found in these investigations, reflect the variation in *C. perfringens* strains. Determination of these values of a larger number of strains would not only make this variation more apparent, but would also provide more information on the 'extremes' among strains. These investigations were carried out in the scope of formulating measures to reduce the number of food borne disease cases by *C. perfringens*. When doing so, the variation among strains must be taken into consideration, and preferably data referring to 'extreme' strains must be obtained and used. Strain Cp 9 as used in this study appears to be a very heat resistant strain with a D-value at 90 °C of 193 minutes for spores and 171 minutes at 45 °C for vegetative cells.

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<sup>1</sup> Data will be published in report describing prevalence of Cpe-gene in strains from the Food and Consumer Product Safety Authority

## Tables and Figures

**Table 3 D-values (minutes), Log D-values and Z-values of spores in PBS from duplicate experiments**

Strain	Temp (°C)	D <sub>A</sub>	D <sub>B</sub>	D <sub>average</sub>	Log D <sub>average</sub>	Z
Cp 3	90	68	69	69	1.84	5.44
	95	8	9	8	0.90	
	100	1	1	1	0.00	
Cp 5	90	155	101	122	2.09	4.79
	95	12	11	11	1.04	
	100	1	1	1	0.00	
Cp 7	90	123	99	109	2.04	4.91
	95	17	16	16	1.20	
	100	1	1	1	0.00	
Cp 8	90	104	108	106	2.03	4.94
	92.5	78	75	77	1.89	
	95	12	11	12	1.08	
	97.5	5	6	5	0.70	
	100	1	1	1	0.00	
Cp 9	90	223	170	193	2.29	4.38
	95	12	11	11	1.04	
	100	1	1	1	0.00	

**Table 4 D-values (minutes), Log D-values and Z-values of vegetative cells in PBS from duplicate experiments**

Strain	Temp (°C)	D <sub>A</sub>	D <sub>B</sub>	D <sub>average</sub>	Log D <sub>average</sub>	Z
Cp 3	45	71	57	63	1.80	7.56
	50	10	8	9	0.95	
	55	3	3	3	0.48	
Cp 5	45	39	32	35	1.54	14.31
	50	18	20	19	1.28	
	55	7	8	7	0.85	
Cp 7	45	41		41	1.61	7.62
	50	11	11	11	1.04	
	55	1	2	2	0.30	
Cp 8	45	41	32	36	1.56	9.27
	47.5	26	22	24	1.38	
	50	5	5	5	0.70	
	55	4	3	3	0.48	
Cp 9	45	145	210	171	2.23	5.70
	50	54	34	42	1.62	
	55	2	3	3	0.48	

**Table 5 D-values (minutes), Log D-values and Z-values of vegetative cells in 10% pea soup from duplicate experiments**

<b>Strain</b>	<b>Temp (°C)</b>	<b>D<sub>A</sub></b>	<b>D<sub>B</sub></b>	<b>D<sub>average</sub></b>	<b>Log D<sub>average</sub></b>	<b>Z</b>
Cp 3	50	22	26	24	1.38	5.62
	55	5	5	5	0.70	
	60	0.4	0.4	0.4	-0.40	
Cp 5	50	40	43	43	1.63	6.12
	55	8	11	11	1.04	
	60	1	1	1	0.00	
Cp 7	50	10	15	15	1.18	8.50
	55	6	8	8	0.90	
	60	1	1	1	0.00	
Cp 8	50	28	20	20	1.30	10.00
	55	6	7	7	0.85	
	60	2	2	2	0.30	
Cp 9	50	9	12	12	1.08	9.27
	55	6	6	6	0.78	
	60	1	1	1	0.00	



