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**Bacillus cereus: characteristics, behaviour in the gastro-intestinal tract, and interaction with Caco-2 cells**

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This investigation has been performed by order and for the account of Food and Consumer Product Safety Authority (VWA), within the framework of project 250912, Quantitative research of *Bacillus cereus* within the scope of hazard characterization and exposure assessment, and within the Fifth European Community Framework Programme, within the framework of project QLK1-CT-2001-00854, *Bacillus cereus*: Preventing *Bacillus cereus* foodborne poisoning in Europe; detecting hazardous strains, tracing contamination routes and proposing criteria for foods.

## Rapport in het kort

### ***Bacillus cereus*: algemene karakteristieken, gedrag in maagdarmkanaal en interactie met Caco-2 cellen.**

Door het eten van voedsel dat besmet is met de bacterie *Bacillus cereus* kan diarree ontstaan. Onderzoek naar de bacterie zelf en het ziekmakend proces hebben geleid tot meer kennis over het optreden van de ziekte.

Allereerst zijn stammen van de bacterie op grond van specifieke karakteristieken ingedeeld. Hierbij is onder andere gebruik gemaakt van kunstmatig darmsap en lichaamstemperatuur om de groeimogelijkheden van de verschillende stammen *Bacillus cereus* goed te kunnen bepalen.

Daarnaast bleek dat slechts een beperkt aantal stammen ziekte kunnen veroorzaken. Dit zijn voornamelijk stammen die goed groeien bij 37°C en die zich goed kunnen aanpassen aan omstandigheden zoals die voorkomen in de dunne darm.

Een essentiële stap in het ziekmakend proces blijkt de hechting van de *Bacillus cereus* aan de wand van de dunne darm te zijn. Dit proces is in het onderzoek nagebootst met Caco-2 cellen. Daarnaast kunnen de cellen van de dunne darm aanzetten tot ontkieming en vermenigvuldiging van *Bacillus cereus* sporen. Sporen zijn bacteriecellen die zich in een soort sluimerstand bevinden.

In een wetenschappelijke publicatie zullen gegevens worden gepubliceerd ten aanzien van het vóórkomen van *Bacillus cereus* bacteriën in voedsel.

*Trefwoorden:* *Bacillus cereus*, algemene karakteristieken, ziekmakend vermogen, dunne darm, ontkieming.

## Abstract

### ***Bacillus cereus*: characteristics, behaviour in gastro-intestinal tract, and interaction with Caco-2 cells.**

The consumption of food contaminated with the bacterium *Bacillus cereus* may lead to diarrhoea. Investigation of characteristics of the bacterium and the pathogenesis increased knowledge regarding the onset of disease.

Strains of *Bacillus cereus* were categorised according to specific characteristics. Besides artificial media to determine the growth characteristics of the strains, simulated intestinal fluid and human body temperature were used.

Diarrhoeal disease is mainly caused by strains which grow well at 37°C and which adapt easily to small intestinal conditions.

Adhesion of *Bacillus cereus* to the epithelium of the small intestine appears to be of vital importance for the onset of disease. Moreover, epithelial cells are able to induce germination of *Bacillus cereus* spores. These are forms of the bacterium able to withstand a variety of adverse conditions, such as dryness and heat.

Data on the occurrence of *Bacillus cereus* in food commodities in The Netherlands will be published elsewhere.

*Key words:* *Bacillus cereus*, general characteristics, pathogenesis, small intestine, germination.



# Contents

<b>Samenvatting</b>	<b>6</b>
<b>Summary</b>	<b>7</b>
<b>1. Introduction</b>	<b>9</b>
<b>2. Characteristics of <i>Bacillus cereus</i></b>	<b>11</b>
2.1 Introduction	11
2.2 Materials and Methods	11
2.3 Results	14
2.4 Discussion	16
2.5 Conclusions	17
<b>3. Behaviour of <i>Bacillus cereus</i> in the gastro-intestinal tract under simulated circumstances</b>	<b>19</b>
3.1 Introduction	19
3.2 Materials and Methods	19
3.3 Results	23
3.4 Discussion	28
3.5 Conclusions	33
<b>4. Interaction between <i>Bacillus cereus</i> and differentiated Caco-2 cells (as a model for the epithelium of the small intestine)</b>	<b>35</b>
4.1 Introduction	35
4.2 Materials and Methods	36
4.3 Results	38
4.4 Discussion	40
4.5 Conclusions	42
<b>5. General discussion and conclusion</b>	<b>43</b>
<b>References</b>	<b>45</b>
<b>Appendix 1 Simulated gastric and intestinal fluid</b>	<b>49</b>
<b>Appendix 2 Characteristics of <i>Bacillus cereus</i></b>	<b>53</b>
<b>Appendix 3 Behaviour of <i>Bacillus cereus</i> in the gastro-intestinal tract under simulated circumstances</b>	<b>63</b>
<b>Appendix 4 Interaction between <i>Bacillus cereus</i> and differentiated Caco-2 cells (as a model for the epithelium of the small intestine)</b>	<b>83</b>

## Samenvatting

*Bacillus cereus* is een bacterie die bij vóórkomen in voedsel twee typen aandoeningen kan veroorzaken: het braaksyndroom en het diarreesyndroom. De bacterie kan voorkomen als vegetatieve cel, maar ook als spore. Dit rapport handelt voornamelijk over het diarreesyndroom.

Omdat de ziekteverschijnselen van het diarreesyndroom over het algemeen vrij mild van aard zijn en vrij kort duren, wordt zelden een arts geraadpleegd. Daardoor is het moeilijk een goede schatting van het optreden van dit syndroom te maken. Om een betere schatting van het vóórkomen te maken zijn gegevens nodig omtrent de aanwezigheid van *B. cereus* in voedsel en gegevens ten aanzien van het ziekmakend mechanisme. Ook gegevens over karakteristieke eigenschappen van *B. cereus* zijn van belang.

Gegevens ten aanzien van het vóórkomen van *B. cereus* in voedsel in Nederland zullen elders worden gepubliceerd. In dit rapport wordt voornamelijk ingegaan op het ziekmakend mechanisme.

In hoofdstuk 1 van dit rapport wordt een aantal karakteristieken van circa 30 *B. cereus* stammen op een rij gezet. Dit betreft vooral gegevens over groeisnelheden en aanpassingsfasen bij diverse temperaturen. Op basis van deze gegevens is een begin gemaakt om de stammen in te delen in psychrotrofe (i.e. stammen die kunnen groeien bij temperaturen < 10°C, maar niet zo goed bij 37°C) en mesofiele stammen (i.e. stammen met goede groei bij 37°C, maar geen groei bij temperaturen < 10°C). Het bleek echter moeilijk op basis van deze gegevens een duidelijk onderscheid te maken tussen psychrotrofe en mesofiele stammen. Groeisnelheden en aanpassingsfasen gemeten in media die de *in vivo* situatie zo dicht mogelijk benaderen, zoals het gebruik van gesimuleerd darmsap in plaats van artificieel cultuurmedium, maken een beter onderscheid tussen psychrotrofe en mesofiele stammen mogelijk. Tevens blijkt uit ons onderzoek dat de hittegevoeligheid van sporen van psychrotrofe stammen groter is dan die van sporen van mesofiele stammen.

Hoofdstuk 2 van het rapport behandelt het gedrag van *B. cereus* in het maagdarmkanaal. Experimenten met gesimuleerd maag- en darmsap wijzen uit dat mesofiele stammen beter bestand zijn tegen de omstandigheden in het maagdarmkanaal dan psychrotrofe stammen. Niet alleen de temperatuur is daarbij van belang, ook de samenstelling van de inhoud van de dunne darm speelt een rol.

In hoofdstuk 3 van dit rapport wordt nader ingegaan op de interactie tussen *B. cereus* en het epitheel van de dunne darm, het voornaamste doelwit van de enterotoxinen van *B. cereus*. Dit onderzoek is uitgevoerd met een cellijn die het epitheel simuleert. Cellen van *B. cereus* blijken te kunnen hechten aan het epitheel van de dunne darm, hetgeen een voorwaarde lijkt voor het optreden van ziekteverschijnselen. Daarnaast kunnen dunne darmcellen sporen van *B. cereus* aanzetten tot ontkieming, een noodzakelijke stap om tot groei en de daaraan gerelateerde productie van toxinen over te kunnen gaan.

Samenvattend kan worden gesteld dat gegevens met experimenten, die de *in vivo* situatie zoveel mogelijk benaderen, leiden tot andere uitkomsten in risicoschattingen dan wanneer gegevens worden gebruikt die verkregen zijn in experimenten met algemene media.

## Summary

*Bacillus cereus* may cause two types of food borne disease: an emetic syndrome and a diarrhoeal syndrome. Complicating factor is that the organism may occur as vegetative cell, and as heat resistant spore. In this report mainly the diarrhoeal syndrome will be dealt with.

The symptoms of the diarrhoeal syndrome are not very severe and of short duration. Therefore, few ill people will see a physician. This leads to a too low estimation of incidence of the disease. To improve this estimation data on occurrence of *B. cereus* in food commodities and data on the pathogenic mechanism are necessary. Also data on the characteristics of *B. cereus* are of importance. Data on occurrence will be published elsewhere, this report deals mainly with the pathogenic mechanism and characteristics.

Chapter 1 is a compilation of data on growth speed and lag phase of circa 30 *B. cereus* strains determined in artificial culture medium. These data were used to discriminate between psychrotrophic (i.e. strains able to grow at temperatures below 10°C, but with poor growth at 37°C) and mesophilic strains (i.e. strains that do not grow at temperatures below 10°C, but grow well at 37°C). Determination of growth speed and lag phases in simulated intestinal fluid proved to show better discrimination between psychrotrophic and mesophilic strains. Our researches show heat sensitivity of spores to be related to the growth temperature profile of the strains. Spores of psychrotrophic strains appear to be more heat sensitive than spores of mesophilic strains.

The behaviour of spores and vegetative cells in the gastro-intestinal tract is the subject of research in chapter 2 of this report. Using simulated fluids, the mesophilic strains appear better to withstand the gastro-intestinal environment than psychrotrophic strains. Not only temperature plays a role, also the composition of the intestinal fluid is of importance.

In the last part of this report the interaction between the epithelium of the small intestine (simulated by using a human cell line) and *B. cereus* is investigated. Spores and vegetative cells are able to adhere to the epithelial cells, apparently a prerogative for the onset of diarrhoeal disease. Moreover, the epithelial cells are able to induce germination of spores of *B. cereus*, an important step before start of growth and subsequent enterotoxin production.

Overall can be concluded that the outcome of a risk assessment is more realistic using data from experiments closely relating the *in vivo* situation instead of data from a more artificial design.





# 1. Introduction

The genus *Bacillus* consists of Gram positive, spore forming microorganisms that are ubiquitously distributed in the environment. The genus is subdivided in three groups based upon cellular morphology and physiological properties. Group 1, the *Bacillus cereus* group, consists of *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. anthracis*, and *B. weihenstephanensis*. Several species from this group receive special attention as either suspect or proven pathogens. *B. thuringiensis* is well known and widely used as insecticidal agent; *B. anthracis* is well known as the agent that causes anthrax, a disease that may affect mammals; *B. cereus* is known to cause food borne disease.

The close relationship of this group is emphasized by their similarity on DNA-level (Daffonchio *et al.*, 1998; Helgason *et al.*, 2000).

*B. cereus* can cause two forms of food borne disease, an intoxication or emetic syndrome, and a toxico-infection or diarrhoeal syndrome (Kramer and Gilbert, 1989). For both syndromes the incubation time and the duration are relatively short. As a consequence many patients do not see physicians, which leads to an underestimation of the number of cases. Nevertheless cases of food borne disease caused by *B. cereus* comprise yearly around 4% of the total number of food borne diseases, as reported through the Food and Consumer Safety Authority (Duynhoven *et al.*, 2005). The majority of these reports concern the emetic syndrome, since outbreaks of this syndrome usually involve two or more individuals at the same time. The diarrhoeal syndrome is thought to involve usually individual cases, but no hard figures on this are available.

The incubation time of the emetic syndrome varies between 30 minutes and 5 hours, the duration of symptoms usually is 24 hours. The pathogenesis of this syndrome is well known: emetic toxin (or cereulide) is produced by *B. cereus* in the incriminating food commodity prior to consumption. After ingestion of the contaminated food, the toxin reaches the small intestine, activating a 5HT-3 receptor and thus triggering emesis (Agata *et al.*, 1995).

The incubation time of the diarrhoeal syndrome varies between 6 – 24 hours, and the duration of symptoms is around 24 hours (Kramer and Gilbert, 1989; Granum, 1994). The pathogenesis of the diarrhoeal syndrome is more complicated. *B. cereus* cells (spores and/or vegetative cells) are ingested with contaminated food. After passing the stomach and reaching the small intestine *B. cereus* must grow and produce enterotoxins that are responsible for the diarrhoeal symptoms. Many aspects concerning the pathogenesis of this syndrome, however, need more clarification. These include growth characteristics from *B. cereus* in food, survival and growth of *B. cereus* in the gastro-intestinal tract and the interaction between *B. cereus* and the epithelium of the small intestine. This report is concerned solely with the diarrhoeal syndrome, the emetic syndrome is discussed elsewhere.

This report is a compilation of various investigations regarding: 1) characteristics of *B. cereus* that are of importance to survival during preparation of food, 2) behaviour of *B. cereus* in simulated gastro-intestinal conditions in order to estimate the influence of physical conditions on the survival and growth of *B. cereus*, and 3) the interaction between *B. cereus* and differentiated Caco-2 cells as a model for epithelial cells of the small intestine being the target site for the enterotoxins. Data regarding the prevalence of *B. cereus* in food commodities in the Netherlands will be published elsewhere (Wijnands *et al.*, in preparation a).

Most investigations have been carried out with a set of twelve strains to mimic the diversity of *B. cereus*.

## 2. Characteristics of *Bacillus cereus*

### 2.1 Introduction

*Bacillus cereus* is a microorganism that can grow at a great variety of temperatures, ranging from psychrophilic to thermophilic strains, strains able to grow at very low or very high temperatures, respectively (Kramer and Gilbert, 1989).

Within the framework of food safety and human disease, only a limited number of strains is of importance. After processing, food is often stored at refrigerator temperatures (4 - 10°C), and strains able to grow or survive at these temperatures may be of importance. Since the main steps in the diarrhoeal syndrome occur in the gastro-intestinal tract, strains able to grow at 37°C and higher are also of importance.

The potential of strains to grow at certain temperatures is most obviously determined by assessing growth rate at the temperature of interest. However, recently a molecular biological method has been described (Stetten *et al.*, 1998), which is able to discriminate between strains able to grow below 7°C (psychrotrophic stains) and strains unable to grow below 7°C (mesophilic strains). This PCR method is based on small differences in base sequence in the 16S rDNA of the strains. Such differences have not only been found in *B. cereus* strains, but also in *B. thuringiensis*, and *B. mycoides*.

The ability of *Bacillus* strains to form spores plays an important role in the concept of food safety. Spores are highly resistant to environmental influences, and therefore attention must be paid to the type and duration of processes in food preparation. Heating is a process step often included in food preparation. Where bacterial vegetative cells do not survive proper heating, spores are much more heat resistant and their elimination requires more drastic measures. Therefore, more knowledge on the heat resistance of spores is of importance.

### 2.2 Materials and Methods

#### 2.2.1 Strains

The strains that were selected for research are described in Table A.2.1 (see Appendix 2). The “Sensor project” (De Wit *et al.*, 2001) was a population-based cohort study on gastroenteritis in the Netherlands. NVH is the Norwegian School on Veterinary Science, INRA is the French National Institute for Agronomic Research, DSMZ is the German Collection of Microorganisms and Cell Cultures, NCTC is the British National Collection of Type Cultures.

#### 2.2.2 Spore production

Following overnight growth at 30°C in Brain Heart Infusion (BHI) broth, all selected

*B. cereus* strains (see Table A.2.1) were grown on polymyxine pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (Holbrook and Anderson 1980) for 48 hours at 30°C. Colonies were collected, suspended in and washed with sterile demineralized water until milky white. The spores were suspended in physiological salt solution, heated 10 minutes at 80°C and stored at -70°C or -20°C in physiological salt solution in 1.5 ml vials (1 ml per vial), or stored in 30% glycerol in physiological salt solution in 1.5 ml vials containing glass beads at -70°C or -20°C.

### **2.2.3 Growth characteristics**

#### **2.2.3.1 Growth characteristics in Brain Heart Infusion broth**

##### **Growth at 10°C**

A glass bead from the -20°C spore stock of each strain was transferred to a tube containing 10 ml BHI-broth. Following incubation at 30°C (18-24 hours), 100 µl BHI-culture was transferred to 50 ml BHI-broth in a 60 ml infusion bottle (precooled at 10°C), and placed in a 10°C water bath (the water bath was placed in a room conditioned at 4°C). Strains were checked every 24 hours for turbidity. The optical density at 600 nm (OD600) was measured from the moment the first signs of turbidity were visible. When the OD600 was more than 0.7, the sample was diluted in physiological salt solution before measurement. The OD600 was measured until no changes were seen for more than two days. Purity of the culture after the experiment was checked by culture on Columbia agar with sheep blood (Oxoid) at 30°C during 18-24 hours. Experiments were conducted in duplicate.

Since most of the OD 600 values in the experiments at 10°C did not exceed the 0.100 value, growth characteristics were also assessed in separate experiments by making decimal dilutions of samples, plating these on Trypton Soy Agar (TSA), and incubating the plates 18-24 hours at 30°C. These experiments were carried out twice, with 12 strains out of the 30 strains of the initial selection, based on their growth at 28 and 37°C.

##### **Growth at 28°C, 37°C, and 42°C**

The method at these temperatures differed from the growth experiments at 10°C at the following points: 1) the temperatures of the water bath were 28°C, 37°C, or 42°C, and 2) the duration of the experiments was 10 hours at 28°C, and 8 hours at 37°C and 42°C. Most experiments were conducted in duplicate.

#### **2.2.3.2 Growth characteristics in simulated intestinal fluid**

##### **Growth at 37°C**

Growth of *B. cereus* strains at 37°C in complete simulated intestinal fluid (SIF, including bile salts and pancreatic juice, described in Appendix 1 was carried out with 12 strains (see 2.2.3.1, Growth at 10°C). These strains have been selected from the strains used for the growth characteristics in BHI based on their growth profiles and PCR results. These twelve strains were also used for further experiments regarding investigations into the pathogenic pathway of the diarrhoeal syndrome. Simulated intestinal fluid was prepared based on a

previously described method (Rotard *et al.*, 1995), and pipetted in 60 ml infusion bottles (50 ml per bottle).

After thawing of the spores (from the  $-70^{\circ}\text{C}$  stock in physiological salt solution) at room temperature, 100  $\mu\text{l}$  spore suspension was added to 50 ml SIF (preheated at  $37^{\circ}\text{C}$ ). Bottles were kept at  $37^{\circ}\text{C}$  in a water bath. Samples were taken at  $t=0^1$ , 1, 2, 3, and 4. From the samples the number of colony forming units were determined by plating 10-fold dilutions in duplicate on Trypton Soy Agar (TSA), and incubating the plates 18-24 hours at  $30^{\circ}\text{C}$ . Purity of the spores was assessed by plating samples at  $t=0$  and  $t=4$  on Columbia agar with sheep blood (Oxoid).

### **2.2.3.3 Evaluation of results and calculations**

All OD600 and culture data of the experiments were collected in Excel-files. The OD600 values were transformed to LN (natural logarithm). These data were plotted, and a straight line was calculated. The generation time was calculated as follows:  $\text{LN}2/\text{RC} = \text{generation time}$ . The lag phase was calculated as follows:  $\text{LN}(\text{intersection with the y-axis})/\text{RC} = \text{lag phase}$ .

When using the culture data, the data were plotted, and preferably in the logarithmic part of the curve Excel calculated the linear equation. Both the generation time and the lag phase were determined as described for the OD 600 data.

The generation times and lag phases for all strains are shown in this report.

### **2.2.4 Growth temperature profile**

Two methods were used to determine whether strains were psychrotrophic or mesophilic, namely a PCR-method based on a difference in 16S rDNA signature between psychrotrophic and mesophilic strains (Stetten *et al.*, 1998) and a culture method in which growth at  $10^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  were the main parameters.

#### **2.2.4.1 Determination of growth temperature profile by PCR**

The method was carried out as previously described (Stetten *et al.*, 1998). Briefly, *B. cereus* strains were grown in Brain Heart Infusion (BHI) broth, overnight at  $30^{\circ}\text{C}$ . A PCR mixture was made as follows: 5  $\mu\text{l}$  10x PCR-buffer (Roche Molecular Biochemicals), 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  of each of the deoxynucleoside triphosphates, 300  $\mu\text{M}$  of each primer [MF, UR, UF, and PR, according to von Stetten (Stetten *et al.*, 1998)], 2.5 units AmpliTaq polymerase (Roche Molecular Biochemicals), 1  $\mu\text{l}$  template DNA (= 1  $\mu\text{l}$  BHI-culture), and sterile Milli-Q water to a final volume of 50  $\mu\text{l}$ . The PCR was carried out in a Hybaid thermal cycler (Thermo Electron Corporation) with the following program: 2 minutes at  $94^{\circ}\text{C}$  (1 cycle), 30 cycles of 15 seconds at  $94^{\circ}\text{C}$ /15 seconds at  $55^{\circ}\text{C}$ /15 seconds at  $72^{\circ}\text{C}$ , and finally 2 minutes at  $72^{\circ}\text{C}$ . The PCR products were analyzed by electrophoresis in a 2 % agarose gel, using a 100 bp ladder. The psychrotrophic strains display a band at 130 bp, the mesophilic strains a band of 250 bp. Intermediate strains may display both bands.

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<sup>1</sup> hours

#### **2.2.4.2 Determination of growth temperature profile by culture at various temperatures**

For these determinations the data on growth characteristics at 10°C, 37°C, and 42°C, as described under chapter 1.2.3.1, were used. These temperatures are most important in determining whether strains are mesophilic or psychrotrophic. The generation time data were plotted in bar-graphs.

#### **2.2.5 Heat resistance of *Bacillus cereus* spores**

The heat resistance of *B. cereus* spores was determined as previously described (Dufrenne *et al.*, 1994). Briefly, six glass tubes with screw cap with rubber inlay, each containing 7 ml phosphate buffered physiological salt solution (0.07M, pH 7.2), were submerged in a water bath at the desired temperature. Spores, stored at -20°C and thawed at room temperature, were added and incubated for 25, 20, 15, 10, 5 and 0 minutes (for each time period a different tube was used). After the indicated time, all tubes were placed on melting ice until further research. Samples from each tube were investigated by plating 100 µl of decimal dilutions in duplicate on Trypton Soy Agar (TSA), and incubating the plates 18-24 hours at 30°C.

The temperatures at which the heat resistance was measured were 80°C, 85°C, 87.5°C, 90°C, 95°C, and 97.5°C. From the colony counts plotted against time, D-values at each temperature were determined for each strain. Not each temperature was applied to each strain, but for each strain at least three different temperatures were used to obtain an inactivation curve from which the D-value could be calculated. Also, dependant on the results or expected results, the time periods, in which measurements were made, were varied.

#### **2.2.6 Statistical analysis**

Statistical analyses were performed in Excel (Microsoft) using the t-test: two-sample assuming unequal variances.

### **2.3 Results**

For figures and tables mentioned in this chapter see Appendix 2.

#### **2.3.1 Determination of generation times using BHI**

The results of the calculations of the experiments for determining the growth characteristics are shown in Tables A.2.2 to A.2.6. The Tables A.2.2, A2.2.4, A2.2.5, and A2.2.6 are based on OD 600 measurements at 10°C, 28°C, 37°C, and 42°C in BHI, respectively. The OD values at 10°C in BHI did not exceed the 0.100, and were regarded as unreliable. Therefore, the experiments at 10°C were repeated, growth was monitored by culturing samples on TSA-plates, and the results of these experiments are shown in Table A.2.3. Also the generation

times at 10°C, 37°C and 42°C are shown in Figures A.2.1, A.2.2 and A.2.3, in which the strains with immeasurable generation times are shown as having a very long generation time. The results for some strains have not been shown (see Tables A.2.2 and A.2.6, strains with the indication “n.d.”), since growth of these strains took so long that these experiments were stopped and the results were not recorded. Therefore, the generation times and lag phases could not be determined reliably. In Figures A.2.1, A.2.2 and A.2.3, that show the results at 10°C, 37°C and 42°C respectively, these unreliable generation times are shown as bars exceeding the generation time used for the Y-axis.

