Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

A study of the abiotic factors influencing the biofilm and spore formation of dairy isolates of *Geobacillus stearothermophilus* and characterisation of spores based on their heat resistance

MASSEY
UNIVERSITY

A thesis presented in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

in

Food Safety & Microbiology

At Massey University, Palmerston North, New Zealand

Murali Kumar

2021

கற்றது கைமண் அளவு, கல்லாதது உலகளவு

What one knows is only a handful, whereas the unknown is size of the world

Abstract

Geobacillus stearothermophilus, a Gram-positive thermophilic bacterium is an important contaminant in the dairy industry during milk powder manufacture. This bacterium is capable of survival and growth in sections of the milk powder manufacturing plant where higher temperatures (40-65 °C) prevail. They form biofilms on industrial processing surfaces and their spores are heat resistant. Although, these bacteria are not pathogenic, their presence is an indicator of poor plant hygiene and diminishes the quality of end products thereby incurring economic loss. Previous studies have focused on designing control strategies to prevent the biofilm and spore formation during milk powder manufacture but with limited success. This is largely attributed to the limited knowledge we possess on the biofilm and spore forming capacity of these thermophiles and the impact of abiotic factors.

This study focussed on the role of abiotic factors viz. incubation temperature and total dissolved solids on the biofilm and spore formation of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980. The heat resistance of the spores was characterized and the effect of phosphate on the heat resistance of spores was discussed. The effect of temperature and total dissolved solids on the biofilm and spore formation was evaluated using a Centre for Disease Control (CDC) reactor in a milk environment. The results from the CDC study demonstrated that 65 °C is the most preferred temperature for biofilm and spore formation. Milk proteins influence the attachment of bacteria to the stainless steel surface with caseins having the greatest effect. The three strains were characterized based on their heat resistance and the effect of phosphate on the heat resistance of these spores was determined using atomic absorption spectroscopy (AAS). AAS demonstrated that phosphate when present in the heating medium causes release of cations from within the spore thereby lowering their heat resistance. In addition, the Calcium and Dipicolinic acid (DPA) content of these spores was determined and the DPA content was found to be associated with the heat resistance of spores. The heat resistance of spores obtained by a milk biofilm system under

continuous flow was determined and compared with previous values obtained from spores prepared in a static laboratory medium. There is no significant difference ($p \leq 0.05$) in the heat resistance of spores obtained by the biofilm in milk and by a sporulation medium. The results from spore formation under different temperatures and total solids will aid in the design of thermal processing steps aimed towards controlling the biofilm and spore formation of *G. stearothermophilus* during milk powder manufacture. The effect of phosphate on the heat resistance of spores, will aid in the design of a phosphate based cleaning agent to minimise product spoilage caused by the presence of spores of *G. stearothermophilus*. The results showing the similarity between the heat sensitivity of spores prepared in sporulating medium and a continuous flow CDC reactor using milk provide confidence in results on heat resistance taken from laboratory sporulating medium in terms of their relevance to the dairy industry.

This thesis is dedicated to my parents, Kumar and Vijayalakshmi and to the almighty God

Acknowledgements

To my supervisors Steve Flint and Jon Palmer thank-you for your support over the 3.5 years it took to complete this journey. Steve, you always have an amazing amount of curiosity and enthusiasm in coming up with new ideas. I have always admired Jon for your eye on fine details. I would also like to extend my gratitude to Paul Plieger and Mark Waterland from the School of Fundamental Sciences for their help with analytical techniques. Special thanks to Chris Hall and Sawatdeenaruenat Chanapha for their help at the pilot plant. Anne-Marie Jackson, Kylie Evans, Baizura Zain and Hoaran Wang, I really appreciate your efforts in making me feel at ease in the SEAT microbiology lab and helping me out with equipment. John Edwards and John Sykkes a special thanks to you both for your support with the ancillary equipment.

Declaration

This thesis is comprised of seven chapters. Some sections in the materials and methods are repeated, however the results and discussion are different for each chapter.

Table of Contents

Abstract 2

Acknowledgements 5

Declaration 6

List of Publications... 15

List of Conference Presentations... 16

1 General Introduction 18

1.1. Rationale and importance 18

1.2. Research questions and hypotheses.. 20

1.3. References 21

2 Literature Review.. 23

2.1. Introduction to *Geobacillus* sp. and its role as a dairy contaminant........................... 23

2.1.1. Spoilage of milk and milk related products by *Geobacillus* species 23

2.1.2. Controlling *Geobacillus* species in dairy processing... 24

2.2. Spore formation and germination of *Geobacillus* endospores in dairy processing...... 25

2.2.1. Structure of endospores... 25

2.2.2 Overview of steps involved in spore formation 26

2.2.3. Triggering of spore formation.. 26

2.2.3.1. Nutrient limitation in triggering spore formation……………………………27

2.2.3.2. Role of minerals in triggering spore formation………………………………28

2.2.3.3. Role of incubation temperature in triggering spore formation………………29

2.2.3.4. Role of pH in triggering spore formation……………………………………30

2.2.4. Activation and germination of spores 30

2.2.4.1. Role of germinants in spore activation and germination……………………31

2.3. Resistance of spores 32

2.3.1. Heat resistance 32

2.3.2. Role of pH and temperature of sporulation media on heat resistance…………...33

2.4. Biofilm formation 34

2.4.1. Steps involved in biofilm formation 35

2.4.1.1. Steps involved in the adhesion phase……………………………………..35

2.4.1.2. Role of surface conditioning on bacterial attachment………………………..36

2.4.1.3. Milk fouling and factors impacting them……………………………………38

2.4.1.4. Role of temperature and pH on casein particles…………………………...39

2.4.1.5. Effect of milk proteins on bacterial attachment and biofilm formation on stainless steel surface………………………………………………………………………40