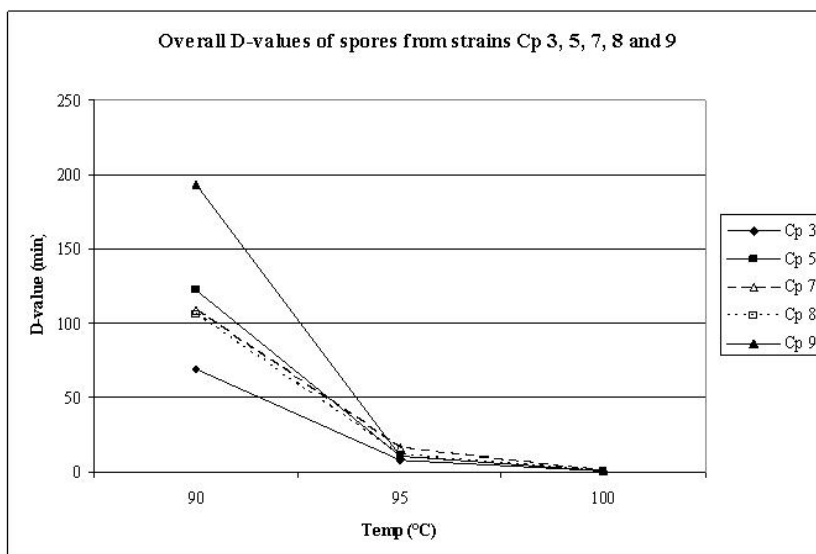


Figure 1 Comparison of average D-values in phosphate buffered saline of spores from *C. perfringens* strain Cp 3, 5, 7, 8 and 9.

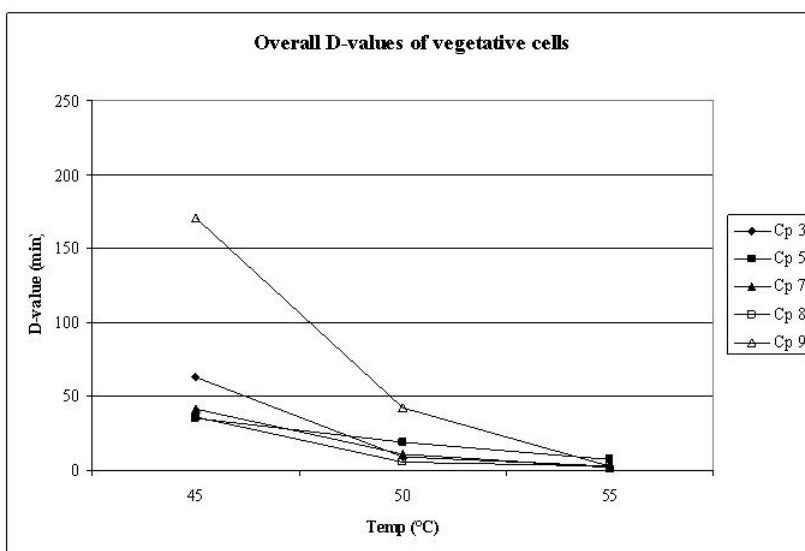


Figure 2 Comparison of average D-values in phosphate buffered saline of vegetative cells from *C. perfringens* strain Cp 3, 5, 7, 8 and 9.

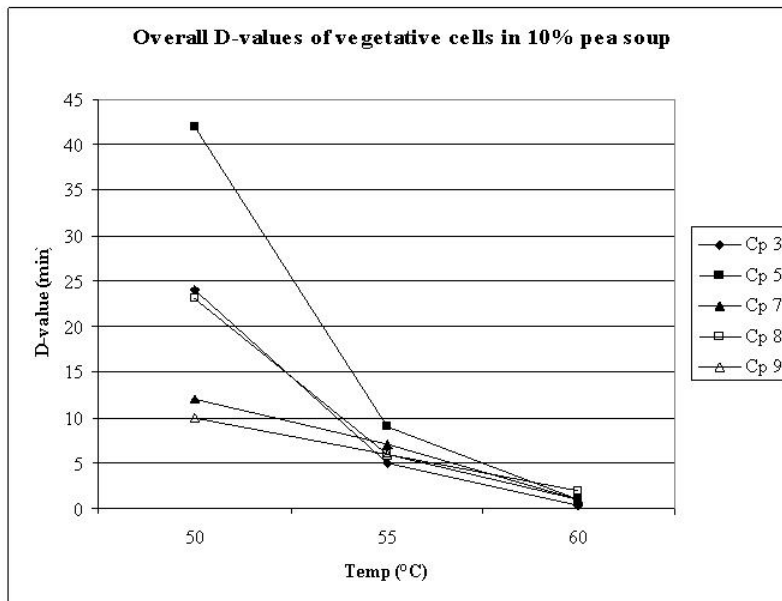


Figure 3 Comparison of average D-values in 10% pea soup of vegetative cells from *C. perfringens* strain Cp 3, 5, 7, 8 and 9.