In the legend at the bottom of Tables A.2.2, A.2.4, A.2.5 and A.2.6 the 95% confidence intervals for the mean generation times and the mean lag phases are mentioned. Strains with generation times smaller than the interval grew well at the indicated temperature, strains with generation times above the interval grew poorer at the indicated temperature. Also the lag phases were taken into consideration. Strains were considered psychrotrophic based upon good growth and short lag phase at 10°C, and poorer growth and long lag phases at 37°C and 42°C. Mesophilic strains were generally characterized by poorer growth and long lag phases at 10°C, and good growth and short lag phases at 37°C and 42°C. For example, strain PAL 2 has a relative short mean generation time and an average lag phase at 10°C (Table A.2.2), at 37°C both generation time and lag phase are on average, and at 42°C both parameters are below average. Combined with the corresponding lag phases PAL 2 was considered to be mesophilic. Therefore, from the tables and the figures the growth temperature profile of the strains can be distinguished. Strains PAL 17, PAL 18, PAL 20, and PAL 22 have relatively low generation times at 10°C, and relatively high generation times at 37°C. The differences with the rest of the strains is not significant ( $P > 0.05$ ), but the tendency is clear. Also, strain PAL 17 has a high generation time at 42°C. These four strains were, based on these results, designated as psychrotrophic. Also in the PCR-method these strains showed to be psychrotrophic.

For the experiments at 10°C in BHI with culture of samples only a limited number of strains has been used. This selection was based on the results in BHI at 42°C, 37°C and 10°C with OD-measurement, where not only the OD 600 was measured with the spectrophotometer, but also growth and turbidity were assessed visually: the OD from bottles with non turbid BHI was not yet measured to prevent the occurrence of cross-contamination.

The results at 28°C are the least discriminative, as they were carried out near the optimal growth temperature of *B. cereus.*, and therefore not taken into consideration for determining the growth temperature profile.

### ***2.3.2 Determination of generation times using simulated intestinal fluid***

In Table A.2.7 the results of the calculations for determining generation time and lag phase are presented for the same limited number of strains used to determine these characteristics at 10°C in BHI with culture of samples. Two strains (PAL 3 and PAL 7) showed no growth at all, and therefore no generation time or lag phase could be determined (indicated by “n.g.”). In general short generation times correspond with short lag phases.

These experiments were conducted later than the growth experiments in BHI, when the limited selection of strains had already been divided in psychrotrophic and mesophilic, based on the growth characteristics and PCR-results.

In Figure A.2.4 the results from the calculations of the generation times have been plotted in a graph. The psychrotrophic strains (PAL 3, PAL 17, PAL 18, and PAL 20) appear to have higher generation times than the mesophilic strain with the exception of strain PAL 25. Two psychrotrophic strains (PAL 7 and PAL 22) did not show growth in simulated intestinal fluid at 37°C, and therefore no bars are shown for these two strains. The P-value of a t-test is slightly higher than 0.05, and therefore the difference between the mesophilic strains (minus strain PAL 25) and the psychrotrophic strains is not significant.

### ***2.3.3 Determination of growth temperature profile by PCR***

From the results of the growth experiments growth temperature profile of the various strains can be derived. Most important temperatures were 10°C, 37°C, and 42°C. Not only the generation time is of importance, also the lag phase adds to determine growth temperature profile. (See also section 3.2).

In Table A.2.8 the PCR-results are shown. A distinction is made on the basis of PCR-signature between psychrotrophic (p) and mesophilic (m) strains.

### ***2.3.4 Heat resistance of spores***

In Table A.2.9 and Figure A.2.5 the results of the determination of heat resistance of spores of various strains at various temperatures are shown, represented as D-values in minutes.

In Figure A.2.5, the data from Table A.2.9 are shown separately for psychrotrophic (x) and for mesophilic strains (▲), irrespective of the strain number. The individual strains are not shown, but this graph shows the difference in D-values between mesophilic and psychrotrophic strains in a different manner. From Figure A.2.5 can be derived that, in general, the mesophilic strains have higher D-values than the psychrotrophic strains. The difference at 90°C, the only temperature with data of all twelve strains, is not significant ( $p > 0.05$ ), but the tendency in Figure A.2.5 is clear.

## **2.4 Discussion**

The generation times of strains PAL 17, PAL 18, PAL 20, and PAL 22 at 37°C in BHI are significantly higher, and based upon these data these strains may be called psychrotrophic.

At 10°C the data are less discriminative. Some strains do indeed show a sufficiently high deviation from the mean generation time to call them mesophilic (PAL 2, PAL 27) or psychrotrophic (PAL 20, PAL 22). But in general the outcomes are not very clear. Strain 3 appears to be mesophilic from the results at 10°C, but has in the PCR-test a clear psychrotrophic signature.

The growth temperature profile of strains PAL 17, PAL 18, PAL 20, and PAL 22, as detected in growth experiments at 37°C, is confirmed by the PCR test, where these strains show the



psychrotrophic signature. Psychrotrophy of strain PAL 3 can be derived from the results at 37°C and 42°C: at both temperatures this strain fails to grow (as indicated in Figures A.2.2 and A.2.3 by the lacking bars). From the results at 42°C, see Figure A.2.3, there is a strong indication that strain PAL 7 is psychrotrophic. The results for these two strains are confirmed by the PCR-test where both strains show psychrotrophic signatures. From the graphs in Figures A.2.2 and A.2.3 can be read that strains PAL 25, PAL 26, PAL 27, and PAL 28 have low generation times, indicating mesophily of these strains. These results are confirmed by the PCR test.

The growth experiments at 37°C in simulated intestinal fluid (SIF) confirm the results obtained with the other techniques and seem to be more discriminative. Although the difference is not significant due to the aberrant behaviour of strain PAL 25, there is a clear tendency for the psychrotrophic strains to grow slower (as indicated by higher generation times, see Table A.2.7 and Figure A.2.4) than the mesophilic strains. Moreover, two psychrotrophic strains, PAL 7 and PAL 22, do not even grow in SIF.

The difference in heat resistance between psychrotrophic spores and mesophilic spores can best be seen in Figure A.2.5. The heat resistance of mesophilic spores is consequently higher at each of the temperatures at which both types of spores have been tested. At only one temperature all strains have been tested; i.e. 90°C. At this temperature the results, and more specific the differences between psychrotrophic and mesophilic strains, are not significant ( $0.05 < p < 0.1$ ). There is however a strong tendency that the D-values of the mesophilic strains are higher at each temperature than the D-values of the psychrotrophic strains.

*Bacillus cereus* is a species with a large variety of strains. This variety is not only expressed in the growth characteristics at different temperatures, as described in this part of the report. The variety is also expressed in the differences in heat resistance of the spores.

Such information is of importance in risk assessment, when conclusions have to be drawn on the potential health risk of, for example, food preparation processes. One always has to bear in mind the large variety of properties of the species.

## 2.5 Conclusions

The results with growth experiments at various temperatures are generally in concordance with the results by PCR growth temperature profile: the same strains are named psychrotrophic or mesophilic. The PCR results present a black and white discrimination, where the classification based upon growth is less discriminative.

The results of the growth experiments in simulated intestinal fluid confirm and emphasize the differences between psychrotrophic and mesophilic strains. We conclude that data from experiments in simulated intestinal fluid, resembling more closely the *in vivo* situation, are better suitable for modeling than data from tests in culture media.

The differences in growth temperature profile are also expressed in the differences in heat resistance of spores. Although the difference is not significant, spores from mesophilic strains are generally more heat resistant than spores from psychrotrophic strains.

### 3. Behaviour of *Bacillus cereus* in the gastro-intestinal tract under simulated circumstances

#### 3.1 Introduction

The mechanism of the diarrhoeal syndrome is believed to be as follows: food contaminated with *B. cereus*, spores and/or vegetative cells, is consumed. Spores pass the stomach, reach the small intestine, germinate, grow, and produce the enterotoxins. Vegetative cells are believed to be very susceptible to stomach conditions, and will therefore hardly reach the small intestine.

However, during food consumption the pH in the stomach rises. The highest pH and the subsequent drop after ingestion of the food depend on the type and amount of food (Russell *et al.*, 1993; Dressman *et al.*, 1990). The lapse of pH after the ingestion of three types of food (solid, intermediate and fluid) and the role of age in the lapse of pH after food intake have been described before (De Jonge *et al.*, 2003). During food uptake the pH in the stomach may reach values at which vegetative cells of *B. cereus* may survive, and subsequently reach the small intestine. There they may grow and thus contribute to the onset of disease. Results concerning the possible contribution of vegetative cells to the pathogenesis of *B. cereus* will be discussed elsewhere (Wijnands *et al.*, in preparation b).

Aim of these investigations was twofold, namely to confirm the hypothesis that spores pass the stomach unharmed and germinate and grow in the small intestine, and to confirm that the gastric conditions have a negative effect on survival of vegetative cells before they are transferred to the small intestine. All studies are to be performed *in vitro*. As the human gastro-intestinal tract is a very complicated organ it is nearly impossible to mimic the exact circumstances and physiological properties. Our set up includes the use of artificial gastric and intestinal fluids. A variety of formulations used to simulate the gastric environment can be found in the literature (Anonymous, 1995; Cooper *et al.*, 1995; Longland *et al.*, 1977). For the experiments described in this report, formulations were used as previously described for the determination of heavy metals after ingestion (Rotard *et al.*, 1995).

#### 3.2 Materials and Methods

##### 3.2.1 Strains.

The twelve *Bacillus cereus* strains used for these experiments are described in Table A.3.1 (Appendix 3). The strains were grown on MEYP<sup>2</sup>-agar (Mossel *et al.*, 1967) and subsequently tested for glucose fermentation, acetylmethylcarbinol production according to Voges-Proskauer, nitrate-reduction and gelatin turn-over. Purity was checked by plating the strains on Columbia agar with sheep blood and incubating overnight at 30°C. The growth

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<sup>2</sup> MEYP = mannitol egg yolk phenol red

temperature profile of the strains was established by a previously described method based on polymerase chain reaction (Stetten *et al.*, 1998).

### **3.2.2 Spore production.**

Pure *Bacillus cereus* strains were grown on PEMBA<sup>3</sup>-agar (Holbrook and Anderson 1980) for 48 hours at 30°C. Spores were collected by scraping colonies from the plates, using distilled water, and centrifugation (10 minutes 1800xg). Washings and centrifugation were continued until the suspension was milky white (generally 4 times). After the final centrifugation step, the spores were suspended in physiological salt solution, heated 10 minutes at 80°C, and stored in 1.5 ml vials (1 ml per vial) at -70°C. Spore counts were performed after the final suspension of spores in physiological salt solution, but the final suspensions were not adjusted to equal spore concentrations before storage and experiments.

### **3.2.3 Production and use of vegetative cells in gastro-intestinal experiments**

Stationary phase vegetative cells were produced by inoculating 10 ml BHI with a glass bead with spores from the -70°C stock and incubating the inoculated BHI at either 30°C or 37°C, dependent on the growth temperature profile of the strain (psychrotrophic strains were incubated at 30°C, mesophilic strains at 37°C). The incubation-time in BHI was circa 5 hours. Subsequently the strains were inoculated in Luria Bertani Glucose broth (LBG) (pH 7.0) and incubated overnight. Psychrotrophic strains at 30°C, mesophilic strains at 37°C.

Log phase vegetative cells were produced by inoculation of 10 ml BHI with a glass bead with spores from the -70°C stock and overnight incubation at 30°C (psychrotrophic strains) or 37°C (mesophilic strains). Subsequently, LBG-broth was inoculated with BHI culture (0.2 ml BHI-culture to 25 ml LBG broth). The strains were incubated for 4 hours at either 30°C or 37°C dependent on their growth temperature profile characteristics and used in the gastro-intestinal experiments. For experiments with gastric fluid 0.2 ml LBG-culture was added to 50 ml SGF<sup>4</sup>, for intestinal experiments 0.2 ml LBG-culture was added to 50 ml SIF<sup>5</sup>. For combined experiments 0.2 ml LBG-culture was added to 50 ml SGF, whereafter 1 ml of the inoculated SGF was added to 50 ml SIF. All experiments involving SGF or/and SIF were performed at 37°C.

### **3.2.4 Simulated gastric and intestinal fluids**

The simulated gastric and intestinal fluids are based on previously described formulations (Rotard *et al.*, 1995) and the composition of (each of the components for) simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) is described in Appendix 1.

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<sup>3</sup> PEMBA = polymyxin pyruvate egg yolk manitol bromothymol blue

<sup>4</sup> SGF = simulated gastric fluid

<sup>5</sup> SIF = simulated intestinal fluid

### ***3.2.5 Evaluation of survival and growth***

Samples, taken during the experiments, were investigated for the total number of colony forming units (vegetative cells and spores, i.e. total count) and for the number of spores (i.e. spore count) after heating the 10x diluted original samples for 10 minutes at 80°C. Ten-fold dilutions of the samples in physiological salt solution containing 0.1% (w/v) peptone were plated in duplicate on Trypton Soy Agar. Agar plates were incubated overnight (i.e. 18 – 20 hours) at 30°C, and numbers of colonies were recorded from countable plates ( $15 < x < 150$  per plate). The means of the duplicates of total and spore counts were calculated and used for further evaluations.

All experiments were carried out with all twelve strains, unless mentioned otherwise.

### ***3.2.6 Behaviour of Bacillus cereus spores and vegetative cells in gastric conditions***

After thawing, spores were suspended in 50 ml simulated gastric fluid (SGF) at pH 2.5 for 60 minutes at 37°C in a water bath. Samples were taken at the start of the incubation (t=0), after 30 minutes (t=30) and at the end of the incubation period (t=60). From the samples the total number of viable cells and the number of spores were determined.

Vegetative cells, both stationary phase and exponential phase cells, were suspended in SGF of various pH, with and without pepsin. At regular time intervals samples were taken for colony counts. The length of the time intervals was adjusted to the expected survival of the cells, i.e. the lower the pH the lower the expected survival the shorter the time intervals. From the colony counts D-values were calculated, being the time in minutes necessary to decrease at a certain pH the colony count with one log-unit.

### ***3.2.7 Behaviour of Bacillus cereus spores and vegetative cells in intestinal conditions***

After thawing, spores were suspended in 50 ml simulated intestinal fluid (SIF) at pH 7.8 +/- 0.3 in a water bath at 37°C for 4 hours. Vegetative cells (stationary or exponential phase), prepared as described before, were suspended in 50 ml simulated intestinal fluid (SIF) at pH 7.8 +/- 0.3 in a water bath at 37°C for 4 hours. Samples were taken at the start of the experiment (t=0) and at each hour afterwards, expressed in minutes (t= 60, 120, 180, and 240). Samples were investigated for total number of viable cells and numbers of spores.

Vegetative cells, stationary phase and exponential phase, were suspended in the various formulations of SIF including complete SIF, and at regular time intervals samples were taken for colony counts. The colony counts were plotted and from the slope of the resulting graphs generation times in minutes were calculated.

### ***3.2.8 Behaviour of spores and vegetative cells in simulated gastro-intestinal transfer***

After thawing, spores were suspended in 50 ml SGF at pH 2.5. After one hour incubation at 37°C, 1 ml SGF suspension was transferred to 50 ml SIF and incubated further for 4 hours at 37°C.

Samples were collected at the start of the experiment in SGF (t=0 SGF), after 60 minutes incubation in SGF (t=60 SGF), immediately after transfer to SIF (t=0 SIF) and every hour during incubation in SIF, expressed in minutes (t=60 SIF, 120 SIF, 180 SIF, and 240 SIF).

Similar experiments, with SGF of pH 1.0 and of pH 4.0, were carried out with one psychrotrophic and one mesophilic strain.

Stationary and exponential phase cells of the mesophilic strains and stationary phase cells of the psychrotrophic strains were exposed to pH 2.5 in 50 ml SGF. After 30 minutes 1 ml SGF was transferred to 50 ml complete SIF. At regular time intervals (see above, but note that exposure to gastric conditions lasted for 30 instead of 60 minutes) samples were collected for colony counts. The colony counts in complete SIF were recalculated to compensate for the 50-fold dilution of the SGF after transfer to complete SIF.

Exponential phase psychrotrophic cells were not included in this experiment because the yield of cells after 4 hours of incubation in LBG-MOPS at pH 7.0 grown at 37°C was too low.

### ***3.2.9 Influence of low temperature storage on behaviour of spores and vegetative cells in simulated gastro-intestinal transfer***

Since food commodities are often stored in refrigerators, the influence of such treatment on the behaviour of spores in simulated gastro-intestinal conditions was investigated. Following incubation at 10°C overnight, spores were exposed to SGF at pH 2.5 and subsequently to SIF. Samples were collected and investigated as described before. These experiments were carried out with spores from psychrotrophic strains 7, 18, and 22 and mesophilic strains 2, 5, and 27. Comparable experiments were carried out stationary phase vegetative cells from the same strains. No exponential phase cells were used for these experiments.

### ***3.2.10 Influence of pH of gastric fluid on the behaviour of spores and vegetative cells in simulated gastro-intestinal transfer***

Most experiments have been carried out at pH 2.5. However, since the pH in the stomach may vary between nearly 1 and nearly 5, preincubation of spores at pH 1.0 and pH 4.0 was used to assess the subsequent behaviour in SIF. For these experiments spores from one psychrotrophic strain (PAL 22) and one mesophilic strains (PAL 27) were used. Apart from the pH of the simulated gastric fluid, the design of the experiments was the same as described before.

Comparable experiments were carried out with stationary phase vegetative cells from psychrotrophic strains PAL 7, 18, and 22, and mesophilic strains PAL 2, 5, and 27.

### **3.2.11 Reproducibility of experiments**

The simulated gastric and intestinal fluids had to be produced freshly for each experiment. In addition, a limited number of strains that could be tested on one day. Therefore, a variety of batches of simulated fluids were used. To assess the reproducibility of results between tests, spores of strain PAL 2 were subjected to the tests in each experiment in which spores were involved, and served therefore as an internal control.

## **3.3 Results**

In Appendix 3 figures and tables referring to this chapter can be found.

In general, when colony counts of  $< 10$  were detected, for graphs and calculations this number of cfu was represented by 1.

### **3.3.1 Behaviour of spores and survival of vegetative cells in SGF**

In Figures A.3.1 and A.3.2 the effect of exposure of spores of psychrotrophic strains and mesophilic strains respectively to pH 2.5 in simulated gastric fluid with pepsin is shown. Only total counts are reported, at  $t=0$ ,  $t=30$  minutes and  $t=60$  minutes. The counts are reported in logarithms of the colony count (log CFU). Since germination and growth are impossible under these circumstances, the small “increases” and “decreases” of the total counts of the strains in Figure A.3.1 and A.3.2 are more an indication of the margin of error in colony count. Therefore, only strain PAL 7 (see Figure A.3.1), with a decrease of nearly one log-unit in the total counts, appears to be slightly affected by pH 2.5 in simulated gastric fluid.

Taking the same margin of error into account as with the psychrotrophic strains, none of the mesophilic strains show increase or decrease of the colony counts during the total time of exposure, indicating that pH 2.5 in simulated gastric fluid does not affect the spores of the mesophilic strains.

The results of survival of vegetative cells, stationary phase and exponential phase, at low pH values in SGF are described in tables 2 and 3 respectively. The results are presented as D-values in minutes, i.e. time in minutes in which the colony count decreases with one log-unit. The stationary phase cells appear to be more acid tolerant. This can be derived from the difference in D-values of the stationary phase cells at pH 4.0 (Table A.3.2) compared with the D-values of the exponential phase cells at pH 4.0 (Table A.3.3). The average D-value of the stationary phase cells at pH 4.0 is about 10 times higher than the average D-value of the exponential phase cells. At pH 3.5 these differences in D-value become less apparent for most of the strains. In general the mesophilic exponential phase cells appear to be more acid tolerant than the psychrotrophic exponential phase cells, because the average D-value without pepsin for the mesophilic strains at pH 4.5 is 62 minutes, whereas for the psychrotrophic strains the average D-value at pH 4.5 is 45 minutes (see Table A.3.3). Also with pepsin there is such difference in average D-values. At pH 4.0 with the stationary phase cells (see Table

A.3.2), the differences in average D-values between the stationary phase mesophilic and psychrotrophic strains are much smaller (see Table A.3.2).

### **3.3.2 Behaviour of spores and vegetative cells in complete SIF**

The effect of exposure of spores to complete SIF during 4 hours (= 240 minutes) is depicted in Figures A.3.3, A.3.4, A.3.5 and A.3.6. The incubation time is set at the X-axis, the colony counts on a logarithmic scale at the Y-axis. Figures A.3.3 and A.3.4 show the results of total and spore counts respectively when spores of the psychrotrophic strains were used. In Figures A.3.5 and A.3.6 the results of total and spore counts respectively for the spores of the mesophilic strains are shown. From the psychrotrophic strains only strains PAL 17 and PAL 18 show a slight increase in total count during the time of the experiment (see Figure A.3.3). These are also the strains that show the highest degree of germination, since the spore count from both these strains after 1 hour of exposure drops more than 1 log unit (see Figure A.3.4). From the mesophilic strains only strain PAL 25 shows hardly any increase in total count (see Figure A.3.5). This is also reflected by the lack of decrease in spore count of this strain indicating that hardly any germination took place (see Figure A.3.6). Also strain PAL 5 does not show any measurable decrease in spore count (Figure A.3.6), although contrary to strain PAL 25 strain PAL 5 does show an increase in total count indicating growth in the complete simulated intestinal fluid (Figure A.3.5).

Mesophilic strain PAL 26 shows a remarkable decrease in spore count over the duration of the experiment (see Figure A.3.5). This spectacular decrease is not reflected in an equal increase in total count (see Figure A.3.6).

The results of the investigations concerning the behaviour of vegetative cells in simulated intestinal fluid (SIF), in SIF with bile salts, in SIF with pancreatin, and in complete SIF (= SIF with bile salts and pancreatin) are shown as generation times, calculated from the colony counts at regular time intervals, in Tables A.3.4 and A.3.5 for stationary phase and exponential phase cells, respectively.

Generation times over 120 minutes were recorded as >>>, since the slope of the graph (and therefore the increase in numbers of cfu) was so small that the errors in the calculations would be too high.

As can be derived from Tables A.3.4 and A.3.5 the addition of bile or pancreatin results in higher generation times for exponential and stationary phase cells. In complete SIF (with bile and pancreatin) the generation times are higher than in SIF (i.e. no bile and pancreatin included).

For the stationary phase cells of the psychrotrophic strains all generation times are over 120 minutes, while only one stationary phase mesophilic strain (PAL 2) has a generation time over 120 minutes (see Table A.3.4). In the exponential phase two psychrotrophic strains show generation times of over 120 minutes (see Table A.3.5). No mesophilic strain in exponential phase shows generation times of more than 120 minutes.



### ***3.3.3 Behaviour of spores and vegetative cells in complete SIF after exposure to pH 2.5 in SGF***

The effect of exposure of spores to complete SIF after exposure to pH 2.5 in SGF (simulating passage through the stomach into the small intestine) is depicted in Figures A.3.7, A.3.8, A.3.9 and A.3.10. Figures A.3.7 and A.3.8 show the results of total and spore counts of the psychrotrophic strains. The results of the total and spore counts of the mesophilic strains are depicted in Figures A.3.9 and A.3.10. In all figures the colony counts in the complete SIF are calculated values and shown on a logarithmic scale at the Y-axis as a function of exposure time at the X-axis. In the experiment only 1 of 50 ml of SGF was transferred to 50 ml complete SIF, causing a 50-fold drop in actual colony count. In order to compensate for this drop in colony count the colony counts for the complete SIF were recalculated by multiplying them with a factor 50. As in the experiment in complete SIF, in this combination experiment the psychrotrophic strains PAL 17 and 18 show an increase in total colony count during the time of the experiment (see Figure A.3.7). These are also the strains that show the highest degree of germination, as can be derived from the decrease in spore count throughout the course of the experiment (see Figure A.3.8).

From the mesophilic strains strain PAL 5 shows hardly any increase in total count (see Figure A.3.9). This corresponds to the lack of decrease in spore count of this strain indicating that hardly any germination took place (see Figure A.3.10). Also strain PAL 25 does not show any decrease in spore count (Figure A.3.10), although contrary to strain PAL 5 strain PAL 25 does show an increase in total count indicating growth in the complete SIF (Figure A.3.9). All other mesophilic strains show both increase in total counts and decrease in spore count.

The vegetative cells were exposed to complete SIF after 30 minutes of exposure to pH 2.5 in SGF. In Figures A.3.19 and A.3.20 the results are shown for the stationary phase cells (psychrotrophic and mesophilic strains respectively), and in Figure A.3.21 the results for the exponential phase of all the mesophilic strains. In all three figures calculated values for the numbers of cfu in SIF are shown to compensate for the transfer of 1 ml SGF to 50 ml SIF. To indicate the effect of transfer of cells from pre-incubation medium to SGF, the colony counts of the starting material have also been incorporated in the figures. Immediately after transfer of cells from the pre-incubation medium to SGF a sample was taken of the SGF to determine the loss of viability upon exposure to SGF. From the stationary phase cells of the psychrotrophic strains only strain PAL 7 does not seem to recover from the low pH-shock after 4 hours in complete SIF; from the stationary phase mesophilic cells PAL 5 does not recover after 4 hours of exposure in complete SIF.