2.4.1.6. Proliferation and growth of attached population……………………………..41

2.4.1.7. Detachment phase……………………………………………………………41

2.5. Factors impacting biofilm formation 43

2.5.1.Role of nutrient limitation on biofilm formation 44

2.5.2. Role of oxygen on biofilm formation 45

2.5.3. Role of temperature on biofilm formation 46

2.6. Conclusion 46

2.7. References 47

Objectives of this study 71

Preface to Chapter 3 73

3 Effect of incubation temperature and the total dissolved solids on biofilm and spore formation 74

Abstract 74

3.1. Introduction 75

3.2. Materials and Methods 76

3.2.1. Culture preparation 76

3.2.2. Design and assembly of the CDC biofilm reactor 76

3.2.3. Inoculation of the CDC biofilm reactor 77

3.2.4. CDC reactor runs 77

3.2.5. Enumeration of bacterial cells and spores 78

3.2.6. Statistical analysis 78

3.3. Results and Discussion 78

3.3.1. The effect of temperature on the biofilm and spore formation 78

3.3.2. The effect of total dissolved solids on the biofilm and spore formation 82

3.4. Conclusion 92

3.5. Supplementary information 93

3.6. References 104

Preface to Chapter 4 111

4 Heat Resistance and the Effect of Phosphate 112

Abstract 112

4.1. Introduction 113

4.2. Materials and Methods 114

4.2.1. Production of spores 114

4.2.2 Estimation of DPA content of spores 115

4.2.3. Heat treatment of spores 116

4.2.4. Role of the heating medium 116

4.2.5. Effect of phosphate on the release of calcium 117

4.3. Results and Discussion 118

4.3.1. Heat resistance of spores 118

.... 119

4.3.2. DPA content of spores 119

4.3.3. Effect of pH of the heating medium on the heat resistance of spores 121

4.3.4 Effect of phosphate in lowering the heat resistance of spores 122

4.3.5. Effect of phosphate on the release of calcium 125

4.4. Conclusion 127

4.5. Supplementary information 128

4.6. References 131

Preface to Chapter 5 138

5 Comparison of the heat resistance of spores obtained by CDC reactor and a sporulation medium 139

Abstract 139

5.1. Introduction 140

5.2. Materials and Methods 140

5.2.1. Microorganisms and media 140

5.2.2. Design and assembly of the biofilm reactor 141

5.2.3 Inoculation of the biofilm reactor 141

5.2.4 Operation of the CDC reactor 141

5.2.5 Enumeration of spores 141

5.2.6 Heat treatment of spores 142

5.2.7 Estimation of calcium content of spores 142

5.2.8 Estimation of DPA content of spores 143

5.2.9. Statistical analysis 143

5.3. Results and Discussion 143

5.3.1. Heat resistance of spores 143

5.3.2 Calcium content of spores 144

5.3.3 DPA content of spores 145

5.4. Conclusion 146

5.5. Supplementary information 147

5.6. References 150

6 Overall Discussion and Summary 153

6.1. Effect of temperature and total dissolved solids concentration 153

6.2. Heat resistance of spores and the effect of phosphate 154

6.3. Comparison of heat resistance of spores obtained by two different methods 154

6.4. Summary 155

6.5. Practical contribution of this study to the dairy industry 156

6.6. Future recommendations 156

6.7. References: 157

7 Appendix 159

7.1. Ingredients 159

7.2. UHT treatment 159

7.3. Attachment trial 159

7.4. Heat resistance of spores of A1, D1, P3 and ATCC 12980 in MOPS and citrate phosphate buffer at pH 7.0 and 110°C.. 160

7.5. Kinetic model for biofilm and spore formation of A1, D1, P3 and ATCC 12980. Combase software was used for the creation of these models. 160

7.6. Consolidated curve fit parameters for biofilm and spore formation of A1, D1, P3 and ATCC 12980……………………………………………………………………………………..185

List of Figures

Figure 2.1. Spore structure taken from Setlow et al., 2003 14

Figure 2.2. Schematic diagram of sporulation and germination cycle in *B. subtillis* taken from McKenney et al., 2013 26

Figure 2.3. Schematic diagram of sporulation specific gene transcription regulation during nutrient starvation in *B. subtillis* taken from Molecular Genetics-University of Groningen, 2016 28

Figure 2.4. Schematic representation of nutrient germination of spores of *Bacillus* sp. taken from Setlow et al. 2014 31

Figure 2.5. Stages of bacterial biofilm development taken from Stoodley et al. 2002 35

Figure 2.6. Milk componenet deposition on heated surface.. 35

Figure 2.7. Schematic representation of the fouling mechanisms during the heating of milk taken from Juernink et al. 1996……………………………………………………………………..36

Figure 2.8. Diagrammatic representation showing the sloughing of a mature biofilm taken from ASM News, 1992, 58:202-207... 43

Figure 3.1. Total viable cells and spores obtained from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM. The bar graph represents the total spores and line graph represents total viable cells attached to the stainless-steel coupon. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon for total spores and total viable cells respectively. * represents one or more observations below the detection limit... 80