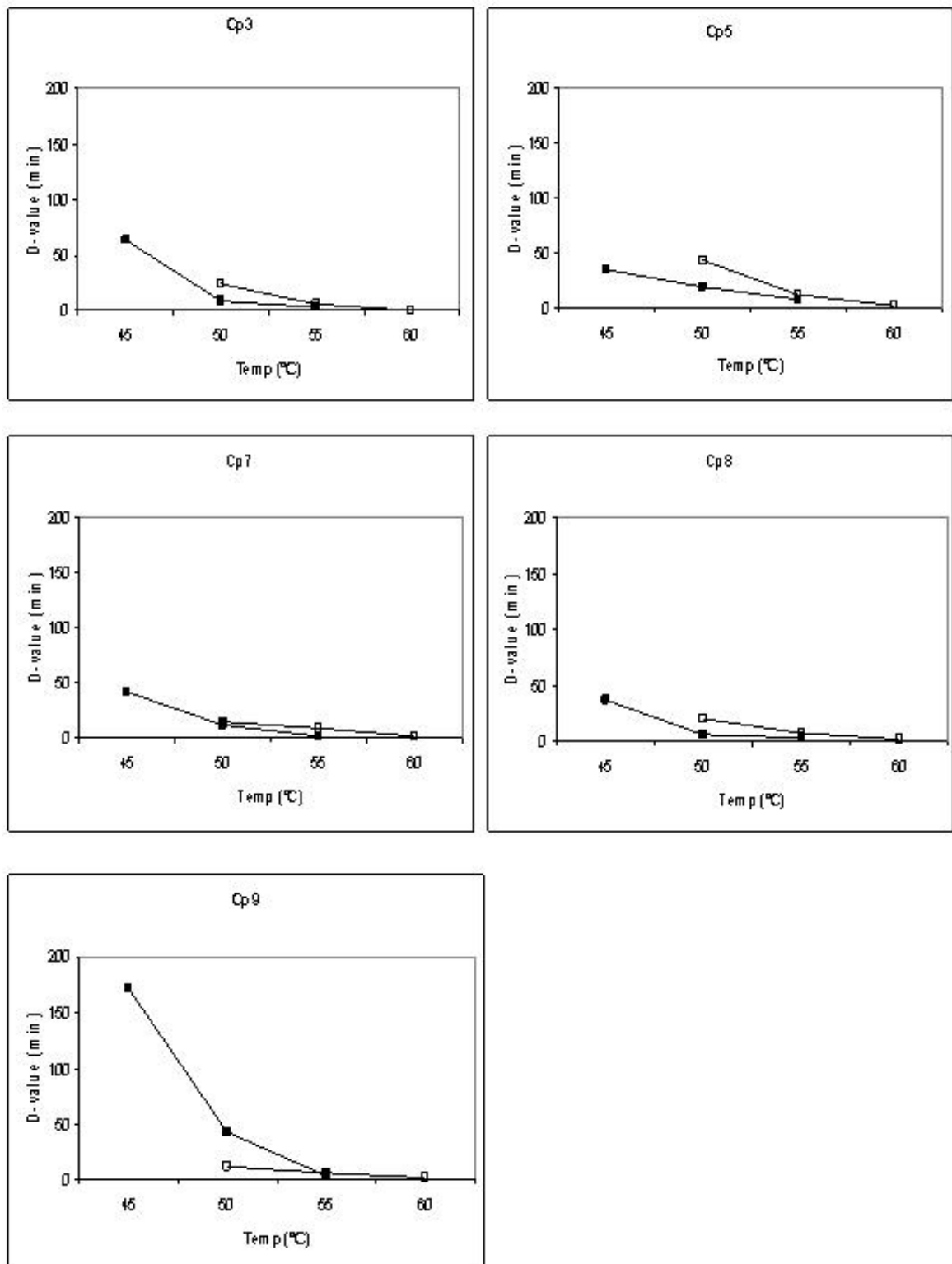


Figure 4 Comparison of D-values of vegetative cells from *C. perfringens* strains Cp 3, 5, 7, 8 and 9 determined in PBS (black squares) and in 10% pea soup (open squares).

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Rijksinstituut  
voor Volksgezondheid  
en Milieu

Postbus 1  
3720 BA Bilthoven  
[www.rivm.nl](http://www.rivm.nl)