The experiments with exponential phase cells in complete SIF after exposure to pH 2.5 in SGF were carried out only with the mesophilic strains since the psychrotrophic strains grew so slow at 37°C prior to exposure to SGF that the numbers were too low to give reliable results (see Figure A.3.21). The experiment involving strain PAL 26 was unsuccessful. Exponential phase cells seem to be more susceptible to low pH than stationary phase cells because only two of the five strains grew after 4 hours of exposure in complete SIF, while

five out of six strains in stationary phase showed growth after 4 hours (compare Figures A.3.20 and A.3.21).

### ***3.3.4 Influence of different pH's in SGF on the behaviour of spores in complete SIF***

The experiments described in section 2.3.3 have been carried out at pH 2.5 in SGF preceding the exposure to complete SIF. To investigate the influence of other pH's in SGF one psychrotrophic and one mesophilic strain have been exposed in SGF to pH 1.0, 2.5 and 4.0 prior to transfer to complete SIF. In figures 11 and 12 the results are shown. Here too, the colony counts in complete SIF have been recalculated to compensate for the dilution step during transfer from SGF to complete SIF. Only total counts are shown on a logarithmic scale at the Y-axis as a function of exposure time on the X-axis. The colony counts of the psychrotrophic strain (PAL 22) after exposure to pH 1.0 appear to be affected, indicated by the slightly lower colony counts (see Figure A.3.11). Between the colony counts after exposure to pH 2.5 and pH 4 respectively no difference can be observed. All in all no increase in colony count can be observed, indicating that this psychrotrophic strain is unable to germinate and grow in simulated intestinal conditions.

Exposure to pH 1 in SGF appears to decrease the number of viable spores from the mesophilic strain (PAL 27). However, the ability to germinate and grow is not affected (see Figure A.3.12). Preincubation at pH 1 appears to lead to lower generation times after germination of the spores, but spore counts are lower than after pre-exposure to pH 2.5. In Figure A.3.12 this phenomenon can be derived from the smaller slope of the curve between  $t=180$  and  $t=300$ . From the colony counts, on which the graphs in figure 12 are based, could be calculated that the generation time between  $t=180$  and  $t=300$  is 45 minutes with pH 1.0 preincubation, versus 29 and 24 minutes with pH 2.5 and pH 4.0 preincubation respectively.

The experiments involving exposure of stationary phase cells to SGF at pH 2.5 and subsequent exposure to SIF were repeated with a limited number of strains at pH 4.0 (60 minutes exposure) and at pH 1.0 (5 minutes exposure) in SGF. The results are shown in Figures A.3.22 to A.3.25, with calculated cfu values at logarithmic scale at the Y-axis due to compensation for the transfer of 1 ml SGF to 50 ml SIF. The calculated cfu values are shown as function of the time of exposure in SGF/SIF. All three psychrotrophic and mesophilic strains that were used for this experiment, show growth after 4 hours in complete SIF following the 60 minute exposure at pH 4.0 in SGF (see Figures A.3.22 and A.3.23). Exposure to pH 1.0 resulted in a lower number of growing strains after transfer to complete SIF, namely two psychrotrophic and two mesophilic strains (see Figures A.3.24 and A.3.25). Also growth appears to be reduced.

### ***3.3.5 Reproducibility of experiments with spores and vegetative cells***

In order to give an indication of the reproducibility of experiments with spores, the results obtained with strain PAL 2 are shown in Figures A.3.13 and A.3.14, total counts and spore counts respectively. The colony counts in SIF are calculated values to compensate for the

transfer of 1 ml SGF to 50 ml complete SIF. The colony counts are shown at a logarithmic scale on the Y-axis as a function of the time of exposure in minutes at the X-axis. To assess the reproducibility of the experiments the total counts and the spore counts of the repeated experiments with strain 2 (Figure A.3.7), involving the exposure of spores to gastric fluid followed by incubation in intestinal fluid, were log transformed. Per sample point in time the average and standard deviation were calculated. The results were considered to be reproducible when the standard deviation per sampling point in time was within 0.25 log unit (95% confidence interval  $\pm 0.5$  log unit). At all time points the standard deviation was  $\leq 0.25$  (the maximum standard deviation found in the total counts was 0.21, in the spore counts 0.25).

Therefore, the reproducibility of the experiments is considered to be satisfactory.

The reproducibility of the experiments with vegetative cells is shown in Table A.3.7. The generation times of strain PAL 2, that was used in each experiment, show for the stationary phase and the exponential phase cells in SIF and complete SIF good correlations. Although for the stationary phase cells in complete SIF nothing can be concluded, since all generation times are over 100 minutes. From these results can be read that repeated production of the intestinal fluid does not influence the outcome of the experiments.

All experiments in SIF were always carried out together with SIF without additions as control experiment. Therefore from all twelve strains there are triplicate results in SIF. Here too, from the numbers of cfu over the exposure time, growth rates and generation times were calculated. The generation times can be read from Table A.3.8. Using linear regression in the statistical program SAS, straight lines were drawn through the log cfu/time points, using the least squares method. The slopes of the different curves (= rate of growth) were compared and the p-value of the observations under the hypothesis of parallelism calculated. A significance level of 0.05 was used to test this hypothesis. The calculations were carried out twice, once using all the cfu data, and once using only the reliable data, i.e. data from counts between 15 and 150 colonies per plate (see chapter 3.2.5). The results in terms of p-value per strain were exactly the same for both calculations. Therefore, the result of only one calculation [= p-value per strain per growth phase using all data points plus the indication whether parallelism of the different growth curves ( $\equiv$  generation time) per strain could be proven] is shown in Table A.3.9. From this table can be read that only the p-value for the exponential phase cells from strain PAL 20 is smaller than 0.05, and therefore the hypothesis of parallelism is rejected for this strain. As can be seen in Table A.3.8 the calculated generation time separate lines are not parallel.

### ***3.3.6 Influence of storage at 10 °C on the behaviour of spores and vegetative cells in SGF and SIF***

In Figures A.3.15 to A.3.18 the results are shown of the experiments involving incubation of spores at 10°C prior to exposure to pH 2.5 in SGF and subsequent exposure to complete SIF. Figures A.3.15 and A.3.16 depict the results for the psychrotrophic strains, total counts and spore counts respectively. In Figures A.3.17 and A.3.18 the results for the mesophilic strains

are shown, also total counts and spore counts respectively. Again, the colony counts in SIF, total and spore, are calculated values to compensate for the transfer of 1 ml SGF to 50 ml SIF. The colony counts are shown at logarithmic scale at the Y-axis, as a function of the time of exposure in minutes at the X-axis. The spore counts for both the psychrotrophic and the mesophilic strains have not been performed for all time points, as indicated at the X-axis.

As can be seen from Figures A.3.17 and A.3.18 the behaviour of the mesophilic strains is comparable to their behaviour in the experiment without preincubation at 10°C (see Figures A.3.9 and A.3.10). From the psychrotrophic strains (see Figures A.3.15 and A.3.16) strain PAL 7 reacts very different compared to the experiment without preincubation at 10°C (see Figures A.3.7 and A.3.8). The other psychrotrophic strains (PAL 18 and PAL 22) show comparable results without and with preincubation at 10°C (compare figures A.3.7 and A.3.8 with A.3.15 and A.3.16).

Like spores stationary phase vegetative cells were subjected to overnight exposure to 10°C prior to exposure to pH 2.5 in SGF and subsequent transfer to complete SIF. The results of these experiments are shown in Figures A.3.24 and A.3.25, for the psychrotrophic and the mesophilic strains respectively. Again, on the Y-axis calculated values for the numbers of cfu at a logarithmic scale are shown due to compensation for the transfer of 1 ml SGF to 50 ml SIF. The X-axis shows the time of exposure in SGF/SIF. From the three psychrotrophic strains that were used for this experiment only strain 18 appeared to grow after 4 hours of incubation in complete SIF (see Figure A.3.24). From the three mesophilic strains two grew after 4 hours of incubation in complete SIF (see Figure A.3.25).

### **3.3.7 Comparison of generation times in BHI and SIF**

Differences between growth of vegetative cells in BHI and SIF at 37°C are shown in Table A.3.6. The generation times are longer in SIF than in BHI for both the psychrotrophic and the mesophilic strains.

## **3.4 Discussion**

### **3.4.1 Discussion of the results**

The diarrhoeal syndrome caused by *Bacillus cereus* is a toxico-infection. In other words, *Bacillus cereus* (vegetative cells or spores) must be ingested, must grow in the small intestine and subsequently produce enterotoxins that are responsible for the onset of the disease symptoms. Because *Bacillus cereus* may occur as vegetative cells and as spores, the fate of both types of cells during passage through the gastro-intestinal tract has been subject of this study.

In food both psychrotrophic and mesophilic strains can be found, therefore both types of strains have been used for the current investigations. At first, the results will be discussed for the spores and vegetative cells separately, and finally the impact of the obtained results on the risk of disease will be discussed.

In Figures A.3.1 and A.3.2 the behaviour of spores of psychrotrophic and mesophilic strains during exposure to pH 2.5 in simulated gastric fluid (SGF) is shown. The variation in counts at the various time points is all within one log-unit without any regular trend, not upward nor downward. From this can be concluded that during the 60 minute exposure the total number of spores measured as colony forming units does not change.

Two notable observations from the experiments regarding survival of vegetative cells in SGF (see Tables A.3.2 and A.3.3) can be made. Firstly, a sharp drop in survival with the decrease of 0.5 unit in pH. And secondly, the pH below which the D-values sharply drops, differs for stationary phase cells and exponential phase cells. The stationary phase cells appear to be more acid-tolerant than the exponential phase cells, regardless of the growth temperature profile (psychrotrophic or mesophilic) of the strains.

Comparison of Tables A.3.2 and A.3.3 (columns on D pH 4.0 and D pH 4.5) also shows the influence of the addition of pepsin to the SGF. When taking a closer look at the stationary phase cells at pH 4.0 (Table A.3.2), the D-values of the investigated psychrotrophic strains appear to drop more dramatically upon addition of pepsin than the D-values of the mesophilic strains. The influence of pepsin on the exponential phase cells at pH 4.5 appears to be different: pepsin appears to increase the D-values of circa 60% of the tested strains. It should be mentioned that other phenomena also may be of influence here. Firstly, pH 4.5 is a condition at which *Bacillus cereus* may actually grow instead of staying alive or even die. And secondly, the influence of pepsin decreases with increasing pH (the optimal working pH is 1.5). At the other pH values used for these researches the influence of the addition of pepsin to SGF is less apparent.

After passing the stomach spores reach the small intestine. The pH of the small intestine is near neutral, and, even before food reaches the small intestine, bile salts and pancreatic juice are secreted into the small intestine (Marieb, 1998). In Figures A.3.3 to A.3.6 the results are described of exposure of spores to complete simulated intestinal fluid (complete SIF). Total cell- and spore counts have been performed in order to discover what happens to the spores. From the graphs in Figure A.3.3 can be concluded that spores from two of the six psychrotrophic strains (namely PAL 17 and PAL 18) germinate in complete SIF and grow slightly. In Figure A.3.4 can be seen that the spore counts of all six strains show some degree of decrease; this can be interpreted as germination of spores of all strains. However, for four of the six psychrotrophic strains the conditions in the small intestine appear to be unfavorable for growth of germinated spores (see Figure A.3.3).

The spores of the mesophilic strains, however, all show growth during exposure to complete SIF, although strains PAL 5 and PAL 25 lag behind (see Figure A.3.5). Contrary to the other four mesophilic strains the spore counts of the samples of strains PAL 5 and PAL 25 show hardly any decrease. This means that the germination efficiency was very small, The spore count of strain PAL 26 (see Figure A.3.6) drops more dramatic than of the other strains, while the total count does not increase proportionally. No explanation can be given for this phenomenon.

From these results it can be concluded that spores of both types of strains (psychrotrophic and mesophilic) appear to be capable to germinate under conditions simulating the small intestine. Subsequently all mesophilic strains appear to be able to grow, whereas only two of six psychrotrophic strains appear to show growth. This is another report of differences between psychrotrophic and mesophilic strains, comparable to differences in heat susceptibility between the two types of strains (Dufrenne *et al.*, 1995, chapter 1 of this report).

The effect of exposure to simulated intestinal fluid with and without the addition of pancreatin and bile (either separately or in combination) is expressed in generation times in minutes in Tables A.3.4 and A.3.5. The addition of additives (pancreatic juice, bile salts, pancreatic juice plus bile salts) to simulated intestinal fluid (SIF) has an adverse effect on the stationary phase cells of psychrotrophic and mesophilic origin although the effect on the latter is less dramatic. This can be deduced from the fact that the generation times of all six psychrotrophic strains reach values of over two hours (see Table A.3.4). The effect on exponential phase cells is less dramatic (see Table A.3.5). The generation times of two of the six psychrotrophic strains increase to over 2 hours, whereas the generation times of the other four strains also show an increase, which is however less dramatic. The increases in generation times for the mesophilic exponential phase cells are, apart from strain PAL 2 with the addition of bile, less than with the mesophilic stationary phase cells. From the separate additions bile seems to induce the largest increase in generation time. For all strains, psychrotrophic as well as mesophilic in exponential and in stationary phase, the generation times increase upon the addition of bile. The addition of pancreatin, on the contrary, appears to have less influence. For most strains an increase in generation time can be observed. Apart from the stationary phase psychrotrophic strains, the effect of co-exposure to bile salts and pancreatin, as in complete SIF, appears to be smaller than the effect of each of the additions separately.

The previously described differences between spores of psychrotrophic and mesophilic strains are confirmed by the experiments in which spores were first exposed to low pH in SGF and subsequently transferred to complete SIF. The results of these experiments are shown in Figures A.3.7 and A.3.8 for the psychrotrophic strains, and in Figures A.3.9 and A.3.10 for the mesophilic strains. Again, like in the experiment with complete SIF alone, the psychrotrophic strains PAL 17 and PAL 18 are able to germinate and grow in SIF after exposure to SGF (Figure A.3.7), while the other psychrotrophic strains show no increase in total numbers.

However, pre-exposure to pH 2.5 of spores from the mesophilic strains appears to be favorable for subsequent growth in SIF. Comparing Figures A.3.5 and A.3.9, the total counts at t=300 SIF in figure 9 appear to be higher than the total counts at t=240 in figure 5. However, contrary to the findings in complete SIF alone, PAL 25 shows proper growth after prior exposure to low pH in SGF. Due to the missing value at t=300 in SIF it is hard to draw conclusions on the behaviour of this strain in complete SIF after exposure to SGF at low pH.

From these results it may be concluded that the spores of strain PAL 25 are somehow affected by the low pH, resulting in better growth in complete SIF.

Although only one psychrotrophic and one mesophilic strain were used, variation of the pH of the SGF used for exposure of spores before transfer to complete SIF does show an effect for the experiments involving pH 1.0 in SGF (see Figures A.3.11 and A.3.12). The total counts in complete SIF of the psychrotrophic strain after preincubation at pH 1.0 in SGF are lower than those for pH 2.5 and pH 4.0. The total counts of the mesophilic strain at pH 1.0 result in a slower growth after transfer to complete SIF, compared to the preincubation pH's 2.5 and 4.0. This can be derived by calculating the generation time from the data: with pH 1.0 preincubation the generation time in complete SIF between  $t=180$  and  $t=300$  becomes 45 minutes, while with the pH 2.5 and 4.0 preincubation the generation times are 29 and 24 minutes, respectively.

As with the spores, the ultimate test was the exposure of the vegetative cells to low pH in SGF followed by incubation in complete SIF, mimicking the passage through the stomach and the small intestine. Exponential phase cells were prepared by incubating the strains for four hours. As within that time span only the mesophilic strains reached sufficiently high cell numbers, experiments mimicking the passage through the stomach and the small intestine were not carried out with exponential phase psychrotrophic cells.

It takes a long time, often over 4 hours, in complete SIF for all psychrotrophic and some mesophilic strains in stationary phase at pH 1.0 and 2.5 to reach countable numbers in complete SIF. At pH 4.0, countable numbers of most of the mesophilic strains are obtained within three hours while the psychrotrophic strains again trail behind. This can be concluded from Figures A.3.19, A.3.20, and A.3.22 to A.3.25. Therefore the assumption that vegetative cells alone will be able to cause diarrhoeal symptoms after ingestion of around  $10^7$  cells/ml is quite unlikely. And if vegetative cells would contribute to disease, the data in Figures A.3.19 and A.3.20 give an indication that the mesophilic strains again are of greater importance than the psychrotrophic strains. However, one has to bear in mind that these experiments were carried out in complete SIF without the addition of any food component, solid or fluid. For the *Bacillus cereus* cells it can be described as the worst case scenario, since they are directly exposed to low pH in the SGF and all ingredients in the intestinal fluid and do not have the protection of any kind of, for example, solid food ingredients.

Finally, the exposure of stationary phase vegetative cells to low temperature did neither bring any protection to the low pH in the SGF nor the opposite, as can be derived by comparing Figures A.3.19 and A.3.20 with Figures A.3.23 and A.3.24, respectively.

SIF is a complex medium to work with and the experiments were that elaborate that only a very limited number of strains, either as spores or as vegetative cells, could be tested each day. Instead of performing all experiments in duplicate or triplicate, one strain was used as a control strain in each experiment. The results for this strain were analyzed statistically, and for experiments with spores or vegetative cells, the reproducibility appeared to be good. Therefore, we concluded that the experiments could be compared to one another.

Since 37°C is (near) the maximum growth temperature and 28 to 30°C is the optimal growth temperature for the psychrotrophic strains, the question arose whether the changes in generation times were not just due to temperature instead of to the changed environment. As can be seen from Table A.3.6, the increase in generation time between BHI and SIF for the mesophilic strains is, apart from strain PAL 2, less than 1.5 fold. For the psychrotrophic strains, however, the difference is a factor 1.5 to 2 for all strains. This led to the conclusion that the differences in generation times as found in the previous experiments are mainly due to the use of SIF and not to the temperature used in the experiments. And thus can be concluded that the mesophilic strains appear to be less affected by the intestinal environment.

### 3.4.2 General discussion

Based on the results of all experiments, the mesophilic strains appear to be more important for the onset of diarrhoeal disease, because they appear to be less affected by intestinal conditions. But some additional remarks are justified.

The described experiments were carried out in stationary simulated fluids, with nothing of the intestinal epithelial layer present. The intestinal epithelial layer forms an important part of the pathogenic mechanism for the diarrhoeal syndrome, since damage to the epithelial layer leads to diarrhoea. So only part of the influence of the intestinal environment has been studied. Experiments regarding the interaction between *Bacillus cereus* and differentiated Caco-2 cells as model for the epithelial layer of the small intestine are described in part three of this report.

In the small intestine *in vivo* the addition of pancreatic juice and bile salts to the intestinal fluid is regulated by a number of factors such as the smell or sight of food and the amount of food that enters the small intestine (Guyton and Hall, 1996; Marieb, 1998). Also, *in vivo* the influence of the additives will decrease with ongoing transport of material through the small intestine from the duodenum to the ileum, leading to decreased influence on the *B. cereus* spores/vegetative cells. In the experiments described here only a single dose of the additives to intestinal fluid has been tested, so no data are available on the influence of varying concentrations of bile and/or pancreatic juice.

As stated in the introduction, the pH in the stomach will change during the consumption of food, dependent on the type of food and the individual. The pH will increase when food starts entering the stomach, and subsequently the pH will decrease to the low values that indicate the non-fed state of the individual (De Jonge *et al.*, 2003). The pH may even reach values higher than 4.0, a pH at which vegetative cells can survive. In small amounts of food passing through the stomach the concentration of *B. cereus* vegetative cells may be very high. The amount of vegetative cells that can pass the stomach will decrease with transit time through the stomach, which is estimated to have a mean value of 2 hours (Marieb, 1998). Therefore the participation of vegetative cells to the pathogenesis of the diarrhoeal syndrome cannot be excluded, especially during the first moments of food ingestion when the digestive system still has to start working optimally. Data with respect to the contribution of vegetative cells to the diarrhoeal syndrome will be reported elsewhere (Wijnands *et al.*, in preparation b).



Results, involving the occurrence of *B. cereus* in food commodities, show that sometimes a single food commodity is contaminated with more than one strain of *B. cereus* (Wijnands *et al.*, in preparation a). Co-ingestion of various types of *B. cereus* will not be uncommon, and should therefore be considered as future research topic. For example, when thinking of co-ingestion of psychrotrophic and mesophilic spores, one has to bear in mind the following situation. Although the total number of spores may seem high enough to cause diarrhoea, no symptoms might occur due to the inability of psychrotrophic spores to grow in the small intestine after germination. Also, when considering the hypothesis that adhesion of spores to epithelial cells is believed to be a very important factor in the pathogenic mechanism, even more important than mentioned in the literature (Andersson *et al.*, 1998), the psychrotrophic spores might block binding space on the epithelial cells, causing less adhesion of mesophilic spores and therefore less growth and less chance for disease symptoms. However, this is still speculative and should be investigated after further research of adhesion of spores to epithelial cells of the small intestine.

The researches described in this report contribute to the clarification and understanding of the pathogenesis of the diarrhoeal syndrome caused by *B. cereus* as far as passage through the stomach and survival and growth in the small intestine are concerned. This is of importance in assessing the hazard and the subsequent description of the risk assessment concerning *B. cereus* in food. Eventually these results may contribute to a better description of the dose-response relation. It has to be taken into consideration, however, that these researches were carried out under simulated conditions without the addition of any food component, representing the “worst case” for *B. cereus*. This means that not all is clarified yet.

### 3.5 Conclusions

Spores and vegetative cells from 6 psychrotrophic and 6 mesophilic strains of *B. cereus* were used to study the behaviour of *B. cereus* in the gastro-intestinal passage under simulated conditions. These studies demonstrated that:

- Spores were not affected by exposure to simulated gastric conditions; vegetative cells were rapidly inactivated with lowering pH. Stationary phase cells were more resistant to lowering of the pH than exponential phase cells.
- Mimicking the gastro-intestinal passage learned that spores from 5 out of the 6 mesophilic strains germinated and grew in the simulated intestinal fluid after exposure to pH 2.5 in gastric fluid. From the spores of the psychrotrophic strains 2 out of 6 germinated and grew after exposure to low pH in simulated gastric fluid. Pre-exposure to pH 1.0 in SGF led to a lower number of spores transferred to the SIF and lower growth rates in SIF.

Short during pre-exposure of spores and vegetative cells at a temperature of 10°C had no effect on the subsequent behaviour in the gastro-intestinal transfer. The effect of long time pre-exposure was not investigated.

- The generation times of psychrotrophic vegetative cells increased more in simulated intestinal fluid than those of mesophilic vegetative cells, when compared to the generation times in brain heart infusion broth.
- Mesophilic strains appear to cope better with the conditions during the gastro-intestinal passage than the psychrotrophic strains. Therefore, the mesophilic strains appear to be more important for the onset of diarrhoeal disease than the psychrotrophic strains.

## 4. Interaction between *Bacillus cereus* and differentiated Caco-2 cells (as a model for the epithelium of the small intestine)

### 4.1 Introduction

The pathogenesis of the toxico-infection is still largely unknown, but is more complicated than the pathogenesis of the emetic syndrome. It is believed to be as follows: food contaminated with spores and/or vegetative cells is consumed; spores will survive the harsh conditions in the stomach, germinate and grow in the small intestine, and produce enterotoxins. Vegetative cells will partly pass the stomach, grow in the small intestine and produce enterotoxins as well.

More and better understanding of the events happening in the gastro-intestinal tract is of importance for better risk assessment on *B. cereus*.

We started investigations regarding several parts of the pathogenesis of the toxico-infection. Items of research are: 1) the behaviour of spores and vegetative cells during the simulated gastro-intestinal transfer (see chapter 2 of this report), 2) the interaction of *B. cereus* with the epithelial layer of the small intestine (this chapter) and, 3) production of enterotoxins in simulated intestinal fluid (to be published elsewhere).

In chapter 2 of this report we showed that spores and vegetative cells of mesophilic strains (i.e. strains that hardly grow < 10°C, and grow well at 37°C) probably form a greater risk than psychrotrophic strains (i.e. strains that grow easily < 10°C, but have difficulties growing at 37°C). This conclusion was based on the phenomenon that 5 out of 6 mesophilic strains and 2 out of 6 psychrotrophic strains were able to grow in simulated intestinal fluid after exposure to simulated gastric fluid, and on the observation that mesophilic strains grow faster in simulated intestinal fluid.

The diarrhoea caused by *B. cereus* is classified as secretory diarrhoea, in which the water and electrolyte transport between lumen and the epithelial cells is disturbed (Belaiche, 2000). Therefore the investigations, regarding the interaction between *B. cereus* spores/vegetative cells and the epithelial layer of the small intestine, as described in this chapter, are of importance.