Figure 3.2. Total viable cells and spores obtained from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 20% (w/v) RSM. The bar graph represents the total spores and line graph represents total viable cells attached to the stainless-st steel coupon. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log

CFU/coupon for total spore and total viable cells respectively. * represents one or more observations below the detection limit 84

Figure 3.3. Total viable cells and spore released into the milk from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM with 4% (w/v) MPC. The bar graph represents the total spores and line graph represents total viable cells released into the flowing milk respectively. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 0.7 and 2.0 log CFU/mL for total spore numbers and total viable cells respectively. * represents one or more observations below the detection limit... 89

Figure 3.4. The total viable cells and spores of *G. stearothermophilus* A1 in 10% (w/v) RSM with 0.8% (w/v) WPC(a and b) and 4.2% (w/v) Na- Caseinate(c and d) added at 55°C obtained from the biofilms(a and c) and released into the milk (b and d). The bar graph represents the total spores and line graph represents total viable cells respectively. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon (a and c) and 0.7 and 2.0 log CFU/mL (b and d) for total spore numbers and total viable cells respectively. * represents one or more observations below the detection limit 90

Figure 4.1. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ºC in distilled water. Error bars represent standard deviation of triplicates. 119

Figure 4.2. Survival curves of G. stearothermophilus A1, D1, P3 and ATCC 12980 in citrate-phosphate buffer at pH 4 (A), pH 7 (B) and pH 8 (C). Error bars represent standard deviation of triplicates 122

Figure 4.3. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ºC in distilled water containing 0.1% (A), 1% (B) and 2% (C) (w/v) of di-sodium phosphate. Error bars represent standard deviation of triplicates... 123

Figure 4.4. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ºC in distilled water containing 0.05% w/v Na-EDTA. Error bars represent standard deviation of triplicates. 125

List of Tables

Table 2.1. Mineral composition of cow's milk taken from the minerals of milk, HAL, France 2005 29

Table 4.1. Comparison of the D_{110} value and the dipicolinic acid content of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 120

Table 4.2. Effect of phosphate on the percentage release of calcium from spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 126

Table 5.1. Comparison of D_{110} values of spores obtained from sporulation agar and from a CDC reactor.. 144

Table 5.2. Comparison of the total calcium content of spores obtained from sporulation agar and from a CDC reactor 145

Table 5.3. Comparison of the DPA content per spore obtained from sporulation agar and from a CDC reactor.. 146

List of Publications

1. M. Kumar, S.H. Flint, J. Palmer, P.G. Plieger, M. Waterland (2019). The effect of phosphate on the heat resistance of spores of dairy isolates of *Geobacillus stearothermophilus*. *International Journal of Food Microbiology* 309, 1083334.

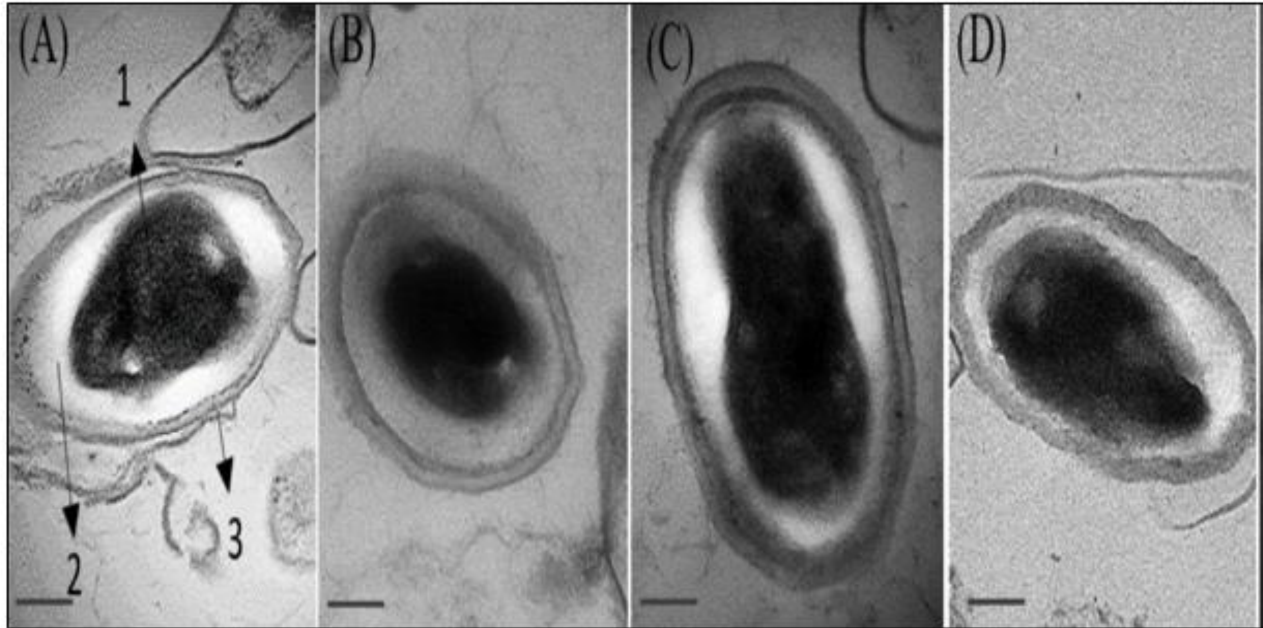
2. **M. Kumar**, S.H. Flint, J. Palmer, S. Chanapha, C. Hall (2020). Influence of the incubation temperature and total dissolved solids concentration on the biofilm and spore formation of dairy isolates of *Geobacillus stearothermophilus*. *Applied and Environmental Microbiology* 87, 02311-20

3. **M. Kumar**, S.H. Flint, J. Palmer, P.G. Plieger (2020). A comparison of the spore heat resistance of dairy isolates of *Geobacillus stearothermophilus* obtained using a CDC biofilm reactor and a sporulating medium. *International Dairy Journal* 116, 105000

List of Conference Presentations

1. M. Kumar, S.H. Flint, J. Palmer, P.G. Plieger, M. Waterland (2018). The effect of phosphate on the heat resistance of spores of dairy isolates of *Geobacillus stearothermophilus*. Presented at: New Zealand Microbiological Society Conference, Dunedin, November 2018.

2. M. Kumar, S.H. Flint, J. Palmer, S. Chanapha, C. Hall (2019). The effect of incubation temperature and total dissolved solids concentration on the biofilm and spore forming capacity of dairy isolates of *Geobacillus stearothermophilus*. Presented at: New Zealand Microbiological Society Conference, Palmerston North, November 2019.



Frontispiece TEM images of spores of *Geobacillus stearothermophilus,* each picture was taken at x60,000 magnification. A: dormant *G. stearothermophilus* A1 spore. B: dormant *G. stearothermophilus* D1 spore. C: dormant *G. stearothermophilus* P3 spore. D: dormant *G. stearothermophilus* ATCC12980 spore. 1: spore core, 2: spore cortex, 3: spore coat. The scale for each image is shown in the lower left corner and corresponds to 200 nm.

Chapter 1

1 General Introduction

1.1. Rationale and importance

Whole and skim milk powder account for half of the global dairy trade with major importers growing in countries like India, China and Brazil (Knips, 2005). Milk powders are used by consumers as a substitute for fresh milk and in the manufacture of a range of processed food products. In a milk powder manufacturing plant, the quality of products can be affected by thermophilic bacteria capable of producing acids or enzymes potentially leading to off flavours. Thermophilic bacteria are also capable of forming heat resistant endospores capable of surviving pasteurization and in some cases UHT treatments (Andre et al., 2013; Scheldman et al., 2006; Scott et al., 2007; Yuan et al., 2012). Among the spore forming bacteria, *Geobacillus stearothermophilus* are well known for their production of heat resistant endospores (Burgess et al., 2010). They form biofilms in milk processing plants and are a potential source of microorganisms leading to spoilage in milk powder (Bremer et al., 2015; Yuan et al., 2018).

There are many gaps in the knowledge and understanding of how *G. stearothermophilus* forms biofilms and spores on industrial processing surfaces and the factors influencing them. The current study addresses some of the gaps to enable a better understanding of the factors influencing the biofilm and spore formation.

The main purpose of this work was to investigate factors that can influence (positively or negatively) the biofilm and spore formation of *G. stearothermophilus* A1, D1, P3, isolates from the dairy industry that have distinct genotypic and phenotypic differences (Burgess et al., 2014). This study included the role of two major abiotic factors namely, incubation temperature and total dissolved solids concentration on the biofilm and spore formation of A1, D1, P3 and ATCC 12980 which has

never been reported earlier for these strains. These two factors were specifically chosen since these three dairy isolates were previously obtained from the evaporator section of the manufacturing facility where a gradient of temperature and milk solids concentration exist (Burgess et al., 2014).

The heat resistance of spores of A1, D1, P3 and ATCC 12980 was determined. Data available in literature suggest that the heat resistance of spores is influenced by the sporulation condition and the heating medium (Wells-Bennik et al., 2019), so this formed the basis for an investigation of the heat resistance of these spores. The role of phosphate on the heat resistance of spores was studied and a hypothesis for the role of phosphate as a chelating agent when present in the heating medium was validated. In the last part of this study, the heat resistance of spores of A1, D1, P3 and ATCC 12980 obtained from a sporulating medium and from a continuous flow CDC were compared for the first time.

This study provides an improved understanding of the factors influencing the biofilm and spore formation of these phenotypically different dairy isolates of *G. stearothermophilus* and characterized the spores based on their heat resistance. The information and knowledge gained is useful for the design of cleaning processes involving a phosphate based cleaning agent in dairy manufacture to avoid contamination of milk with *G. stearothermophilus* and in the design of sterilization and ultra-high temperature (UHT) thermal treatments aimed towards minimising the occurrence of heat resistant *G. stearothermophilus* spores during the production of commercially sterile dairy products.

1.2. Research questions and hypotheses

Research Questions

- What are the roles of abiotic factors i.e. incubation temperature and the total dissolved solids in the biofilm and spore formation of dairy isolates of *Geobacillus stearothermophilus*?
- What is the effect of milk proteins (whey and caseins) on the biofilm and spore formation of dairy isolates of *G. stearothermophilus*?
- How can spore populations of *G. stearothermophilus* be characterised based on their heat resistance and what is the role of the heating medium on the heat resistance of spores?
- What is the role and mode of action of phosphate in lowering the heat resistance of spores of *G. stearothermophilus* A1, D1 and P3?
- Is the heat resistance of spores obtained from the milk biofilm system under continuous flow different from those obtained by sporulating medium?