A number of observations led to the hypothesis that disease may only occur if spores and/or vegetative cells adhere to the epithelium of the small intestine. These observations were:

- The incubation time of the disease is 6 – 24 hours (Kramer and Gilbert, 1989)
- The lag time in simulated intestinal fluid is 2 – 3 hours (see chapter 2)
- Production of enterotoxins starts in the exponential growth phase (Granum *et al.*, 1993)
- Production of enterotoxins is detectable during the exponential growth phase (in *in vitro* experiments) starting at a cell count of  $10^6$  –  $10^7$  colony forming units per millilitre (not published results),

- The mean transit time through the stomach and the small intestine is 6 hours (not published results); a transit time of 2 hours through the small intestine has been mentioned (Moffett *et al.*, 1993), and
- The enterotoxins are highly instable molecules; in vitro the activity vanished within 30 minutes (to be published elsewhere).

For the investigations on the interaction between *B. cereus* and the small intestinal epithelium, Caco-2 cells were used. Although the origin of these cells is the human colon, these cells resemble, after differentiation, the epithelial cells of the small intestine. They have been widely used to investigate adhesive and invasive properties of a large variety of microorganisms (Jaradat and Bhunia, 2003; Bolton *et al.*, 2000; Morita *et al.*, 2002).

## 4.2 Materials and Methods

### 4.2.1 Strains

The *B. cereus* strains and their origin are mentioned in Appendix 3 Table A.3.1.

### 4.2.2 Production and preparation of spores

See section 3.2.2.

### 4.2.3 Production of vegetative cells

Brain heart infusion broth (BHI) was inoculated with glass beads of the twelve strains and incubated overnight (18 – 20 hours) at 30°C.

### 4.2.4 Total and spores counts

See section 3.2.5.

### 4.2.5 Culturing of Caco-2 cells<sup>6</sup> and HEp-2 cells<sup>7</sup>

Caco-2 cells, obtained from ATCC (HTB-37, ATCC, USA), were cultured in DMEM 10% [Dulbecco's Modified Eagle Medium (Gibco, 42430-025) supplemented with heat-inactivated fetal calf serum (10% v/v, Integro B.V., the Netherlands), 5 ml 100x non-essential amino acids (Gibco, 11140-035), 2 mM glutamine (Gibco, 25030-024), and 0.05 mg ml<sup>-1</sup> gentamycine (Gibco, 15750-037)]. Cells were collected every 7<sup>th</sup> day by washing the monolayer twice with 0.022% disodium-EDTA (Acros, 14785) in phosphate buffered physiological salt solution (PBS, 0.07M, pH 7.2) and trypsinising the cells using 50 µg ml<sup>-1</sup> trypsin (Gibco, 25050-014), in 0.022% di-Na-EDTA in PBS. Cells were seeded to a concentration of 1x10<sup>6</sup> cells in 10 ml DMEM 10% in 75 cm<sup>2</sup> culture bottles (Costar, 3376). The bottles were incubated at 37°C with 5% carbon dioxide. The culture medium was refreshed every 4<sup>th</sup> day after passage of the cells.

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<sup>6</sup> Caco-2 cells = human colorectal adenocarcinoma cells

HEp-2 cells, a gift from dr. Erwin Duizer (RIVM, Bilthoven, The Netherlands) were cultured similarly in MEM 10% [Minimal Essential Medium with Hank's salts (Gibco) supplemented with penicillin/streptomycin (100 units ml<sup>-1</sup>, 100 µg ml<sup>-1</sup> respectively), sodium bicarbonate (0.08 % w/v), and heat-inactivated foetal calf serum (10% v/v, Integro B.V., the Netherlands)].

#### **4.2.6 Preparation of differentiated Caco-2 cells**

After washing and trypsinization Caco-cells from passage 25 to 45 (Duizer 1999) were seeded in 12-well plates at a concentration of 1.6x10<sup>5</sup> cells ml<sup>-1</sup> in DMEM 10%. In each well 1 ml of this suspension was pipetted. Plates were incubated at 37°C with 5% carbon dioxide for 23 days before use in adhesion experiments. Each second or third day the medium in the wells was replaced by fresh medium. For microscopy experiments, cover slips were placed in the wells before adding the Caco-2 cell suspension in order to obtain differentiated Caco-2 cells on cover slips.

#### **4.2.7 Adhesion/invasion experiments**

Adhesion/invasion experiments were carried out in 12-well plates containing differentiated Caco-2 cells or HEp-2 cells (1.6x10<sup>5</sup> cells per well) or in 12-well plates without cells. Before the start of the experiment, the Caco-2 cells or HEp-2 cells were washed three times with experimental culture medium (ECM), i.e. DMEM 10% without gentamycin and heat-inactivated foetal calf serum for Caco-2 cells (Caco-2-ECM) or MEM 10% without penicillin/streptomycin and heat-inactivated foetal calf serum for HEp-2 cells (HEp-2-ECM). The required number of vials from the -70°C stock containing 1 ml spore suspension per vial were thawed. The spores were washed once with appropriate ECM (600 µl washing solution, centrifugation at 13,000xg during 5 minutes), and resuspended in appropriate ECM (1 ml per vial). To determine the initial number of viable particles total and spore counts were carried out after resuspending the spores in ECM.

Per well, 40 µl spore suspension in appropriate ECM was added, plates were centrifuged briefly (1 minute at 175xg) and incubated at 37°C with 5% carbon dioxide (t=0). After 1 hour incubation (t=1), the 12-well plates containing either Caco-2 or HEp-2 cells or no cells were washed three times with appropriate ECM. Subsequently 1% Triton X-100 (Merck, Darmstadt) in PBS (0.07 M, pH 7.2) was added, to lyse the Caco-2 or HEp-2 cells and to collect remaining *B. cereus* cells (spores and/or vegetative cells). The lysates were investigated for total counts and spore counts

#### **4.2.8 Microscopic determination of adhesion/invasion**

Differentiated Caco-2 cells on cover slips placed in 12-well plates were exposed during one hour to spores from strain 28, and further incubated in Caco-2-ECM. The cover slips were washed with physiological salt solution, and subsequently the DNA in the Caco-2 cells and the spores was coloured with the fluorescent dye propidium-iodide (Molecular Probes,

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<sup>7</sup> HEp-2 cells = human laryngeal epidermoid carcinoma cells

Eugene, OR USA), according to the manufacturer's instructions. Subsequently the cover slips were placed upside down on a microscope slide for microscopic research with a laser scanning microscope (BioRad, Hercules, CA USA). Combination of pictures taken at various heights gives a top to bottom view through the Caco-2 cells. Pictures were taken after various incubation times. These experiments were carried out three times, with different batches of Caco-2 cells, using strain 28.

#### **4.2.9 Control experiments**

Three 12-well plates with differentiated Caco-2 cells were used. One was incubated in ECM with spores of strain 28, and two plates were incubated with differentiated Caco-2 cells in ECM without spores. After one hour incubation the supernatants from all wells per plate were collected and centrifuged for 10 minutes at 1800xg. Half of the centrifuged supernatant from the plates without spores was heated for 5 minutes at 100°C. All supernatants, heat treated and not heat treated, were pipetted into plates without differentiated Caco-2 cells (one plate per type of supernatant). Subsequently, 40 µl spore suspension were added per well (total 9 wells). Again the plates were incubated for 1 hour, the excess of spores washed away and samples were investigated at T=0, T=1, and T=3, as described under "Adhesion/invasion experiments".

As control for the reproducibility of the method, one 12-well plate with Caco-2 cells and one plate without Caco-2 cells were exposed during 1 hour to spores and subsequently treated as described under "Adhesion/invasion experiments".

### **4.3 Results**

Tables and figures regarding this chapter can be found in Appendix 4.

Microscopic observations did not show any evidence for invasion of *B.cereus* spores in differentiated Caco-2 cells. Therefore, from now on only adhesion will be mentioned and not adhesion/invasion.

An overview of the adhesive properties of spores of the twelve strains used for these experiments is given in Table A.4.1 and Figures A.4.1 and A.4.2. In Table A.4.1, for each strain the total counts at t=0 (start of the experiment) and at t=1 (after 1 hour incubation) are shown. In the left part of the table (and in Figure A.4.1) after spores had been added to Caco-2 cells, in the right part of the table (and in Figure A.4.2) after vegetative cells had been added to the Caco-2 cells. Spores of all strains show some degree of adhesion, as indicated by the percentages in Table A.4.1 (the higher the percentage, the higher the degree of adhesion) and by the differences between the total counts at t=0 and t=1 per strain in Figure A.4.1.

Also the vegetative cells show adhesive properties, as depicted in Table A.4.1 and Figure A.4.2.

In Table A.4.2 the results for adhesion of spores to plates with (t=1 Caco-2) and without Caco-2 cells (t=1 cell free) are compared after the addition of spores at t=0. For each strain at t=1 the total and spore counts were determined, and from these numbers a spore count/ total count ratio was determined. In the right column of the table the t=1 cell free ratio is divided by the t=1 Caco-2 ratio. This gives a germination index. A germination index from  $\leq 1$  means no germination, a ratio  $> 1$  means germination. The higher this germination index the higher the degree of germination.

In Figure A.4.3 the phenomenon of germination influence by Caco-2 cells is depicted for strain 28. The left (darker) bars represent the total counts at the given time points, the right (lighter) bars represent the spore counts at the given time points. At t=1 cell free the difference between total and spore count is a lot smaller than at t=1 Caco-2, indicating that with the Caco-2 cells present a high degree of germination has taken place. At t=1 cell free the degree of germination is a lot smaller.

The various control experiments were carried out to learn more about 1) the production and use of the germination inducing compound (Figure A.4.4A), 2) heat stability of the compound (Figure A.4.4B), and 3) whether the compound is produced commonly by epithelial cells (Figure A.4.4C). The activity of the compound was based upon its germination inducing property. In case germination of spores occurred after the addition of spores to Caco-2 cells, the number of spores should drop and the number of vegetative cells should become high compared to the number of spores. This property was translated into a ratio spore count / total count. In case of germination the ratio should be  $\ll 1$ , no germination would result in a ratio close to 1. In Figure A.4.4 the results of various control experiments are shown. To obtain Figure A.4.4A supernatants from differentiated Caco-2 cells were exposed to spores or not prior to the transfer of the supernatant to a new cell-free plate and addition of spores to this plate. Apparently the germination inducer was readily absorbed by spores that were added to the first plate with Caco-2 cells. After all, the supernatant was no longer able to induce germination in the cell free plate, as indicated by a relatively high germination ratio ( $1.6 \times 10^4 / 3.3 \times 10^4 = 0.46$ ) at “t=1 sup from exposed Caco-2 cells” in Figure A.4.4A. The germination ratio obtained after transfer of non exposed supernatant has become very low ( $1.4 \times 10^2 / 7.5 \times 10^3 = 0.016$ ), indicating that the germination inducer was still present in this supernatant. The left pair of bars in figure 4A represents the starting situation, namely the spore and total count of the spores suspension added to the supernatant, with the spore count equal to the total count.

Exposure of the germination inducer to heat does not influence its inducing capacity. As shown in Figure A.4.4B with and without heat treatment the germination ratio becomes low:  $1.2 \times 10^2 / 3.7 \times 10^4 = 0.003$  at “t=1 untreated sup” and  $1.9 \times 10^2 / 3.3 \times 10^4 = 0.006$  at “t=1 heat-treated sup”.

The germination inducer is not commonly produced by epithelial cells. HEp-2 cells do not induce to germination, the spore count / total count ratio is  $8.0 \times 10^4 / 1.4 \times 10^5 = 0.57$  (see figure A.4.4C “t=1 HEp-2”) which is close to the ratio in a cell free plate  $7.0 \times 10^4 / 7.8 \times 10^4 = 0.90$  (see Figure A.4.4C “t=1 CF”).

## 4.4 Discussion

Differentiated Caco-2 cells are widely used in research as an in vitro method for studying the epithelial cells of the small intestine. The interaction of various microorganisms with the small intestinal epithelium has been investigated using differentiated Caco-2 cells (Jaradat and Bhunia, 2003; Morita *et al.*, 2002).

Since the toxico-infection due to *B. cereus* is a phenomenon that takes place mainly in the small intestine, the use of these differentiated Caco-2 cells appeared to be the logical step for closer evaluation of the pathogenic mechanism.

Adhesion of spores is a subject that is investigated within the scope of safe production of food, particularly with contamination of surfaces that are in contact with food commodities during preparation. Spores of *B. cereus* have been found to adhere to a variety of surfaces, namely metal, glass, and plastics (Peng *et al.*, 2001; Faille *et al.*, 2002; Rönner *et al.*, 1990). Therefore, it is not surprising that spores adhere to differentiated Caco-2 cells, as shown in this report and as described previously (Andersson *et al.*, 1998). However, we found that spores from all strains used for the investigations were able to adhere to differentiated Caco-2 cells. Apart from one, there was no similarity between our and their strains. Another important difference between the two investigations was also the method for detecting adhesion. Where Andersson *et al.* (1998) used microscopy, we employed culture methods for determining the number of adhered *B. cereus* cells.

Also, we found that vegetative cells adhere to differentiated Caco-2 cells. This has been described before (Minnaard *et al.*, 2004) and is an important finding in the explanation of the pathogenic mechanism of the toxico-infection caused by *B. cereus*. Because, after spores and/or vegetative cells have reached the small intestine, as a consequence of the consumption of contaminated food, the circumstances in the small intestine make adhesion to the epithelial cells prerequisite for the onset of the diarrhoeal syndrome.

The nature of adhesion can, based on these results, only be speculated on. Some microorganisms, such as *Listeria monocytogenes* use specific receptors on the epithelial cells for adhesion (Jaradat and Bhunia, 2003). The presence of such adhesins on the surface of *B. cereus* spores and/or vegetative cells has never been established. Therefore, no definite answer can be given.

Another phenomenon is at least as important to mention. When comparing spore adhesion to differentiated Caco-2 cells in 12-well plates and adhesion to the same plates without differentiated Caco-2 cells, the spores in the plate without differentiated Caco-2 cells showed no or delayed germination. The differentiated Caco-2 cells appear to excrete a compound capable of inducing germination of spores in 8 of the 12 *B. cereus* strains that were used in these investigations.

Spores readily absorb the germination inducing compound. After all, supernatant from Caco-2 cells, that had not been exposed to spores, induced germination after transfer to a cell free plate and the subsequent addition of spores. Whereas, supernatant from Caco-2 cells, that had



been exposed to spores, was not able to induce germination. Apparently, the germination inducing compound was no longer available.

The germination inducing compound appears to be heat resistant, since heating of supernatant of non-exposed Caco-2 cells did not reduce the germination inducing potential of the supernatant. The compound will therefore not be a large biological molecule, like a protein, but rather a small molecule.

Induction of germination does not appear to be a property of epithelial cells in general, since spores adhered to HEp-2 cells, but were not induced to germinate.

The experiments with differentiated Caco-2 cells, and all the controls, were carried out in cell culture medium. Such medium is very rich, i.e. it contains a broad spectrum of compounds, including salts, amino acids and vitamins. The germination inducing properties of several compounds, such as alanine and inosine, have been described (Preston and Douthit, 1988). Recent investigations have shown the germination inducing properties of various other compounds such as glutamine and adenosine (Hornstra *et al.*, 2004). The culture medium for Caco-2 cells, and the experimental medium used for the adhesion experiments, both contain glutamine. But despite the presence of glutamine, spores could not germinate. There may be two explanations for this phenomenon. First, the concentration of glutamine in the experimental medium, being circa 6 mM, may be too low. And second, for the induction of germination more than one compound might be necessary, and besides glutamine no other compound is present in the experimental medium.

The differentiated Caco-2 cells appear to produce either a compound that can induce germination by itself or supplement the amount of glutamine to a level necessary for germination.

Further investigations to the nature of the germination inducing compound produced by differentiated Caco-2 cells are therefore most interesting. However, it has to be confirmed that small intestinal epithelial cells also induce germination of spores of *B. cereus*.

The relevance of the induction of germination by differentiated Caco-2 cells can, for the time being, only be speculated on. It is known that spores contain several receptors for germination (Shibata *et al.*, 1986). In our studies we found that some strains did not show induction of germination by differentiated Caco-2 cells. Maybe spores of these strains lack one or more receptors, making them unable to germinate under conditions as posed in the *in vitro* differentiated Caco-2 system. However, we used this system to study adhesion to the small intestinal epithelium, in the absence of intestinal fluid after feeding, i.e. in the absence of bile salts, pancreatic proteolytic activity, and food constituents. Therefore, it is not the best way to investigate the assumption that the presence of germination receptors, or rather the absence, may discriminate between hazardous and non-hazardous strains.

Also, the strains that were used for these investigations do not permit further speculation into this subject. Although some of the strains derive from possible food outbreaks, it is not certain that they were indeed the organisms at fault for the symptoms.

Therefore, further research to investigate the contribution of germination receptors to the pathogenesis of *B. cereus* is recommended.

## 4.5 Conclusions

Differentiated Caco-2 cells were used to mimic the epithelium of the small intestine, in order to study the interaction between *B. cereus* and the small intestinal epithelium. Our studies demonstrate that:

- Both spores and vegetative cells show adherence to the differentiated Caco-2 cells.
- No invasion of *B. cereus* cells in differentiated Caco-2 cells was observed.
- Differentiated Caco-2 cells excrete a compound that is able to induce germination of spores from 8 out of 12 *B. cereus* strains. The compound is readily absorbed or neutralized by the spores, is heat stable, and apparently not commonly produced by epithelial cells.

## 5. General discussion and conclusion

From the two types of food borne disease caused by *B. cereus*, the incidence of the toxicoinfection or diarrhoeal syndrome is difficult to estimate. Important reasons are the relative mild symptoms and the short duration of these symptoms, as a result of which few patients seek medical assistance.

Risk assessment might offer an alternative method to estimate incidence of this disease. For this, information is necessary on the prevalence of the organism and on the factors influencing the onset of disease. Especially some factors influencing the latter have been assessed in this report: factors regarding the organism itself, and factors regarding the environment (= gastro-intestinal tract) where the disease takes place.

*B. cereus* is an organism with very divergent properties. Growth may occur over a wide range of pH and temperature (Kramer and Gilbert, 1989), inactivation of the spores appears to be dependant on the growth temperature profile (see chapter 1 of this report). In other words, the onset of disease depends strongly on the type of *B. cereus* that is ingested with the food.

The data for determination of growth temperature profiles were obtained by techniques using artificial culture media. From these data can be seen that the discrimination between psychrotrophic and mesophilic strains is not black and white. We also determined the generation times and lag phases for a number of strains in simulated intestinal fluid. Whereas these results appear to be more straightforward, the discriminative potential of the simulated intestinal fluid seems to be better than that of artificial culture media. Also, simulated conditions resemble the *in vivo* situation more closely, and are therefore more suitable for studying the pathogenesis. Moreover, results from simulated conditions may improve the quality of models mimicking the pathogenesis. This statement is supported by the difference in survival of *Salmonella* on carrot and in culture media during heating processes. Where *Salmonella* in culture media die readily with increasing heat, on carrot slices the *Salmonella* survives longer (DiPersio *et al.*, 2005). Similar results are found with *Campylobacter*, readily killed in culture media and much more difficult to kill when adhered to chicken meat (De Jonge, unpublished results ).

The pathogenesis of the diarrhoeal syndrome is as follows. *B. cereus* is ingested with contaminated food, passes the stomach and reaches the small intestine. There, spores of *B. cereus* will germinate to become vegetative cells that will grow and produce enterotoxins. The enterotoxins, finally, affect the epithelium resulting in diarrhoea. *B. cereus* can be ingested as spores and as vegetative cells. The final outcome, the diarrhoeal syndrome, is not influenced by the type of cell. For steps in between ingestion and disease, the type of cells may be of importance. We have shown that spores will hardly be affected by the low pH of the stomach, while vegetative cells are dependant on the value of the pH (chapter 2 of this report).

Although the onset of the diarrhoeal syndrome has been characterized earlier as being caused by enterotoxins without any direct interaction of the organism with the host (Granum *et al.*, 1995), interaction between the epithelium of the small intestine and *B. cereus* cells may influence the outcome of the ingestion of the organism. Moreover, ingestion simulations after modeling the various steps of diarrhoeal syndrome indicate that adhesion is an essential step in the pathogenesis. No adhesion would mean that *B. cereus* would flush out of the small intestine without causing any disease (Pielaat *et al.*, 2005). Moreover, enterotoxins are readily inactivated in simulated intestinal fluid. Since these enterotoxins cause the symptoms of the diarrhoeal syndrome, close proximity of *B. cereus* to the small intestinal epithelium is a prerogative for the onset of this disease (Wijnands *et al.*, in preparation c). Therefore, adhesion is not only necessary for *B. cereus* to stay long enough in the small intestine to grow to sufficient numbers, but also to ensure that the enterotoxins are produced close enough to the epithelium to prevent inactivation.

Not only adhesion of *B. cereus* to the epithelial cells seems to play a role, the epithelial cells of the small intestine are even able to induce germination of ingested spores (chapter 3 of this report).

The investigations described in this report contribute to a better understanding of the pathogenesis of the diarrhoeal syndrome. Together with other factors, such as prevalence and dose-response relationship, the risk for diarrhoeal disease can be better estimated, and with that a better estimation of the incidence of the syndrome can be given.

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Investigations into adherence, enterotoxin production and enterotoxin activity in relation to  
the diarrhoeal syndrome by *Bacillus cereus*.



## Appendix 1 Simulated gastric and intestinal fluid

### Simulated gastric fluid (SGF)

#### 1) Materials

- Sodiumchloride, Merck
- Sodiumdihydrogenphosphate, Merck
- Potassiumchloride, Merck
- Calciumchloride, Merck
- 1M and 6M hydrochloride, Merck
- sterile distilled water
- glucose, Merck
- glucuronic acid, Fluka
- urea, BioRad
- glucosamin hydrochloride, Calbiochem
- bovine serum albumin, Roche
- pepsin, Merck
- mucin, Sigma

#### 2) Inorganic component:

- a) 7.85 ml sodiumchloride solution (175.3 g/l)
- b) 1.5 ml sodiumdihydrogenphosphate solution (88.8 g/l)
- c) 4.6 ml potassiumchloride solution (89.6 g/l)
- d) 9 ml calciumchloride solution (22.2 g/l, freshly prepared)
- e) 5.0 ml ammoniumchloride solution (30.6 g/l)
- f) Set the pH at the desired value with hydrochloride solution (1M and/or 6M dependent on pH)
- g) Add sterile distilled water till a volume of 250 ml

#### 3) Organic component

- a) 5.0 ml glucose solution (65 g/l)
- b) 5.0 ml glucuronic acid solution (2 g/l)
- c) 1.7 ml urea solution (25 g/l)
- d) 5.0 ml glucosamin hydrochloride solution 33 g/l)
- e) add sterile distilled water till 250 ml

#### 4) Total gastric fluid

- a) Mix 250 ml inorganic component with 250 ml organic component
- b) Check the pH and adjust if necessary.
- c) Add 0.5 g bovine serum albumin and dissolve completely
- d) Add 0.5 g pepsin (dependent on the experiment)

Add 1.5 g mucin and dissolve completely

## Simulated intestinal fluid (SIF)

### 1) Materials

- a) Sodiumchloride, Merck
- b) Sodiumhydrogencarbonate, Merck
- c) Potassiumdihydrogenphosphate, Merck
- d) Potassiumchloride, Merck
- e) Magnesiumchloride, Merck
- f) Calciumchloride, Merck
- g) Urea, BioRad
- h) Sterile distilled water
- i) Bovine serum albumin, Roche
- j) Lipase, Sigma
- k) Pancreatin, Merck
- l) Bile salts, Sigma

### 2) Composition simulated intestinal fluid

Mix the following ingredients

- a) 20 ml sodiumchloride solution (175.3 g/l)
- b) 20 ml sodiumhydrogencarbonate solution (84.7 g/l)
- c) 5 ml potassiumdihydrogenphosphate solution (88.8 g/l)
- d) 3.15 ml potassiumchloride solution (89.6 g/l)
- e) 4.5 ml calciumchloride solution (22.2 g/l, freshly prepared)
- f) 5 ml magnesiumchloride solution (5 g/l)
- g) 2 ml urea solution (25 g/l)
- h) Add sterile distilled water to a final volume of 500 ml
- i) Add 0.5 g bovine serum albumin
- j) Measure and register the pH
- k) Filter sterilize the solution
- l) Divide the solution in two equal portions
- m) Add to each portion 0.125 g lipase

### 3) Composition simulated intestinal fluid with pancreatin

- a) Add to one of the portion simulated intestinal fluid 0.75 g pancreatin

## 4) Composition of bile solution

- a) Mix the following ingredients
- b) 7.5 ml sodiumchloride solution (175.3 g/l)
- c) 17.1 m sodiumhydrogencarbonate solution (84.7 g/l)
- d) 2.5 ml calciumchloride solution (22.2 g/l)
- e) 1.03 potassiumchloride solution (88.8 g/l)
- f) 2.5 ml urea solution (25 g/l)
- g) 0.45 g bovine serum albumin
- h) Set the pH of the solution to 8.0 using 1N hydrochloric acid
- i) Filter sterilize the solution
- j) Add 1.5 g bile salts
- k) Add sterile distilled water to a final volume of 250 ml

## 5) Composition of simulated intestinal fluid with bile salts

- a) Mix 162 ml simulated intestinal fluid with 54 g bile solution

## 6) Composition of complete simulated intestinal fluid (basic solution with pancreatin and bile)

Mix 162 ml simulated intestinal solution with pancreatin with 54 ml bile solution



## Appendix 2 Characteristics of *Bacillus cereus*

### Tables and Figures

Table A.2.1. Strains and origin (abbreviations are explained in the “Materials and Methods” section of chapter 1).