Hypotheses

- Biofilm and spore forming capacity of *G. stearothermophilus* A1, D1 and P3 is influenced by the incubation temperature and the total dissolved solids of milk
- Individual milk proteins including caseins and whey protein influence the biofilm and spore formation of dairy isolates of *G. stearothermophilus*
- Thermal resistance of spores of dairy isolates of *G. stearothermophilus* is lowered by the presence of phosphate in the heating medium with phosphate acting as a chelating agent, causing excess release of cations from within the spore
- Spores generated on laboratory sporulating medium show similar heat sensitivity to those generating in a continuous flow milk system.

1.3. References

André, S., Zuber, F., & Remize, F. (2013). Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. *International Journal of Food Microbiology*, 165(2), 134–143.

Bremer, P., Flint, S., Brooks, J., & Palmer, J. (2015). Introduction to biofilms: definition and basic concepts. In *Biofilms in the Dairy Industry,* Wiley Balckwell, United Kingdom.

Burgess, S.A., Flint, S., & Lindsay, D. (2014). Characterization of thermophilic bacilli from a milk powder processing plant. *Applied Microbiology*, 116(2), 305-359.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. In *International Journal of Food Microbiology* ,144(2), 215–225.

Knips, V. (2005). Developing countries and the global dairy sector -part 1 global overview. Food & Agriculture Organisation. Italy.

Scheldeman, P., Herman, L., Foster, S., & Heyndrickx, M. (2006). *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology*, 101(3), 542–555.

Wells-Bennik, M. H. J., Janssen, P. W. M., Klaus, V., Yang, C., Zwietering, M. H., & den Besten, H. M. W. (2019). Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. *International Journal of Food Microbiology*, 291(2), 161–172.

Yuan, D. D., Liu, G. C., Ren, D. Y., Zhang, D., Zhao, L., Kan, C. P., Yang, Y. Z., Ma, W., Li, Y., & Zhang, L. B. (2012). A survey on occurrence of thermophilic bacilli in commercial milk powders in China. *Food Control*, 25(2), 752–757.

Yuan, L., Sadiq, F. A., Burmølle, M., Liu, T., & He, G. (2018). Insights into bacterial milk spoilage with particular emphasis on the roles of heat-stable enzymes, biofilms, and quorum sensing. *Journal of Food Protection*, 81(10), 1651–1660.

Chapter 2

2 Literature Review

2.1. Introduction to *Geobacillus* sp. and its role as a dairy contaminant

Thermophilic or heat loving bacteria are capable of growing at elevated temperatures (40-75 °C) and prevail in a variety of industries (Burgess et al., 2010). Their optimum temperature for growth is above the maximum temperature required for the growth of pathogenic bacteria (about 45 °C). Thermophilic bacteria are widely distributed in nature (Bergey, 2007). Among the thermophilic bacteria, *Geobacillus* sp. are well known to be common contaminants in the dairy and food industries (Burgess et al., 2010). In the dairy industry, *Geobacillus* sp. are a primary cause of concern due to their ability to form heat resistant spores and biofilms on processing surfaces (Eijlander et al., 2019; Gopal et al., 2015; Li et al., 2019). Although, not pathogenic, these bacteria are capable of producing acids or enzymes that can cause spoilage of dairy products (De Jonghe et al., 2010). In New Zealand, *Geobacillus* sp. colonise all stages of the milk powder manufacture process except the pre heating stage (Dettling et al., 2020; Scott et al., 2007). Although, *Geobacillus* sp. are known to cause contamination of milk powders, little success has been achieved in completely understanding the ecological niche of these bacteria, their mechanism of sporulation and biofilm formation.

2.1.1. Spoilage of milk and milk related products by *Geobacillus* species

Geobacillus stearothermophilus, formerly referred to as *Bacillus stearothermophillus* (Nazina et al., 2001) is capable of producing heat stable enzymes and acids which can result in off flavours in the final product (Delaunay et al., 2021; Ledenbach & Marshall, 2010). *G. stearothermophillus* has been known to cause flat sour spoilage of evaporated milk, low acid canned vegetables and tomato juice (Heyndrickx & Scheldeman, 2008). The presence of thermophilic bacteria in finished dairy products i.e. milk powders is an indication of poor plant hygiene (Burgess et al., 2014). The source

of thermophilic contamination of milk in dairy industries has been linked with biofilms that are formed on surfaces of processing equipment (Lindsay & Flint, 2009; Scott et al., 2007). Considering the very low levels of thermophiles in raw milk (McGuiggan et al., 2002; Miller et al., 2015) and the short residence of time of milk (<30 min) in a dairy manufacturing plant, it is likely that contamination of end products is caused by microbial growth within the manufacturing equipment. Dispersal of cells from within biofilms into the environment is a natural step in the biofilm cycle (Kaplan, 2010), which may contribute towards microbial contamination of end products.

2.1.2. Controlling *Geobacillus* species in dairy processing

Several modifications in Standard Operating Procedures (SOP) of milk powder manufacturing facilities have been found to be effective against thermophile growth in milk powder manufacturing plants. Such modifications include altering the processing times of fluids, use of sanitizers in Cleaning in Place (CIP) procedures, altering the surface of pipeline, modifying processing temperatures, modifying existing CIP procedures and using quorum sensing inhibitors (QSI) (Ben-Ishay et al., 2017; Flint et al., 2020; Friedlander et al., 2019; Lee Wong, 1998; Meesilp & Mesil, 2019; Ostrov et al., 2019; Vlková et al., 2008; Yuan et al., 2018). The effectiveness of using commercially available sanitizers during CIP procedures is debatable considering bacteria within biofilms are protected from chemicals due to multispecies co-operation and by the Extracellular Polysaccharide (EPS) matrix (Marchand et al., 2012). Knight *et al.* (2004), demonstrated that temperature disruption could control biofilm formation of *Streptococcus thermophilus* in Plate Heat Exchangers (PHE) pasteurizers used for cheese production. Burgess et al. (2009), demonstrated that lowering the growth temperature of *A. flavithermus* from 55 to 48 °C prevented the formation of spores within biofilms. Chmielewski et al. (2003), concluded that altering topography of contact surfaces could control biofilm formation in food processing facilities.

2.2. Spore formation and germination of *Geobacillus* endospores in dairy processing

The genetic pathway concerning the initiation of spore formation and the general stress response are interconnected among *Bacillus* spp (Reder et al., 2012). In dairy processing, spore formation among *Geobacillus* sp. occurs rapidly but the factors contributing towards the initiation of sporulation remains largely unknown. In addition, the spore properties i.e. heat resistance and DPA concentration among *Geobacillus* sp. is known to be influenced by the sporulation conditions (Wells-Bennik et al., 2019). Understanding the role of various physiological factors on the spore forming capacity of *Geobacillus* sp. is important to design methods to control spore formation in milk powder manufacture.

2.2.1. Structure of endospores

Endospores of bacteria comprise of a core region containing the nuclear material surrounded by a dual membrane cortex, which is enclosed by a spore coat (Figure 2.1). Seale et al. (2008), suggested that some species of *Geobacillus* sp. and *Bacillus cereus* might have an additional layer over the spore coat called the exosporium. Apart from the nuclear material, the core contains calcium-dipicolinic acid complex (Ca- DPA) and small acid soluble spore proteins (SASP).

Figure 2.1. Spore structure taken from Setlow et al., 2003

The presence of an exosporium in a dairy strain, *G. stearothermophillus* was confirmed through transmission electron microscopy (Seale et al., 2010). Ankolekar et al. (2010), determined the presence of appendages on the surface of spores of *B. cereus*, which may play a role in the survival of these spores through attachment of spores to fouling layers and biofilms.

2.2.2 Overview of steps involved in spore formation

Most of the work pertaining to spore formation is focused on mesophilic spp. namely *B. cereus* and *B. subtillis*. It is presumed that the structure, sporulation, germination and resistance mechanisms of spores are identical for thermophilic bacilli (Burgess et al., 2010). Sella et al. (2014), concluded that spore formation is a multi-step process consisting of: pre-septation, formation of a septum, formation of fore spore followed by the formation of spore components namely the cortex, inner and outer spore membranes followed by the maturation of spores before being released (Figure 2.2).

2.2.3. Triggering of spore formation

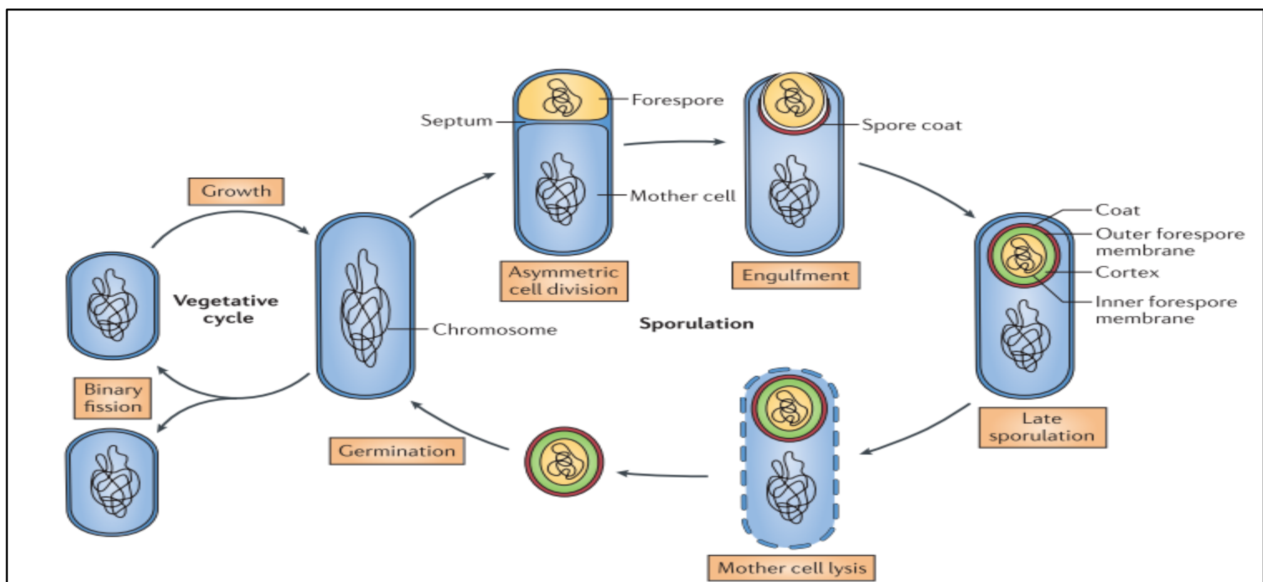


Figure 2.2. Schematic diagram of sporulation and germination cycle in *B. subtillis* taken from McKenney et al., 2013

Unlike the conditions that trigger spore formation in mesophilic bacteria, conditions triggering spore formation in thermophilic bacteria in a dairy environment are less studied. Starvation of cells is considered a likely reason for spore formation among *B. subtillis* (Grossman & Losick, 1988). Spore

formation in a dairy environment is associated with biofilms and the inability of nutrients to reach subpopulations within a biofilm may act as a trigger for spore formation within biofilms (Bremer et al., 2009). Apart from biotic factors, several abiotic factors also play a major role in triggering sporulation. Kolodokin-Gal et al. (2013), recently demonstrated that reduced oxygen levels triggered sporulation of *B. subtillis*. The formation of spores within a biofilm will be discussed in more detail in the forthcoming sections.