Strain	Original number	Origin
PAL 1	9900325-3	Sensor project
PAL 2	9901672	Sensor project
PAL 3	9901909-1	Sensor project
PAL 4	9901909-2	Sensor project
PAL 5	9902169-2	Sensor project
PAL 6	9902187	Sensor project
PAL 7	9902187-1	Sensor project
PAL 8	9902195	Sensor project
PAL 9	9902196	Sensor project
PAL 10	9902632	Sensor project
PAL 11	9902651	Sensor project
PAL 12	9902679	Sensor project
PAL 13	9902891	Sensor project
PAL 14	9902900	Sensor project
PAL 15	9902965	Sensor project
PAL 16	9903009	Sensor project
PAL 17	9903073-2	Sensor project
PAL 18	9903295-4	Sensor project
PAL 19	P21S	INRA Avignon
PAL 20	(AV)TZ 415	INRA Avignon
PAL 21	(AV)Z 421	INRA Avignon
PAL 22	Z4222	INRA Avignon
PAL 23	L2104	INRA Avignon
PAL 24	CIP05127	INRA Avignon
PAL 25	1143	NCTC
PAL 26	1145	NCTC
PAL 27	B4ac	DSMZ
PAL 28	1230-88	NVH

*Table A.2.2. Growth characteristics at 10°C determined by OD 600 measurement  
Generation time and lag phase in hours*

Strain	10°C		10°C		Mean at 10°C	
	gen. time	lag phase	gen. time	lag phase	gen. time	lag phase
PAL 1	n.d.	n.d.	n.d.	n.d.		
PAL 2	28.50	66.00	26.70	59.20	27.60	62.60
PAL 3	64.10	106.00	61.90	96.60	63.00	101.30
PAL 4	49.50	93.00	22.40	59.00	36.00	76.00
PAL 5	n.d.	n.d.	n.d.	n.d.		
PAL 6	43.30	65.60	63.00	52.90	53.20	59.30
PAL 7	20.80	50.00	18.70	44.90	19.80	47.50
PAL 8	45.00	130.40	inf.	inf.	45.00	130.40
PAL 9	n.d.	n.d.	n.d.	n.d.		
PAL 10	52.50	136.70	56.80	137.20	54.70	137.00
PAL 11	36.90	94.60	31.10	81.90	34.00	88.30
PAL 12	36.90	59.80	18.70	44.00	27.80	51.90
PAL 13	n.d.	n.d.	n.d.	n.d.		
PAL 14	n.d.	n.d.	n.d.	n.d.		
PAL 15	20.20	68.00	18.10	61.50	19.20	64.80
PAL 16	n.d.	n.d.	n.d.	n.d.		
PAL 17	47.80	97.10	45.90	96.10	46.90	96.60
PAL 18	18.30	45.70	23.40	45.10	20.90	45.40
PAL 19	51.70	103.60	38.90	87.20	45.30	95.40
PAL 20	27.30	53.60	75.30	74.20	51.30	63.90
PAL 21	n.d.	n.d.	n.d.	n.d.		
PAL 22	31.50	56.60	84.50	77.60	58.00	67.10
PAL 23	61.30	132.90	59.20	128.20	60.00	130.60
PAL 24	n.d.	n.d.	n.d.	n.d.		
PAL 25	36.30	107.30	36.30	100.70	36.30	104.00
PAL 26	n.d.	n.d.	n.d.	n.d.		
PAL 27	37.30	87.00	23.30	67.70	30.30	77.40
PAL 28	32.90	75.10	53.70	95.50	43.30	85.30

n.d. = not able to determine generation times or lag phases reliably

inf. = not able to determine generation time or lag phase due to infection

95% confidence interval      Gen. Time      Lag phase  
33.26 - 47.58      67.65 - 93.95

*Table A.2.3. Growth characteristics at 10°C determined by culture experiments  
Generation time and lag phase in hours*

Strain	Gen time	Lag phase
PAL 2	25.86	90.92
PAL 3	30.4	108.31
PAL 5	-	-
PAL 7	18.78	69.72
PAL 17	15.13	50.92
PAL 18	1.41	47.37
PAL 20	10.16	37.29
PAL 22	10.42	38.13
PAL 25	-	-
PAL 26	-	-
PAL 27	26.97	93.67
PAL 28	12.58	41.11

- = no data due to infection

*Table A.2.4 Growth characteristics at 28°C determined by OD-600 measurement  
Generation time and lag phase in hours*

Strain	28°C		28°C		Mean at 28°C	
	gen. time	lag phase	gen. time	lag phase	gen. time	lag phase
PAL 1	0.73	2.23	0.62	1.85	0.68	2.04
PAL 2	0.69	2.08	0.73	2.09	0.71	2.09
PAL 3	1.12	2.83	1.11	2.75	1.12	2.79
PAL 4	0.91	2.61	0.80	2.08	0.86	2.35
PAL 5	0.89	2.61	0.89	2.11	0.89	2.36
PAL 6	0.91	2.42	0.82	2.07	0.87	2.25
PAL 7	0.94	2.56	0.79	2.23	0.87	2.40
PAL 8	0.80	2.31	0.77	2.05	0.79	2.18
PAL 9	0.80	2.31	0.78	2.08	0.79	2.20
PAL 10	0.78	2.27	0.94	2.27	0.86	2.27
PAL 11	0.79	2.30	0.80	2.07	0.80	2.19
PAL 12	0.78	2.32	0.77	2.21	0.78	2.27
PAL 13	0.79	2.36	0.79	2.11	0.79	2.24
PAL 14	0.79	2.37	0.83	1.95	0.81	2.16
PAL 15	0.95	2.65	0.78	2.01	0.87	2.33
PAL 16	0.93	2.51	0.77	2.01	0.85	2.26
PAL 17	0.93	2.57	0.88	2.12	0.91	2.35
PAL 18	0.92	2.54	0.79	2.11	0.86	2.33
PAL 19	0.90	2.42	0.78	2.05	0.84	2.24
PAL 20	0.96	2.54	0.89	2.15	0.93	2.35
PAL 21	0.96	2.54	1.11	2.37	1.04	2.46
PAL 22	1.04	2.57	0.81	2.11	0.93	2.34
PAL 23	0.83	2.43	0.80	2.06	0.82	2.25
PAL 24	0.88	2.22	0.78	2.09	0.78	2.16
PAL 25	0.83	2.34	0.82	2.19	0.83	2.27
PAL 26	0.78	2.31	0.79	2.19	0.79	2.22
PAL 27	1.08	2.60	inf.	inf.	1.08	2.60
PAL 28	0.89	2.16	inf.	inf.	0.89	2.16

inf. = not able to determine generation times or lag phases due to infection

95% confidence interval      Gen. Time      Lag phase  
0.812 - 0.886      2.226 - 2.339

*Table A.2.5. Growth characteristics at 37°C determined by OD measurement  
Generation time and lag phase in hours*

Strain	37°C		37°C		Mean at 37°C	
	gen. time	lag phase	gen. time	lag phase	gen. time	lag phase
PAL 1	0.60	1.52	0.54	1.45	0.57	1.49
PAL 2	0.54	1.51	0.69	1.66	0.62	1.59
PAL 3	n.d.	n.d.	n.d.	n.d.		
PAL 4	0.69	1.83	0.66	1.62	0.68	1.73
PAL 5	0.61	1.55	0.77	1.62	0.69	1.59
PAL 6	0.68	1.67	0.70	1.66	0.69	1.67
PAL 7	0.64	1.75	0.69	1.80	0.67	1.78
PAL 8	0.64	1.54	0.69	1.58	0.67	1.56
PAL 9	0.61	1.55	0.81	1.61	0.71	1.58
PAL 10	0.63	1.55	0.55	1.45	0.59	1.50
PAL 11	0.64	1.63	0.75	1.65	0.70	1.64
PAL 12	0.59	1.64	0.60	1.59	0.60	1.62
PAL 13	0.66	1.59	0.69	1.62	0.68	1.61
PAL 14	0.60	1.52	0.71	1.56	0.66	1.54
PAL 15	0.66	1.66	0.64	1.57	0.65	1.62
PAL 16	0.61	1.53	0.81	1.63	0.71	1.58
PAL 17	0.73	1.94	0.93	2.03	0.83	1.99
PAL 18	0.99	2.61	1.08	2.67	1.04	2.64
PAL 19	0.61	1.56	0.66	1.55	0.64	1.60
PAL 20	0.91	2.30	1.05	2.42	0.98	2.36
PAL 21	0.63	1.50	0.62	1.53	0.63	1.54
PAL 22	0.98	2.42	1.20	2.68	1.09	2.55
PAL 23	0.57	1.56	0.66	1.57	0.62	1.57
PAL 24	0.62	1.54	0.61	1.53	0.62	1.54
PAL 25	0.53	1.47	0.62	1.54	0.58	1.51
PAL 26	0.54	1.47	0.53	1.45	0.54	1.46
PAL 27	0.54	1.46	0.61	1.52	0.58	1.49
PAL 28	0.62	1.58	0.65	1.57	0.64	1.58

n.d. = not able to determine generation times or lag phases reliably

95% confidence interval      Gen. Time      Lag phase  
0.637 - 0.746      1.577 - 1.826



*Table A.2.6. Growth characteristics at 42°C determined by OD measurement  
Generation time and lag phase in hours*

Strain	42°C		42°C		Mean at 42°C	
	gen. time	lag phase	gen. time	lag phase	gen. time	lag phase
PAL 1	0.59	1.46	0.63	1.54	0.61	1.50
PAL 2	0.88	2.21	0.63	1.83	0.76	2.02
PAL 3	n.d.	n.d.	n.d.	n.d.	2.50	
PAL 4	2.93	6.38	inf.	inf.	2.93	6.38
PAL 5	0.72	1.72	0.76	1.63	0.74	1.68
PAL 6	1.08	2.42	0.91	2.08	1.00	2.25
PAL 7	n.d.	n.d.	1.58	3.43	1.58	3.43
PAL 8	0.55	1.43	0.59	1.41	0.57	1.42
PAL 9	0.72	1.73	0.70	1.60	0.71	1.67
PAL 10	0.54	1.42	0.55	1.37	0.55	1.40
PAL 11	0.58	1.52	0.57	1.53	0.58	1.53
PAL 12	0.94	2.53	0.73	2.07	0.84	2.30
PAL 13	0.71	1.75	0.72	1.65	0.72	1.70
PAL 14	0.62	1.61	0.72	1.45	0.67	1.53
PAL 15	0.62	1.65	0.57	1.53	0.60	1.59
PAL 16	0.71	1.72	0.73	1.62	0.72	1.67
PAL 17	1.12	2.94	n.d.	n.d.	1.12	2.94
PAL 18	n.d.	n.d.	n.d.	n.d.	2.50	
PAL 19	0.72	1.74	0.64	1.57	0.68	1.66
PAL 20	n.d.	n.d.	n.d.	n.d.	2.50	
PAL 21	0.56	1.45	0.53	1.34	0.55	1.40
PAL 22	n.d.	n.d.	n.d.	n.d.	2.50	
PAL 23	0.88	2.13	0.72	1.67	0.80	1.90
PAL 24	0.55	1.46	0.56	1.37	0.56	1.42
PAL 25	0.48	1.37	0.52	1.25	0.50	1.31
PAL 26	0.62	1.63	0.55	1.47	0.59	1.55
PAL 27	0.49	1.32	inf.	inf.	0.49	1.32
PAL 28	0.57	1.54	inf.	inf.	0.57	1.54

n.d. = not able to determine generation times or lag phases reliably

inf. = not able to determine generation time or lag phase due to infection

95% confidence interval                      Gen. Time    Lag phase  
0.611 - 0.731    1.524 - 1.792

*Table A.2.7. Growth characteristics in Simulated Intestinal Fluid  
Generation time and lag phase in hours*

Strain	Gen. Time	Lag phase
PAL 2	0.89	2.97
PAL 3	41.02	41.02
PAL 5	7.06	7.06
PAL 7	n.g.	
PAL 17	17.16	17.16
PAL 18	13.84	13.84
PAL 20	51.15	51.15
PAL 22	n.g.	
PAL 25	22.95	22.95
PAL 26	2.59	2.59
PAL 27	3.39	3.39
PAL 28	4.02	4.02

n.g. = no growth

Table A.2.8 Growth temperature profile signatures measured by PCR

m = mesophilic, p = psychrotrophic

Strain	Signature
PAL 2	m
PAL 3	p
PAL 5	m
PAL 7	p
PAL 17	p
PAL 18	p
PAL 20	p
PAL 22	p
PAL 25	m
PAL 26	m
PAL 27	m
PAL 28	m

Table A.2.9. D-values of different strains expressed in minutes.

Psychrotrophic strains						
	D 80°C	D 85°C	D 87.5°C	D 90°C	D 95°C	D 97.5°C
PAL 3	14.0	6.7	2.6	1.6		
PAL 7	28.0	13.0		5.1		
PAL 17	> 100	22.4	6.8	5.5		
PAL 18	75.0	22.8		7.3		
PAL 20	28.7	8.6		3.8		
PAL 22	20.5	8.4		3.3		
Mesophilic strains						
	D 80°C	D 85°C	D 87.5°C	D 90°C	D 95°C	D 97.5°C
PAL 2	> 100	> 100		8.3	3.3	
PAL 5	> 100			14.4	6.1	
PAL 25	> 100			20.7	8.0	4.0
PAL 26		80.6		15.0	2.6	
PAL 27				71.4	8.9	5.3
PAL 28		77.0		12.4	3.8	

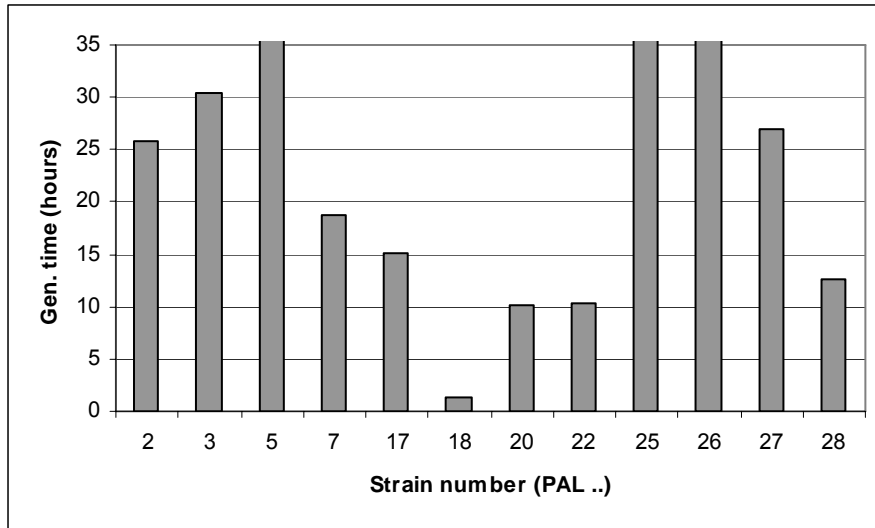


Figure A.2.1 Generation time (hours) at 10°C determined by culture

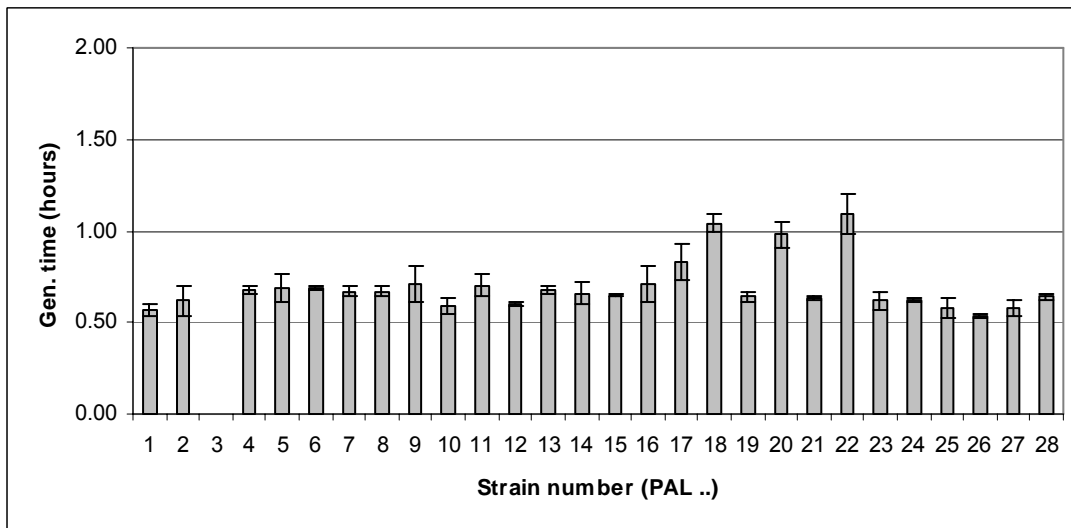


Figure A.2.2 Generation time at 37°C determined by OD 600 measurement

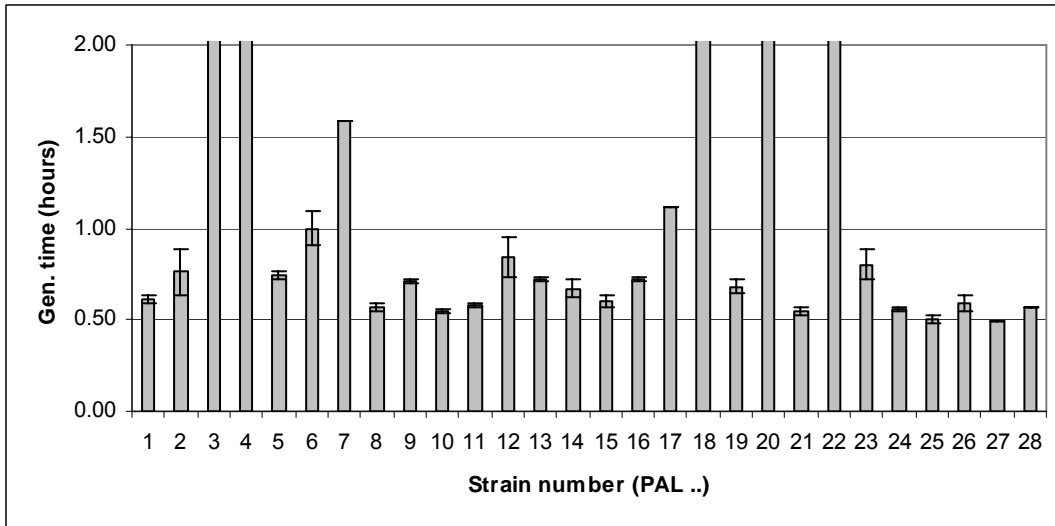


Figure A.2.3 Generation time at 42°C determined by OD 600 measurement

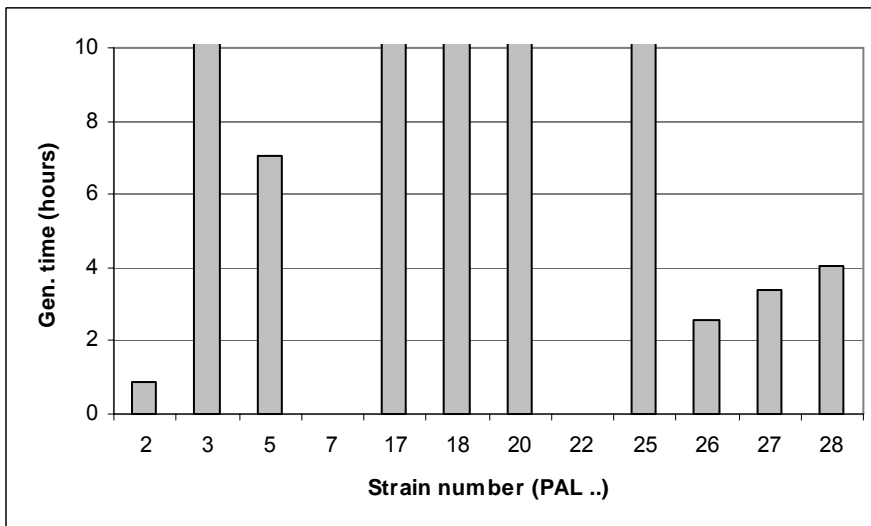


Figure A.2.4 Generation times (hours) in Simulated Intestinal Fluid at 37°C

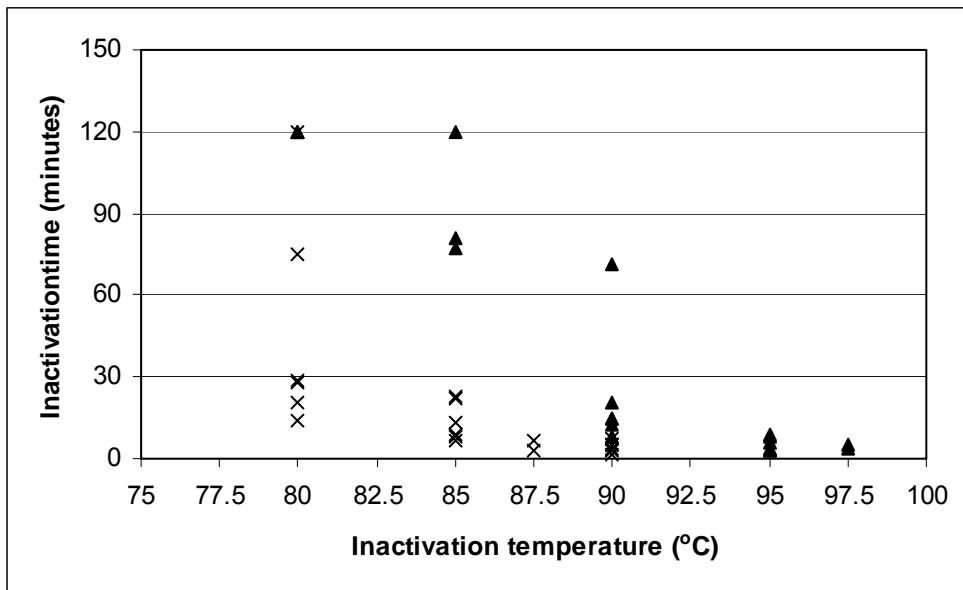


Figure A.2.5 Distribution of D-values from psychrotrophic strains (x) and mesophilic strains (▲).



## Appendix 3 Behaviour of *Bacillus cereus* in the gastrointestinal tract under simulated circumstances

### Tables and Figures

Table A.3.1 Strains used for these investigations

Strain	Original number	Origin	Mesophily	Psychrotrophy
PAL 2	9901672	RIVM*	x	
PAL 3	9901909-1	RIVM		x
PAL 5	9902169-2	RIVM	x	
PAL 7	9902187-1	RIVM		x
PAL17	9903043-2	RIVM		x
PAL 18	9903295-4	RIVM		x
PAL 20	TZ 415	INRA Avignon		x
PAL 22	Z 4222	INRA Avignon		x
PAL 25	NCTC 11143	NCTC	x	
PAL 26	NCTC 11145	NCTC	x	
PAL 27	B4ac	DSMZ	x	
PAL 28	1230-88	NVH	x	

NCTC = National Collection of Type Cultures

DSMZ= Deutsche Sammlung von Mikroorganismen und Zellkulturen  
(German Collection of Microorganisms and Cell cultures)

NVH = Norwegian School of Veterinary Sciences

INRA = L'Institut National de la Recherche Agronomique

\*= RIVM project on the incidence and pathogens of gastroenteritis

Table A.3.2. *Survival of stationary phase cells in SGF without and with pepsin.*  
*D-values in minutes.*

Strain	D pH 4.0		D pH 3.5		D pH 3.0	
		pepsine		pepsine		pepsine
Psychrotrophic strains						
Pal 3	45	30	2	2	1	1
Pal 7	17	12	3	3	1	1
Pal 17	28	20	2	3	1	1
Pal 18	30	18	8	4	2	3
Pal 20	31	10	7	6	1	1
Pal 22	23	11	6	5	1	1
Mesophilic strains						
Pal 2	29	20	6	6	2	5
Pal 5	37	38	20	10	2	3
Pal 25	51	31	7	3	3	3
Pal 26	11	9	3	3	2	2
Pal 27	22	19	3	3	2	2
Pal 28	33	37	20	10	5	5

Table A.3.3. *Survival of exponential phase cells in SGF without and with pepsin.*  
*D-values in minutes.*

	D pH 4.5		D pH 4.0		D pH 3.5	
		pepsine		pepsine		pepsine
Psychrotrophic strains						
Pal 3	42	43	3	3	2	2
Pal 7	43	56	9	7	2	2
Pal 17	48	55	5	4	2	2
Pal 18	54	62	8	3	2	2
Pal 20	41	40	8	3	2	1
Pal 22	40	42	7	4	2	2
Mesophilic strains						
Pal 2	75	106	8	5	1	1
Pal 5	69	95	6	5	2	2
Pal 25	65	64	13	7	2	2
Pal 26	45	33	7	7	2	2
Pal 27	45	44	6	3	1	1
Pal 28	75	77	4	3	2	2



Table A.3.4. Behaviour of stationary phase cells (psychrotrophic and mesophilic), expressed in generation times, in SIF with various additions. Generation times in minutes.