2.2.3.1. Nutrient limitation in triggering spore formation

Spore forming bacteria produce highly resistant cell types called endospores in response to un-favourable conditions. Nutrient limitation in the growth medium is one of the major factors contributing towards the trigger of spore formation among thermophilic bacteria. In response to lack of availability of nutrients, cells can gain mobility in search of nutrients or sporulate to survive harsh conditions. However, Kolter et al. (2011), determined that the stage of sporulation is the last resort of survival strategy among bacteria. In *B. subtillis*, the onset of nutrient limitation causes activation of the Spo0A regulon, responsible for the initiation of spore formation in cells. The activation further leads to a cascade of events which leads to activation of the sporulation factor σ^H (Figure 2.3) (Molecular Genetics, University of Groningen, 2016). Spore forming bacteria require a wide array of macro and micro molecular nutrients to facilitate growth and the lack of availability of one or more of these nutrients could trigger spore production. De Vries et al. (2005), studied the influence of amino acids on spore formation in *B. cereus*. A specific strain of *B. cereus*, ATCC 14579, that prefers amino acids as an energy source produced spores following depletion of these amino acids in the growth medium.

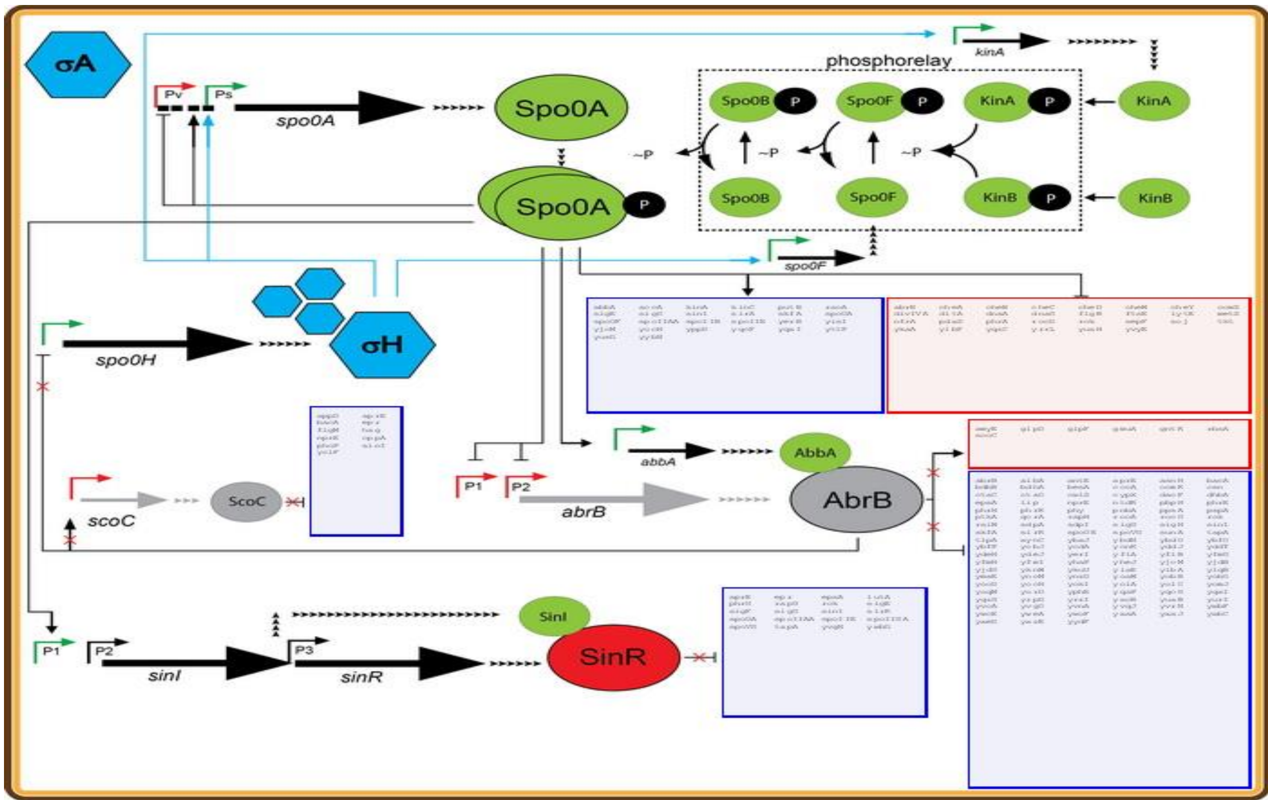


Figure 2.3. Schematic diagram of sporulation specific gene transcription regulation during nutrient starvation in *B. subtillis* taken from Molecular Genetics-University of Groningen, 2016

2.2.3.2. Role of minerals in triggering spore formation

The mineral fraction of milk is about 8-9 gL^{-1} consisting of cations (calcium, magnesium, sodium, manganese and potassium) and anions (phosphate, citrate and chlorides) (Gaucheron, 2005). The calcium and phosphate content are high in protein rich cow's milk (Table 2.1)

The role of these minerals on spore formation is poorly defined. However, the presence of these minerals may play a major role in spore formation. Mineral salts are commonly used in sporulation media (Yao & Walker, 1967). The presence of these minerals in milk (Gaucheron, 2005) raises the possibility of the role of these minerals on spore formation of thermophilic bacteria in dairy environment. The possibility of minerals being important in the spore formation of *Geobacillus* is indicated through sporulation in media containing salts (Seale et al., 2008). Spore yields of 10^5-10^7 spores/ml of *Geobacillus* was demonstrated by Seale et al. (2008) in tryptone medium containing salts i.e. $CaCl_2$, $MnSO_4$, $FeSO_4$ or $MgCl_2$.