Stationary phase cells				
	SIF	SIF +	SIF +	SIF
		panc.juice	bile	complete
Psychrotrophic strains				
PAL 3	74	>>>	>>>	>>>
PAL 7	44	70	>>>	>>>
PAL 17	43	49	>>>	>>>
PAL 18	42	52	>>>	>>>
PAL 20	49	>>>	>>>	>>>
PAL 22	43	>>>	>>>	>>>
Mesophilic strains				
PAL 2	46	64	>>>	>>>
PAL 5	26	27	68	38
PAL 25	27	25	58	37
PAL 26	23	27	86	29
PAL 27	24	62	44	28
PAL 28	21	32	64	27

>>> = >120 minutes.

Table A.3.5. Behaviour of exponential phase cells (psychrotrophic and mesophilic), expressed in generation times, in SIF with various additions. Generation times in minutes.

Exponential phase				
	SIF	SIF +	SIF +	SIF
		panc.juice	bile	complete
Psychrotrophic strains				
PAL 3	56	77	>>>	>>>
PAL 7	45	64	>>>	>>>
PAL 17	20	24	74	30
PAL 18	20	20	59	36
PAL 20	29	>>>	>>>	62
PAL 22	30	>>>	>>>	55
Mesophilic strains				
PAL 2	18	23	51	24
PAL 5	27	22	35	24
PAL 25	23	22	27	27
PAL 26	24	22	36	31
PAL 27	21	39	24	33
PAL 28	21	31	29	23

>>> = >120 minutes.

*Table A.3.6. Comparison of generation times of stationary phase cells in BHI and SIF.*

	BHI	SIF
Psychrotrophic strains		
Pal 3	49	74
Pal 7	31	44
Pal 17	22	43
Pal 18	24	42
Pal 20	32	49
Pal 22	32	43
Mesophilic strains		
Pal 2	22	46
Pal 5	27	26
Pal 25	19	27
Pal 26	19	23
Pal 27	19	24
Pal 28	21	21

Generation times in minutes.

*Table A.3.7. Reproducibility of results expressed in generation times in SIF.*

*Strain PAL 2, stationary and exponential phase cells, in SIF and complete SIF.*

Strain PAL 2			
Stationary phase		Exponential phase	
SIF	SIF	SIF	SIF
	complete		complete
45	>100	17	24
48	>100	18	25
47	>100	17	23
46	>100	19	27
		17	26

Generation times in minutes.

Table A.3.8. Reproducibility of results expressed in generation times in SIF.  
Generation times in minutes.

Stationary phase			Exponential phase					
	SIF	SIF	SIF	SIF	SIF	SIF	SIF	SIF
<b>Psychrotrophic strains</b>								
<b>PAL 3</b>	>>>	>>>	>>>	>>>	>>>	>>>		
<b>PAL 7</b>	54	44	51	49	52	58		
<b>PAL 17</b>	45	61	47	27	21	24		
<b>PAL 18</b>	61	65	44	28	25	25		
<b>PAL 20</b>	64	60	62	26	35	28		
<b>PAL 22</b>	42	63	53	30	34	29		
<b>Mesophilic strains</b>								
<b>PAL 2</b>	62	48	55	22	19	17	17	19
<b>PAL 5</b>	32	23	26	24	24	28		
<b>PAL 25</b>	26	32	31	19	23	24		
<b>PAL 26</b>	27	24	22	18	28	30		
<b>PAL 27</b>	29	25	23	19	23	23		
<b>PAL 28</b>	18	24	22	18	22	26		

Table A.3.9. Analysis for parallelism at log scale using all datapoints

Psychrotrophic strains			Mesophilic strains		
Strainnumber	p-value	parallelism	Strainnumber	p-value	parallelism
PAL 3 stat.	n.r.	n.d.	PAL 2 stat.	0,800	yes
PAL 7 stat.	0,331	yes	PAL 5 stat.	0,757	yes
PAL 17 stat.	0,640	yes	PAL 25 stat.	0,818	yes
PAL 18 stat.	0,244	yes	PAL 26 stat.	0,140	yes
PAL 20 stat.	0,950	yes	PAL 27 stat.	0,343	yes
PAL 22 stat.	0,413	yes	PAL 28 stat.	0,524	yes
PAL 3 exp.	n.r.	n.d.	PAL 2 exp.	0,165	yes
PAL 7 exp.	0,924	yes	PAL 5 exp.	0,229	yes
PAL 17 exp.	0,548	yes	PAL 25 exp.	0,105	yes
PAL 18 exp.	0,820	yes	PAL 26 exp.	0,009	yes
PAL 20 exp.	0,006	yes	PAL 27 exp.	0,589	yes
PAL 22 exp.	0,420	yes	PAL 28 exp.	0,143	yes

stat. = stationary phase cells

exp. = log-phase cells

n.r. = non reliable data

n.d. = not determined

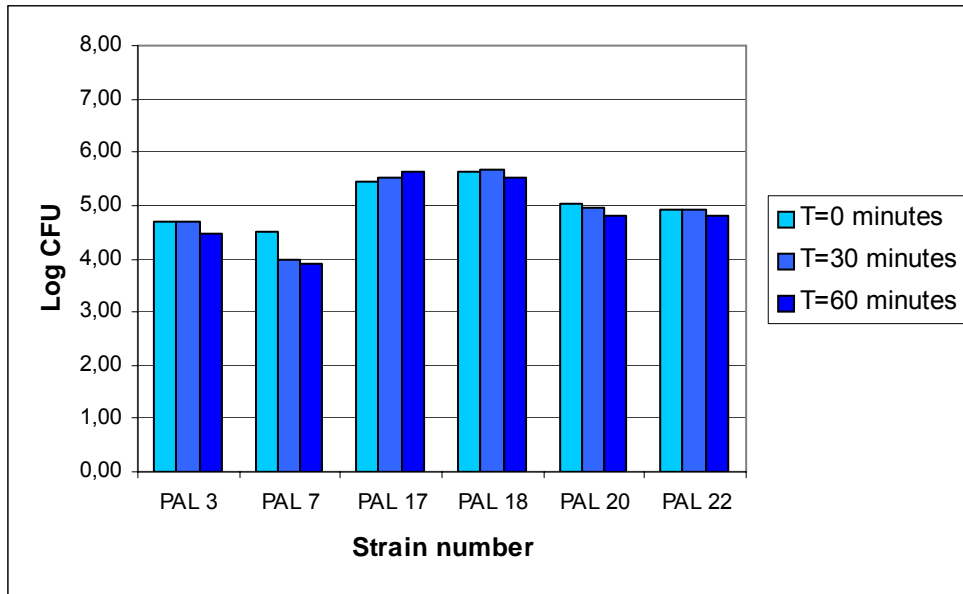


Figure A.3.1. Behaviour of spores from psychrotrophic strains at pH 2.5 in simulated gastric fluid with pepsin.

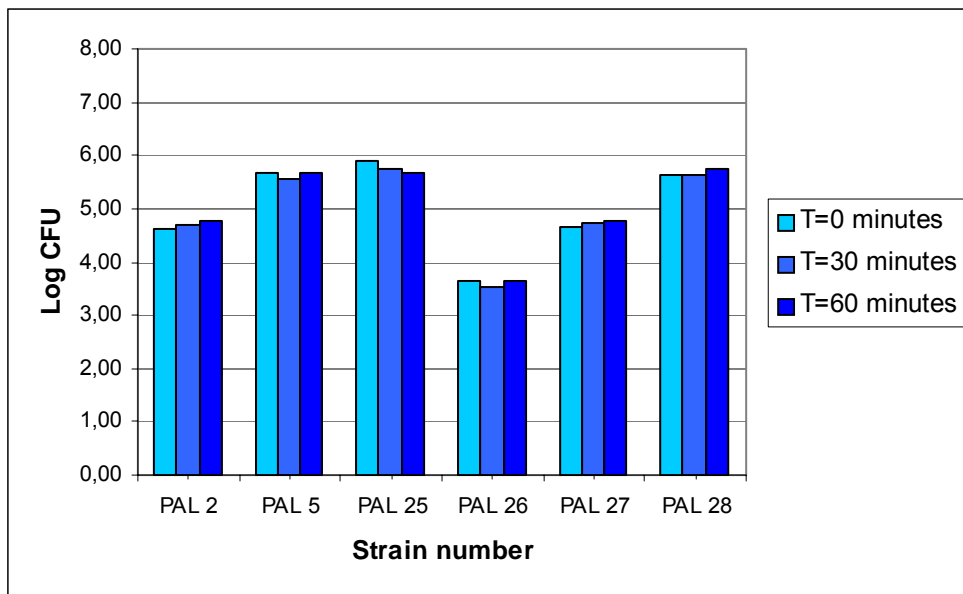


Figure A.3.2. Behaviour of spores from mesophilic strains at pH 2.5 in simulated gastric fluid with pepsin.

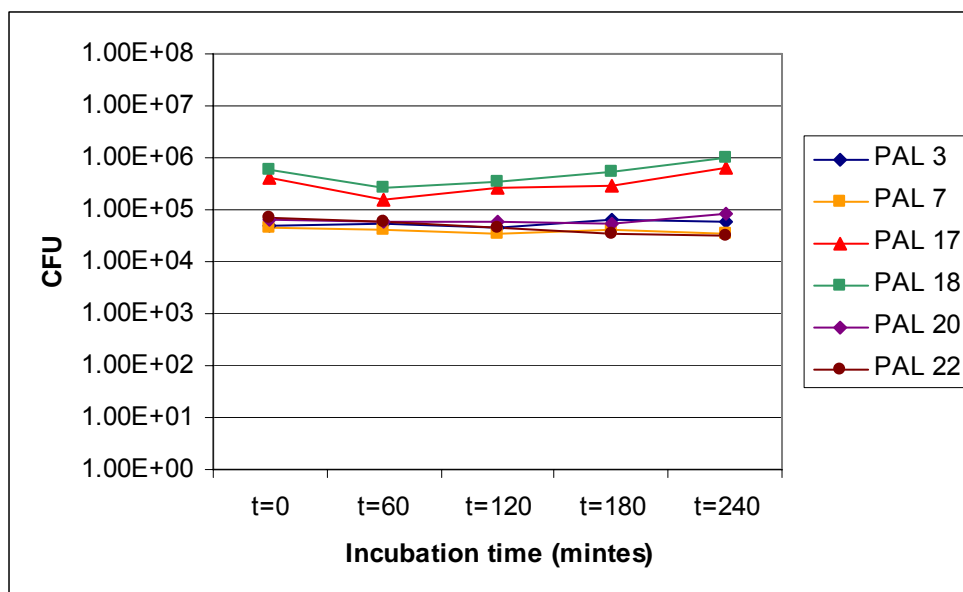


Figure A.3.3. Behaviour of spores from psychrotrophic strains in complete simulated intestinal fluid. Total counts.

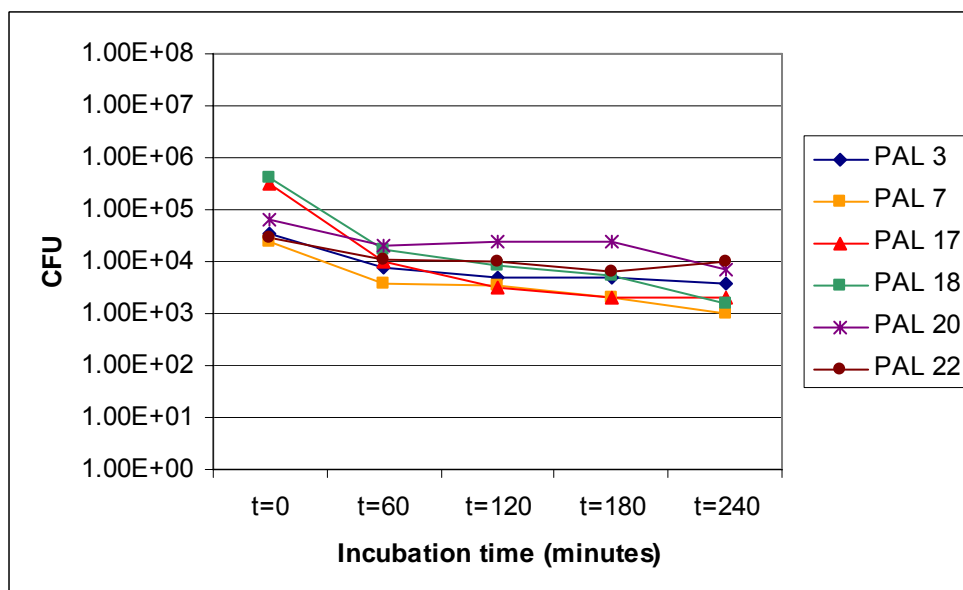


Figure A.3.4. Behaviour of spores from psychrotrophic strains in complete simulated intestinal fluid. Spore counts.

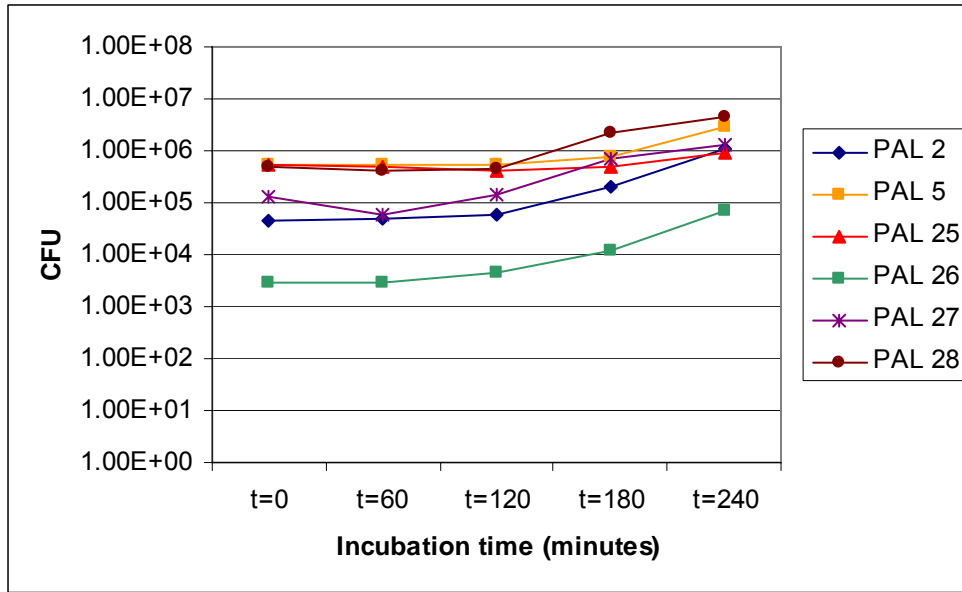


Figure A.3.5. Behaviour of spores from mesophilic strains in complete simulated intestinal fluid. Total counts.

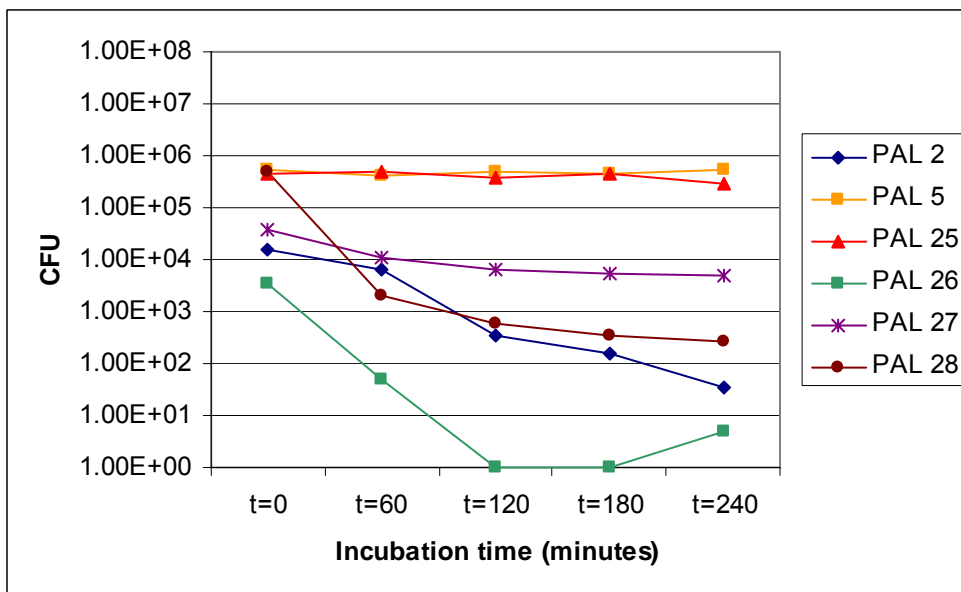


Figure A.3.6. Behaviour of spores from mesophilic strains in complete simulated intestinal fluid. Spore counts.

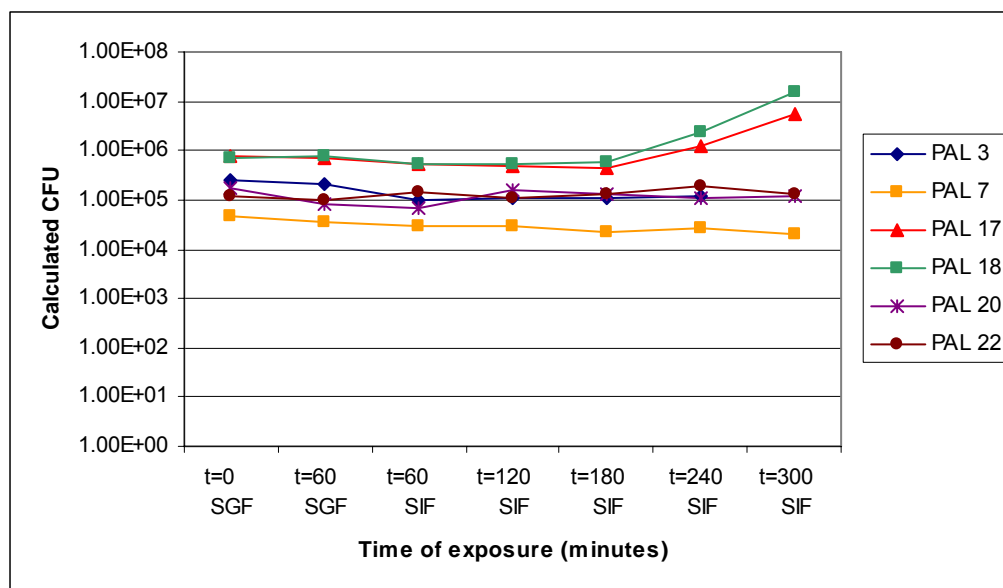


Figure A.3.7. Behaviour of spores from psychrotrophic strains in complete simulated intestinal fluid after exposure to pH 2.5 in simulated gastric fluid. Total counts.

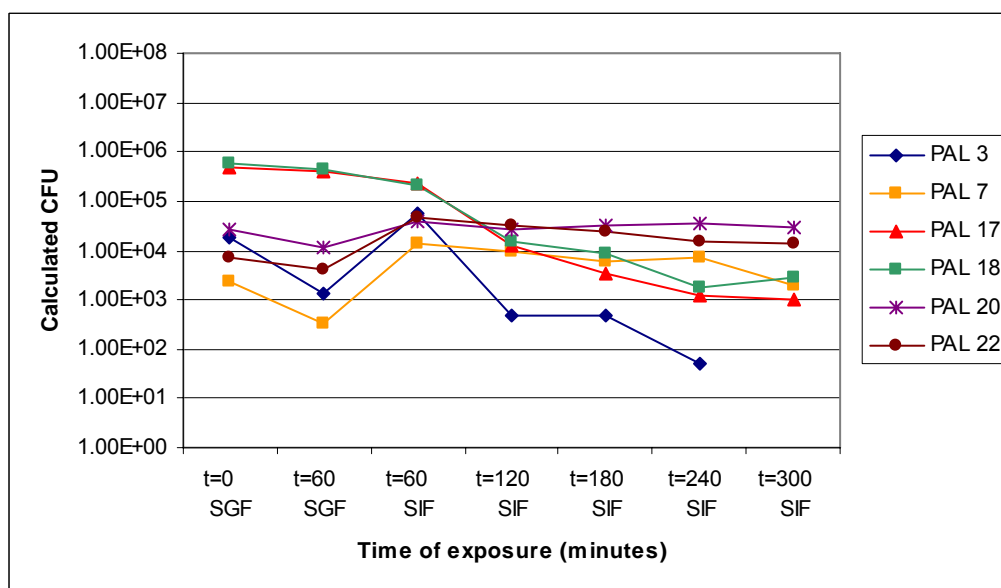


Figure A.3.8. Behaviour of spores from psychrotrophic strains in complete simulated intestinal fluid after exposure to pH 2.5 in simulated gastric fluid. Spore counts.

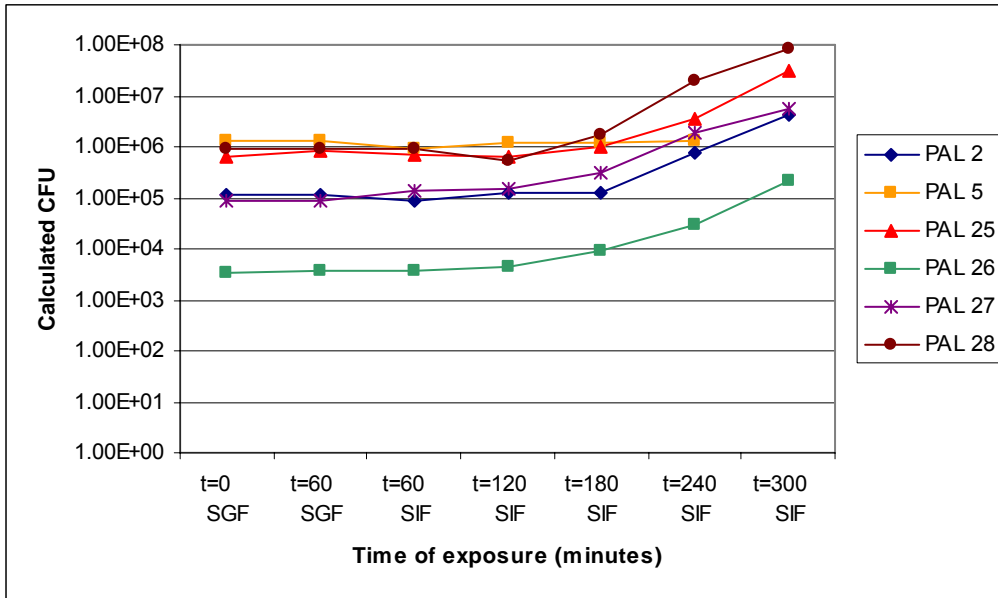


Figure A.3.9. Behaviour of spores from mesophilic strains in complete simulated intestinal fluid after exposure to pH 2.5 in simulated gastric fluid. Total counts.

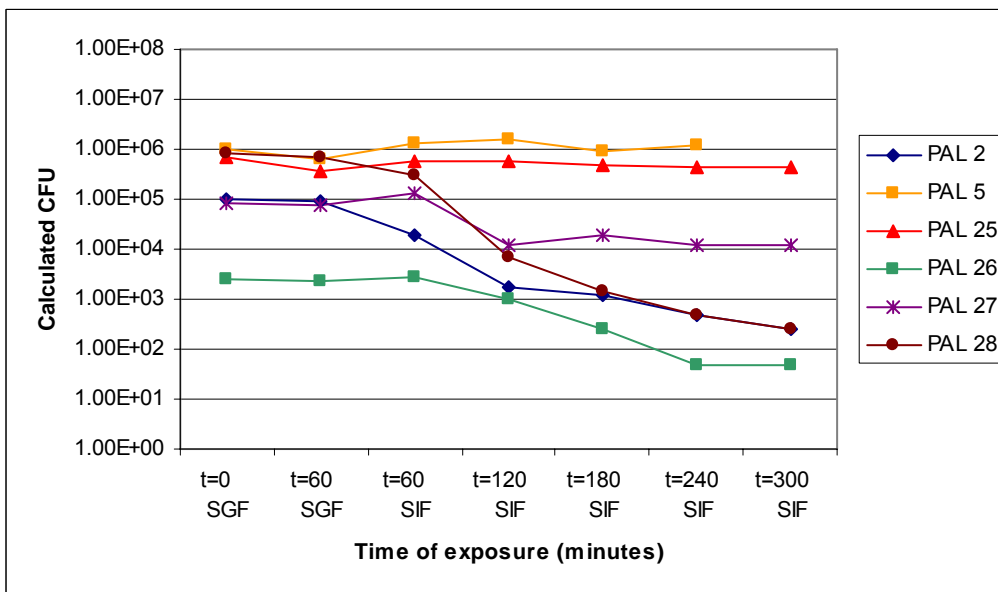


Figure A.3.10. Behaviour of spores from mesophilic strains in complete simulated intestinal fluid after exposure to pH 2.5 in simulated gastric fluid. Spore counts.



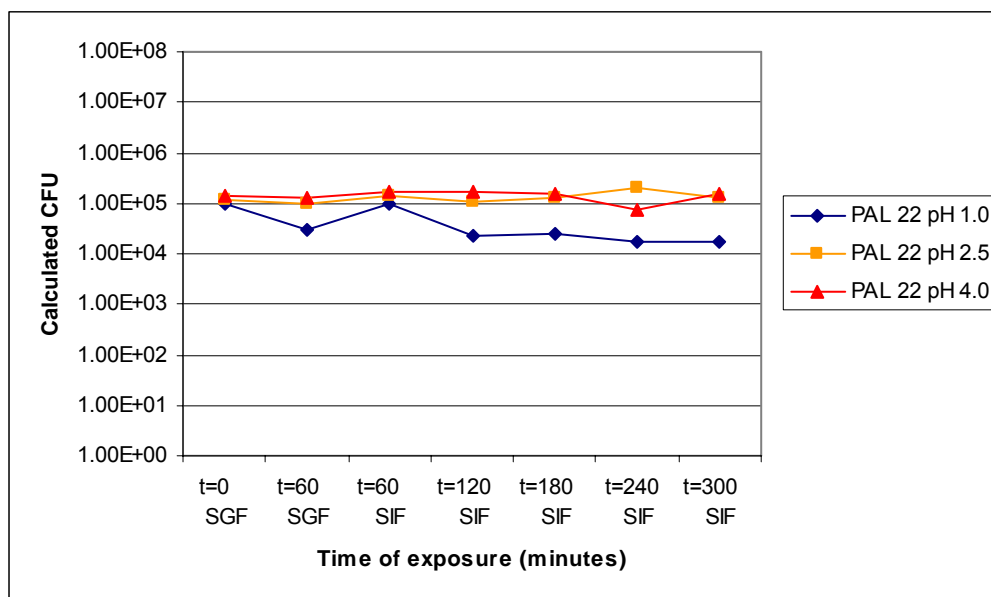


Figure A.3.11. Behaviour of spores of a psychrotrophic strain in SIF after exposure to pH 1.0, 2.5 or 4.0 in SGF. Total counts.

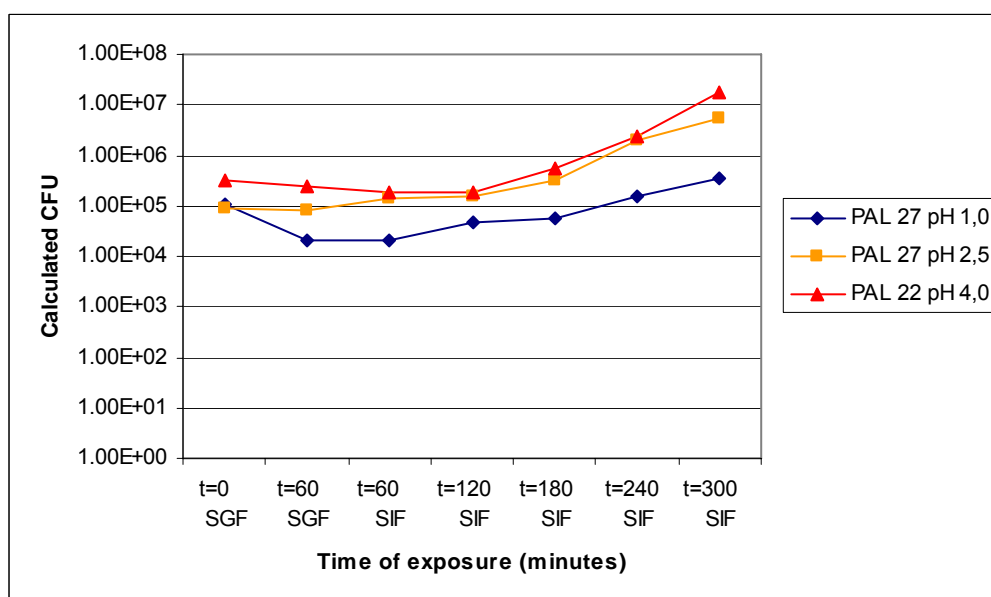


Figure A.3.12. Behaviour of spores of a mesophilic strain in SIF after exposure to pH 1.0, 2.5 or 4.0 in SGF. Total counts.

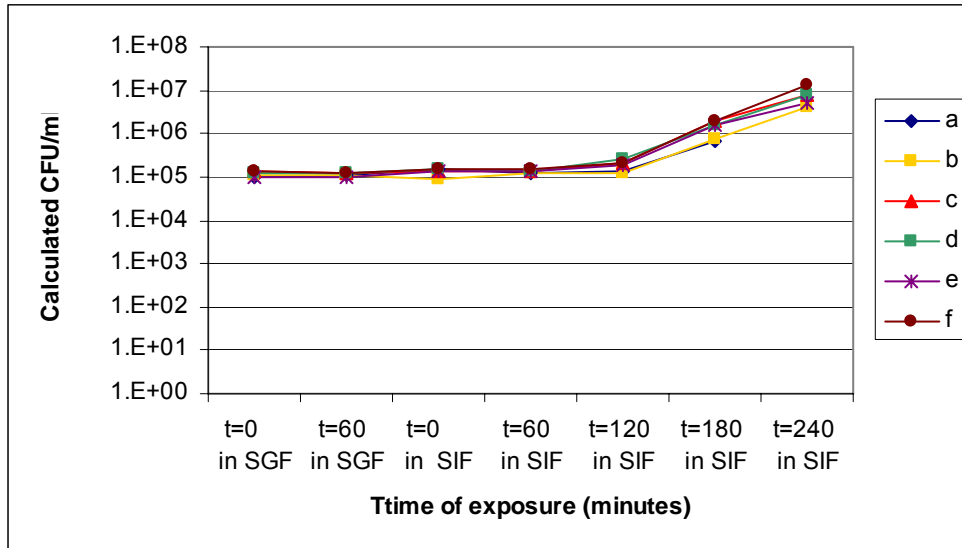


Figure A.3.13. Reproducibility of experiments concerning behaviour of spores of strain PAL 2 in SIF after exposure to pH 2.5 in SGF. Total counts.

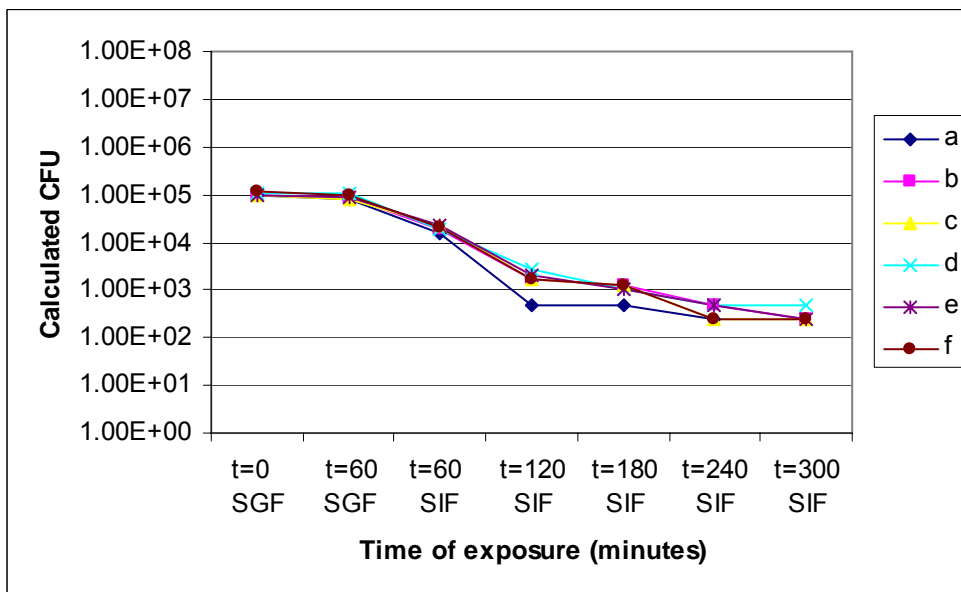


Figure A.3.14. Reproducibility of experiments concerning behaviour of spores of strain PAL 2 in SIF after exposure to pH 2.5 in SGF. Spore counts.

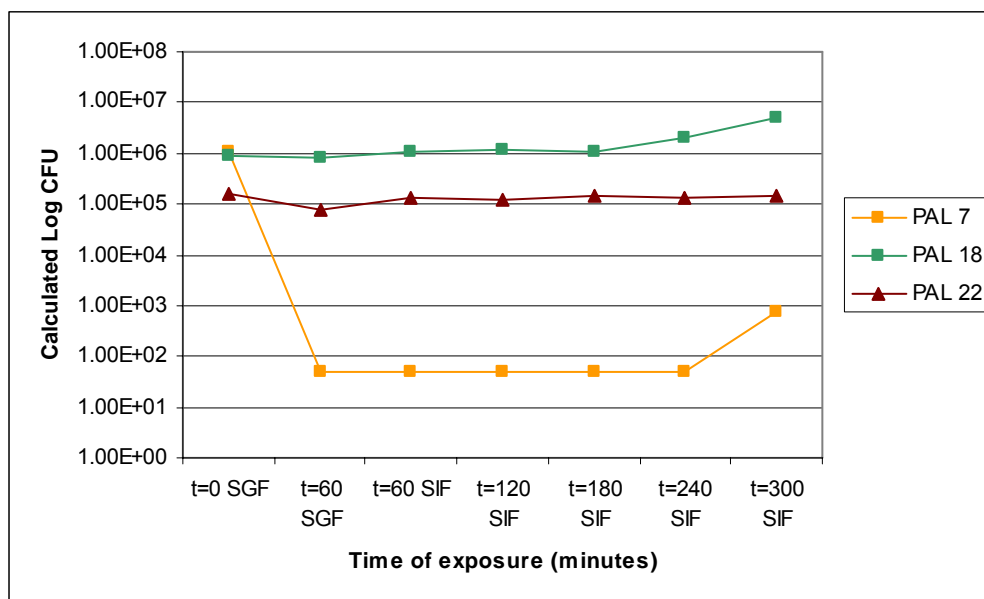


Figure A.3.15. Behaviour of spores of a selection of psychrotrophic strains in SIF after overnight incubation at 10 °C and exposure to pH 2.5 in SGF. Total counts.

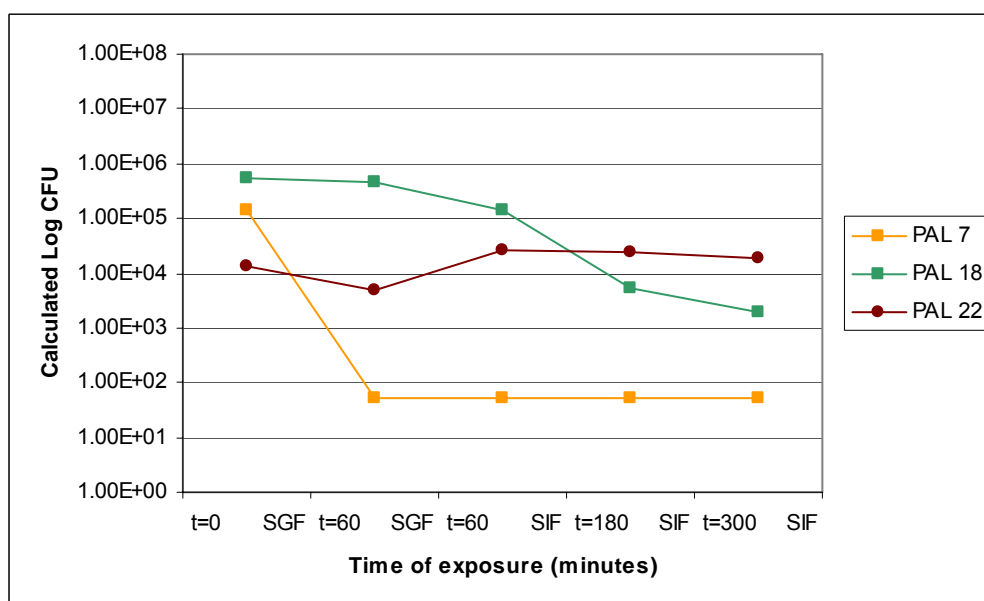


Figure A.3.16. Behaviour of spores of a selection of psychrotrophic strains in SIF after overnight incubation at 10 °C and exposure to pH 2.5 in SGF. Spore counts.

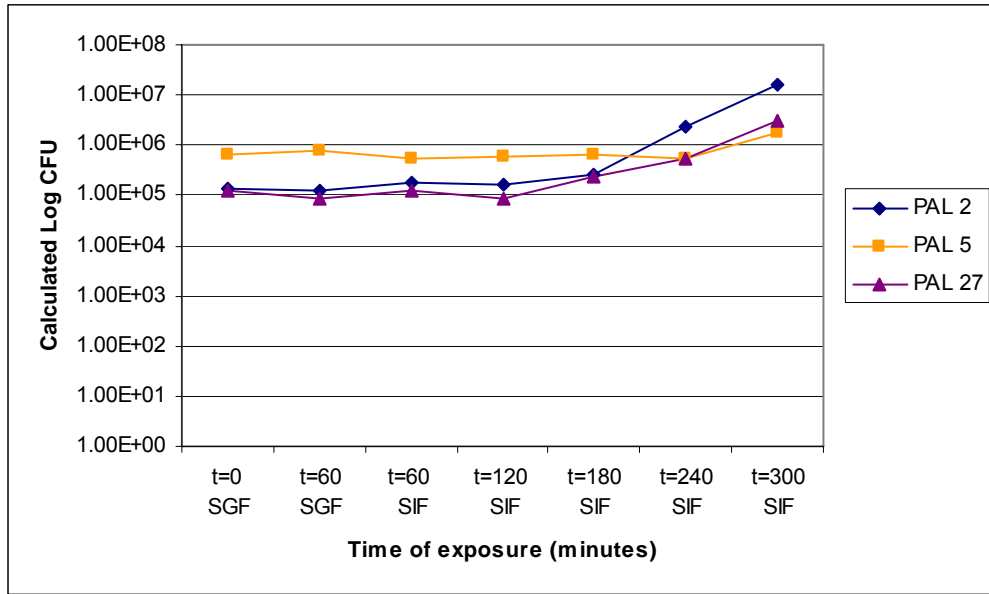


Figure A.3.17. Behaviour of spores of a selection of mesophilic strains in SIF after overnight incubation at 10 °C and exposure to pH 2.5 in SGF. Total counts.

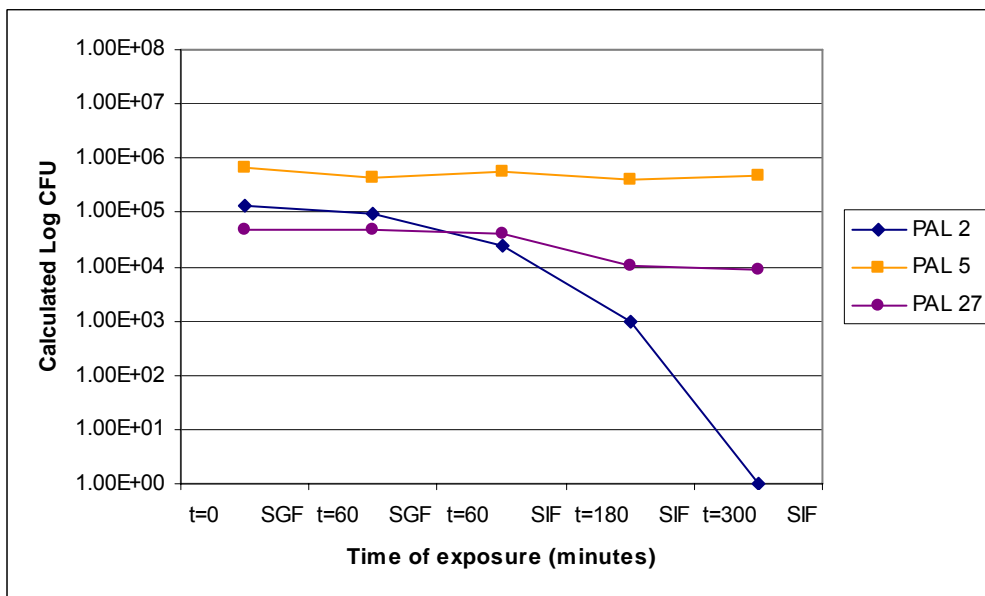


Figure A.3.18. Behaviour of spores of a selection of mesophilic strains in SIF after overnight incubation at 10 °C and exposure to pH 2.5 in SGF. Spore counts.

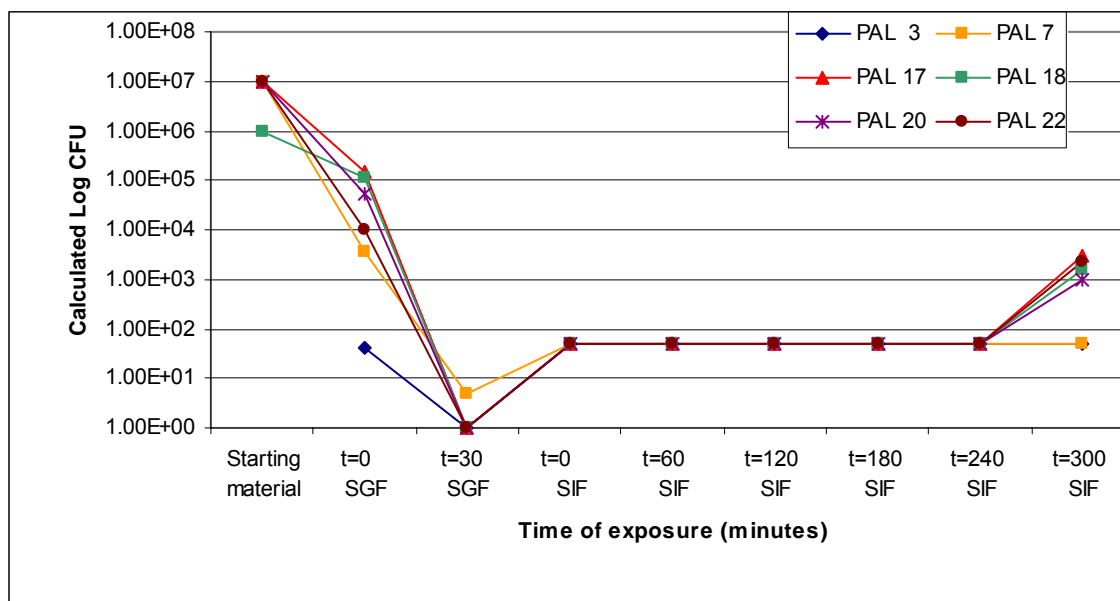


Figure A.3.19. Behaviour of stationary phase cells of psychrotrophic strains in SIF after exposure to pH 2.5 in SGF.

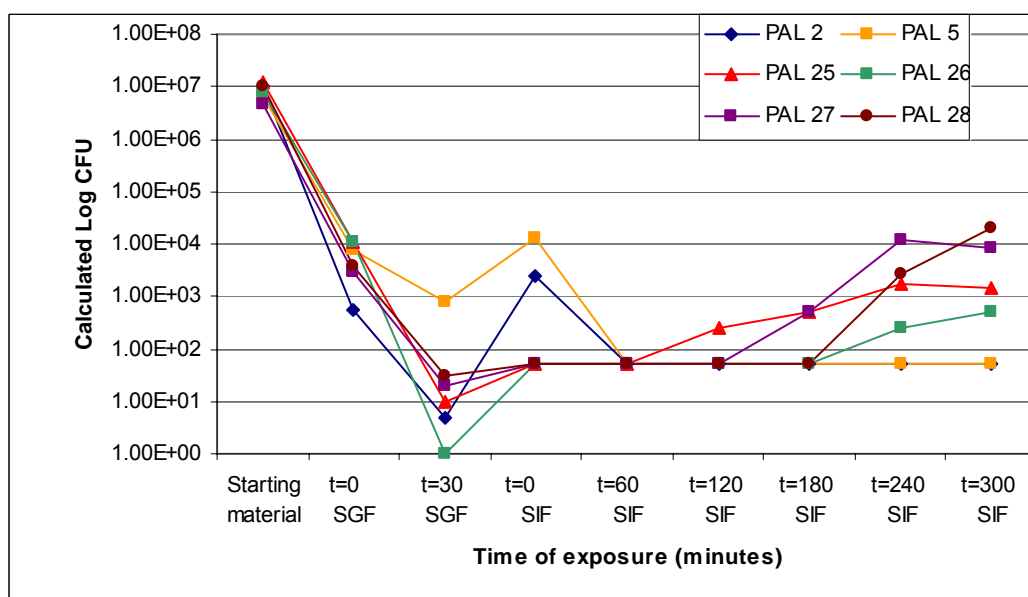


Figure A.3.20. Behaviour of stationary phase cells of mesophilic strains in SIF after exposure to pH 2.5 in SGF.

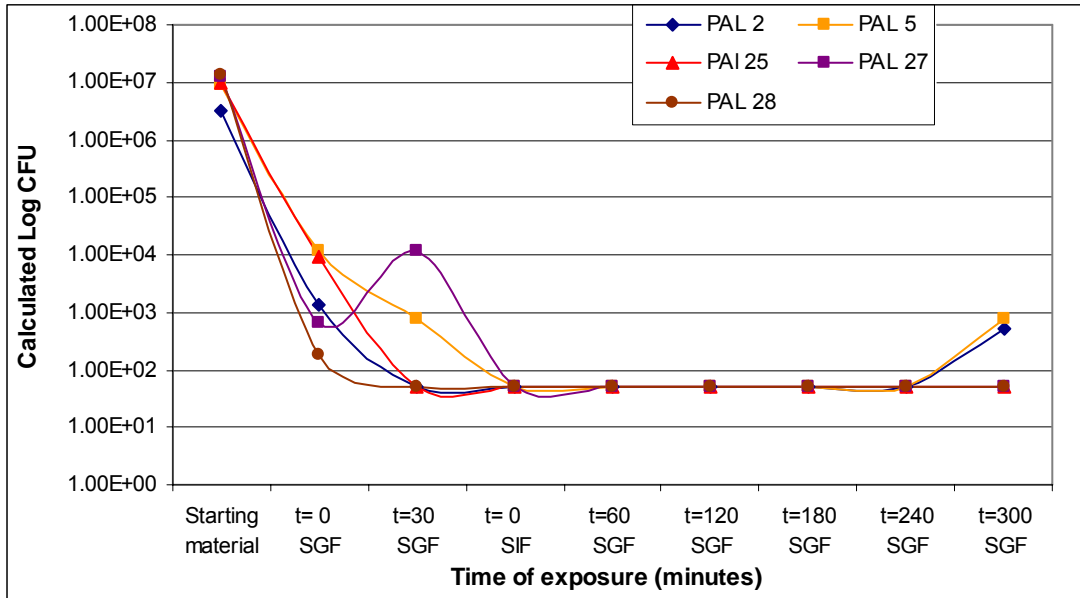


Figure A.3.21. Behaviour of exponential phase cells of mesophilic strains in SIF after exposure to pH 2.5 in SGF.

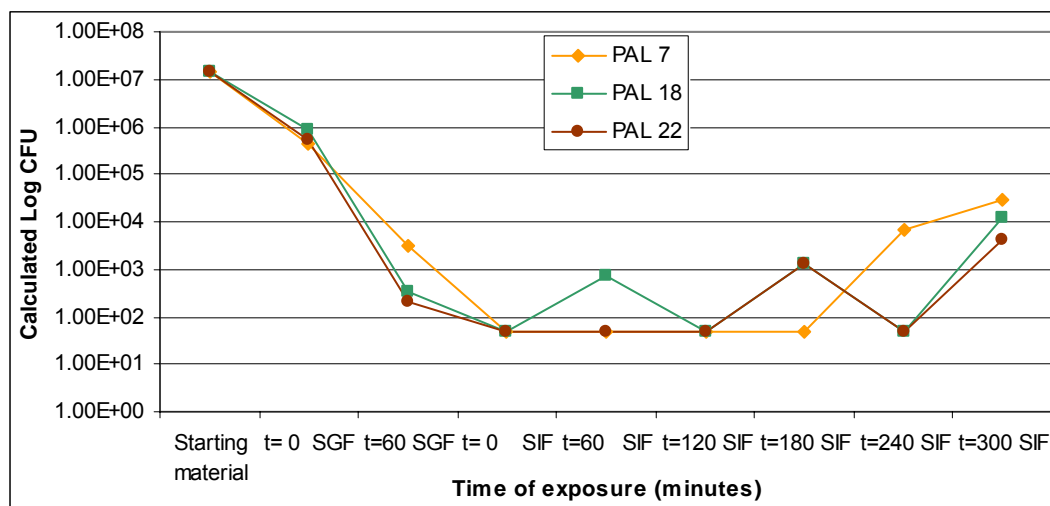


Figure A.3.22. Behaviour of stationary phase cells of psychrotrophic strains in SIF after exposure to pH 4.0 in SGF.

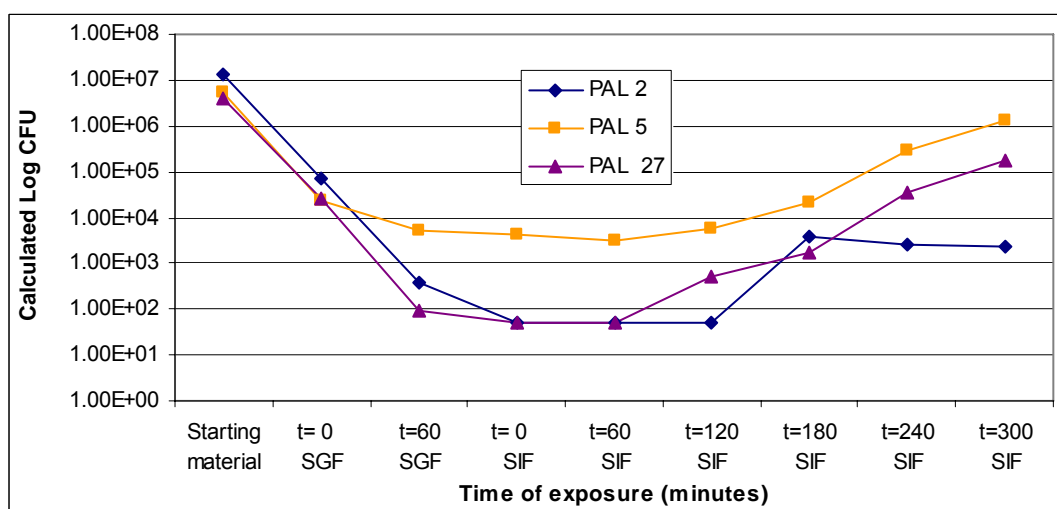


Figure A.3.23. Behaviour of stationary phase cells of mesophilic strains in SIF after exposure to pH 4.0 in SGF.

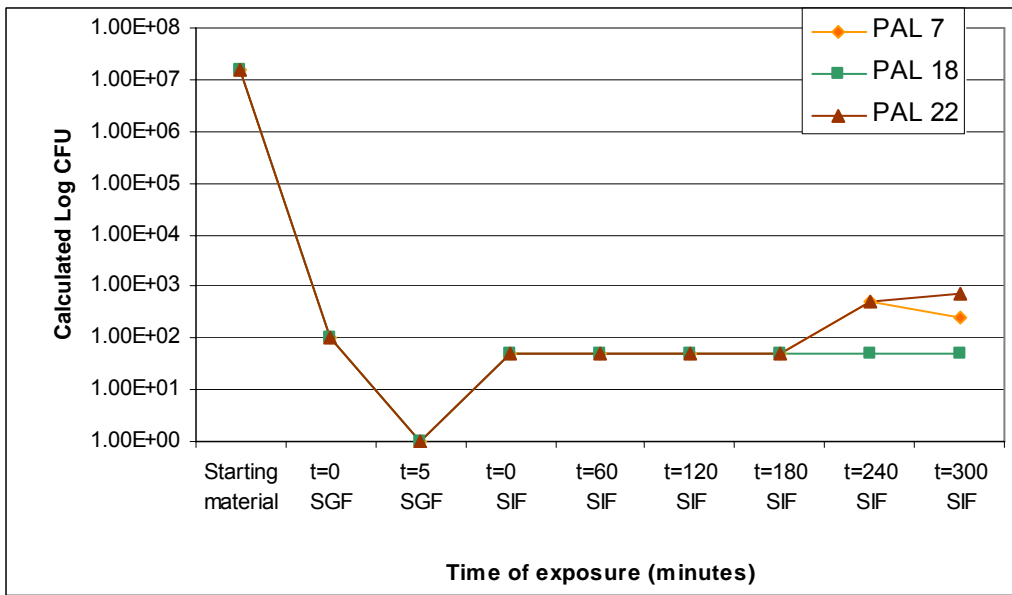


Figure A.3.24. Behaviour of stationary phase cells of psychrotrophic strains in SIF after exposure to pH 1.0 in SGF.

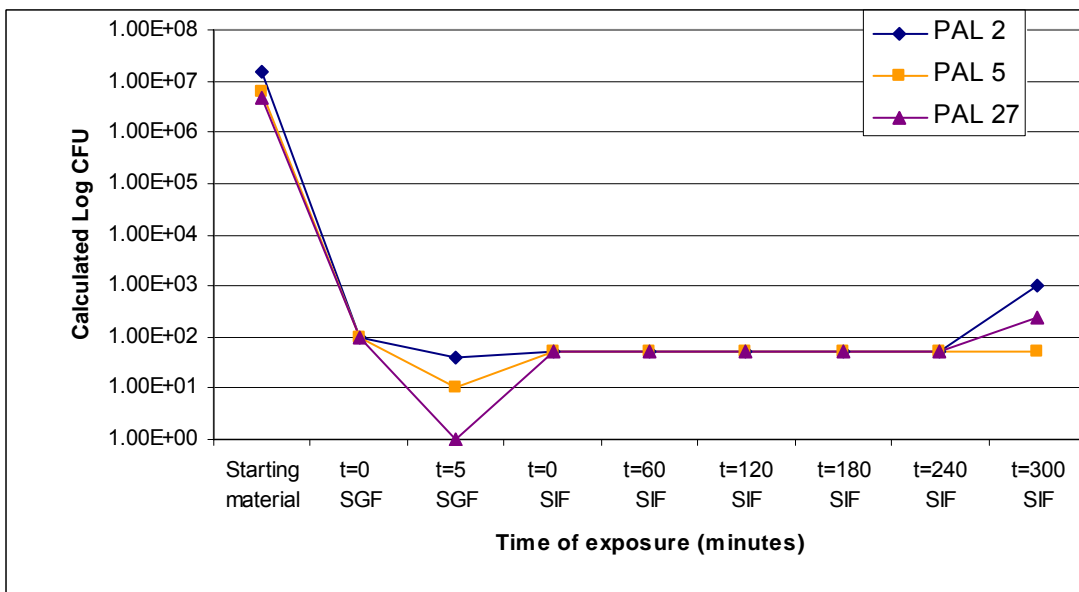


Figure A.3.25. Behaviour of stationary phase cells of mesophilic strains in SIF after exposure to pH 1.0 in SGF.



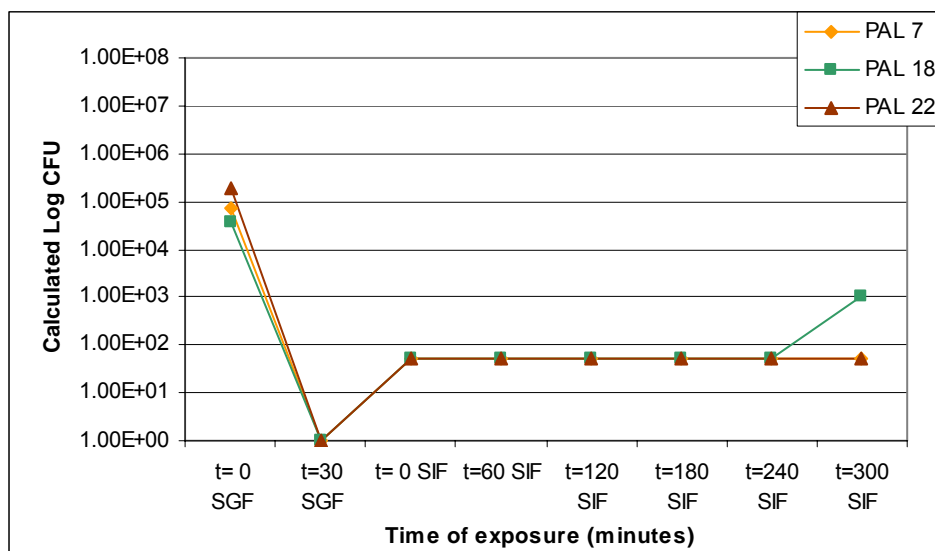


Figure A.3.26. Behaviour of stationary phase cells of psychrotrophic strains in SIF after overnight exposure to 10 °C and exposure to pH 2.5 in SGF.

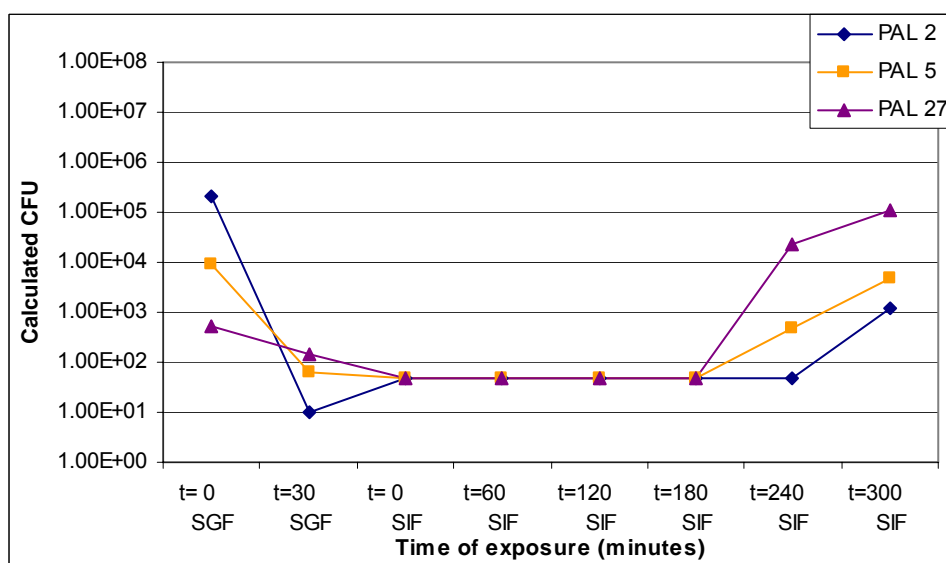


Figure A.3.27. Behaviour of stationary phase cells of mesophilic strains in SIF after overnight exposure to 10 °C and exposure to pH 2.5 in SGF.



## Appendix 4 Interaction between *Bacillus cereus* and differentiated Caco-2 cells (as a model for the epithelium of the small intestine)

### Tables and Figures

Table A.4.1. Adhesion percentages starting from spores (left) and starting from vegetative cells (right).

Spores			Strain	Vegetative cells		
total count at t=0	total count at t=1	% adhesion		total count at t=0	total count at t=1	% adhesion
4,4E+04	2,8E+02	0,63	PAL 2	2,2E+04	2,9E+02	1,32
6,4E+05	2,6E+03	0,41	PAL 3	3,0E+05	1,5E+04	5,00
1,9E+07	3,4E+05	1,8	PAL 5	3,8E+05	5,4E+04	14,06
8,8E+04	2,8E+02	0,32	PAL 7	1,1E+05	1,2E+04	10,71
1,7E+06	2,9E+04	1,7	PAL 17	1,1E+06	1,0E+05	9,26
1,9E+06	2,1E+04	1,1	PAL 18	3,2E+06	1,0E+05	3,09
5,6E+05	4,5E+03	0,8	PAL 20	7,2E+05	2,5E+04	3,47
2,2E+05	1,9E+03	0,86	PAL 22	4,4E+05	9,9E+03	2,25
1,3E+06	6,5E+05	51	PAL 25	2,7E+06	6,0E+04	2,24
6,8E+03	1,0E+01	0,15	PAL 26	9,6E+03	7,0E+01	0,73
1,4E+04	8,5E+01	0,59	PAL 27	2,4E+06	4,0E+02	0,02
1,6E+06	3,0E+03	0,19	PAL 28	2,3E+06	4,2E+04	1,84

Table A.4.2. Adhesion of spores with and without differentiated Caco-2 cells.

Ratios of spore counts and total counts at start of experiment (S) and at t=1 (t=1) plus percentage of t=1/S with and without differentiated Caco-2 cells.

The last column indicates a significant difference in percentage between ratios with and ratios without differentiated Caco-2 cells.

Strain	t=1 Caco-2			t=1 cell-free			Germination index t=1 cell free / t=1 Caco-2
	Total count	Spore count	Ratio	Total count	Spore count	Ratio	
PAL 2	1.30E+02	5.00E+00	0.038	5.60E+02	2.70E+02	0.482	12.5
PAL 3	3.20E+03	7.00E+02	0.219	1.10E+04	2.30E+03	0.209	1.0
PAL 5	1.50E+05	1.70E+05	1.133	3.20E+04	2.30E+04	0.719	0.6
PAL 7	5.40E+03	8.00E+02	0.148	6.00E+03	3.00E+03	0.500	3.4
PAL 17	2.80E+04	5.10E+03	0.182	8.50E+04	9.30E+04	1.094	6.0
PAL 18	5.00E+04	5.30E+03	0.106	1.30E+05	1.20E+05	0.923	8.7
PAL 20	8.60E+03	4.90E+02	0.057	2.90E+04	1.50E+04	0.517	9.1
PAL 22	9.60E+03	9.50E+02	0.099	2.60E+04	8.70E+03	0.335	3.4
PAL 25	5.60E+05	6.80E+05	1.214	5.30E+04	5.20E+04	0.981	0.8
PAL 26	1.70E+02	9.00E+01	0.529	3.00E+01	3.00E+01	1.000	1.9
PAL 27	5.40E+03	1.90E+02	0.035	4.50E+03	4.70E+02	0.104	3.0
PAL 28	6.40E+04	3.10E+02	0.005	7.80E+04	3.40E+04	0.436	90.0

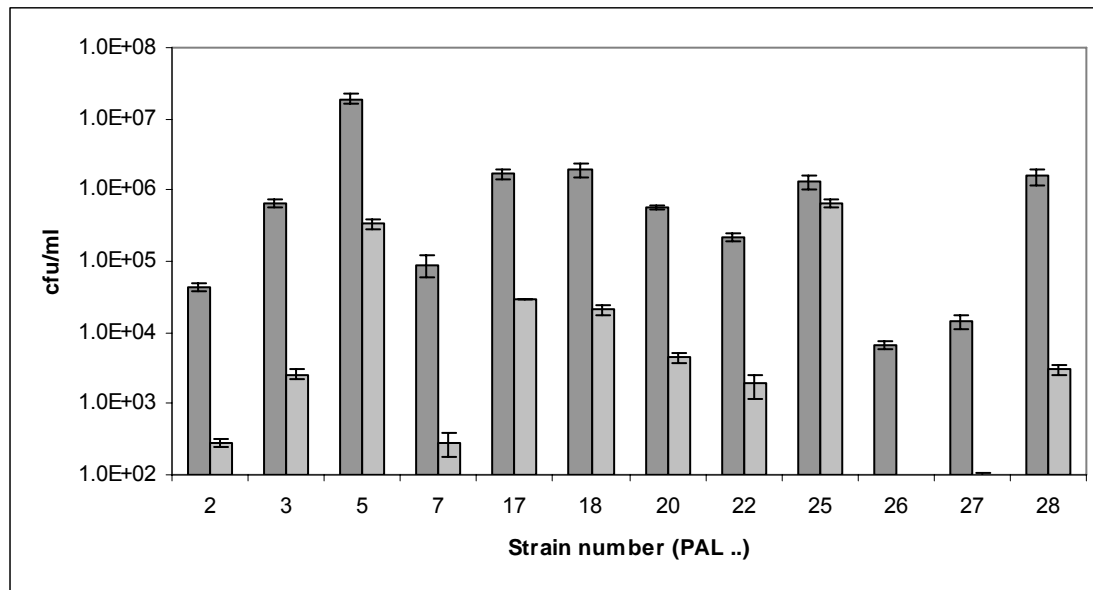


Figure A.4.1. Adhesion of spores to differentiated Caco-2 cells

Dark bars represent the total counts at  $t=0$ . light bars the total counts after 1 hour incubation of Caco-2 cells with spores. The adhesion ratios are shown in Table A.4.1.

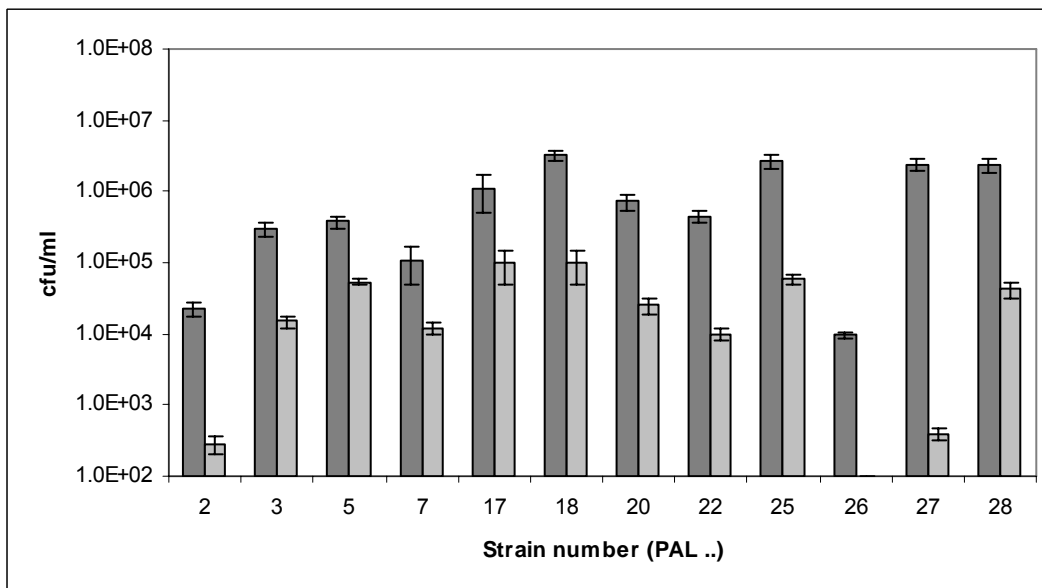


Figure A.4.2. Adhesion of vegetative cells to differentiated Caco-2 cells.

Dark bars represent the total counts at  $t=0$ . light bars the total counts after 1 hour incubation of Caco-2 cells with spores. The adhesion ratios are shown in Table A.4.1.

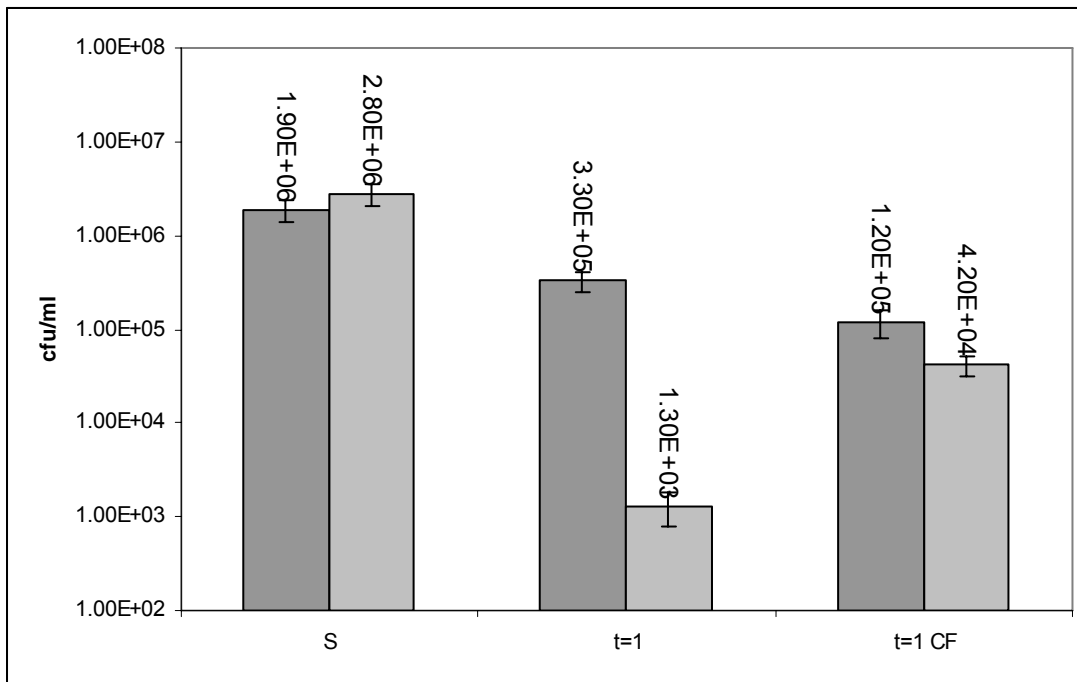


Figure A.4.3. Comparison of total and spore counts of strain 28 in adhesion experiments with and without differentiated Caco-2 cells.

S represents the total count (dark bar) and spore count (light bar) at the start of the experiment. t=1 represents the total count (dark bar) and spore count (light bar) after 1 hour incubation of spores with Caco-2 cells, and t=1 CF the total count (dark bar) and spore counts (light bar) after 1 hour incubation with cell free plates.

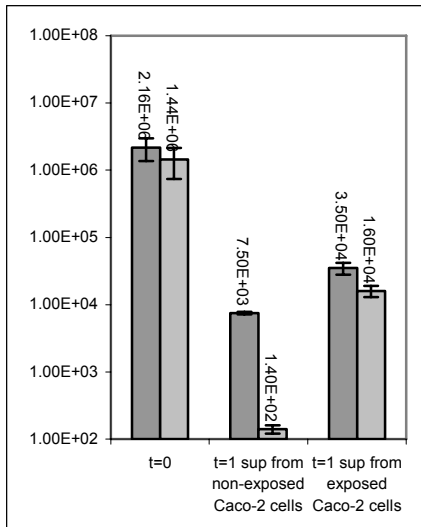


Figure 4.4.A

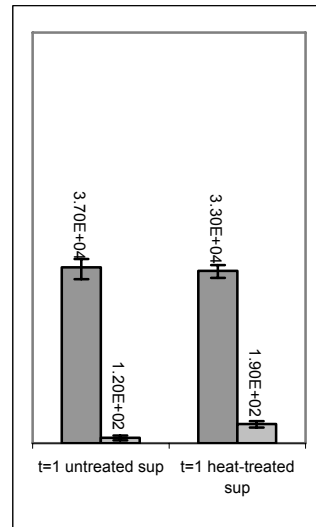


Figure 4.4.B

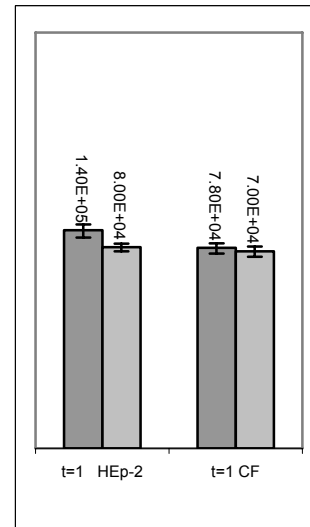


Figure 4.4.C

*Figure A.4.4. Results of control experiments.*

Figure 4.4.A: influence of pre-exposure of supernatant of Caco-2 cells to spores. Bars (+ values) at t=0 is the start of the experiment, and at t=1 with supernatant of non-exposed Caco-2 cells, and at t=1 with supernatant of exposed Caco-2 cells.

Figure 4.4.B: influence of heat treatment on germination inducing compound, left set of bars without heat treatment, right set of bars with heat-treatment.

Figure 4.4.C: influence of HEp-2 cells, left set of bars total and spore counts at t=1 with HEp-2 cells, right set of bars total and spore counts at t=1 in cell-free plates.