Calcium is known to increase the expression of genes involved in spore formation (Oomes et al., 2009). Calcium accumulates in the fore spore during spore formation (Scherrer & Gerhardt, 1972) and higher concentrations of calcium increase the expression of genes involved in the sporulation pathway (Oomes et al., 2009). Calcium present in the spore cortex is chelated with exogenous dipicolinic acid in a 1:1 ratio and plays a vital role in the wet heat resistance of endospores.

Table 2.1. Mineral composition of cow's milk taken from the minerals of milk, HAL, France 2005

Mineral	Concentration ($mg.kg^{-1}$)	Concentration ($mmol.kg^{-1}$)
Calcium	1043-1283	26-32
Magnesium	97-146	4-6
Inorganic phosphate	1805-2185	19-23
Total phosphate	930-992	30-32
Citrate	1323-2079	7-11
Sodium	391-644	17-28
Potassium	1212-1681	31-43
Chloride	772-1207	22-34

Although the role of calcium on sporulation has gained attention in recent years, the role of other minerals i.e. manganese on sporulation is also reported. Sporulation of *B. subtilis* was inhibited in medium lacking manganese salt (Vasantha & Freese, 1979).

2.2.3.3. Role of incubation temperature in triggering spore formation

The effect of incubation temperature on sporulation has been studied previously. Rey et al. (1975), who studied the effect of temperature on growth and sporulation of *Clostridium* concluded that temperature of incubation had a significant effect ($p \leq 0.05$)on the number of spores produced. Baril et al. (2012), who studied the effect of temperature on the spore formation kinetics of *Bacillus* sp. concluded that lower temperatures delayed the sporulation process among *B. licheniformis* and *B.*

weihenstephanesis. Mtimet et al. (2015), concluded that the most efficient temperature for sporulation among *G. stearothermophilus* ATCC 12980 is close to the optimum temperature of growth. Burgess et al. (2009), who studied the effect of temperature on the biofilm and spore formation of *A. flavithermus* concluded that spores were formed within the biofilm at 55°C, 60°C and not at 48°C.

To the authors knowledge, no studies have been undertaken to study the effect of incubation temperature on the spore formation of dairy isolates of *Geobacillus* sp.

2.2.3.4. Role of pH in triggering spore formation

Yazdany and Lashkari (1975), who studied the effect of incubation pH on spore formation among *B. stearothermophilus* concluded that maximum sporulation occurred between pH 7.7 - 8.7 and sporulation decreased below and above the above mentioned pH ranges. Mazas et al. (1997), determined that sporulation rates and the amount of spores of *B. cereus* were only high in the range of pH 6.5-8.0. Baweja et al. (2008), who studied the properties of *B. anthracis* prepared under various environmental conditions demonstrated that sporulation efficiency varies significantly ($p \leq 0.05$) in response to pH alterations. Baril et al. (2012b), concluded that lower incubation temperature and pH delayed sporulation of *B. licheniformis* and *B. weihenstephanesis* and the final spore concentration decreased progressively as pH decreased.

2.2.4. Activation and germination of spores

Bacterial spores can lay dormant for many years until the onset of conditions favourable for growth. Substances causing the activation and germination of spores are termed as “germinants”. Germinants include biotic and abiotic components including nutrients, heat and minerals. The process of changing from a spore to a vegetative cell involves a three step process: activation, germination and outgrowth (Setlow, 2014). Activation of spores can result from heat, chemicals and acidic conditions (Setlow, 2014; Abee et al., 2011).

Spore activation and germination of *B. subtilis* is a three step process involving cation release; Ca-DPA release and lysing of the spore cortex (Setlow, 2014). The step succeeding activation of spores is the first stage of germination, commonly referred to as the commitment stage involving partial cortex hydrolysis and release of monovalent cations upon germinant addition followed by stage 2 involving complete hydrolysis of the spore cortex, thereby compromising the dormant state that was observed previously (Figure 2.4).

Figure 2.4. Schematic representation of nutrient germination of spores of *Bacillus* sp. taken from Setlow et al. 2014

2.2.4.1. Role of germinants in spore activation and germination

Setlow (2014), concluded that spores of *Bacillus* spp. can lay dormant for years, but can rapidly lose their dormancy upon exposure to molecules called germinants. Spores of *Bacillus sp.* are equipped with a specific set of receptors that constantly monitor the environment for germinants. Germinants, capable of spore activation can be organic or inorganic molecules (Moir, 2006). Upon the successful binding of a germinant with the specific germinant receptor, a cascade of reactions results in the loss of heat resistance of spores. McCann et al. (1996), proposed that sometimes more than one germinant receptor is involved in response to single or multiple germinants. Heterogeneity among

spore populations has been observed mainly due to large variation in levels of germinant receptors. Super dormancy, observed among spore populations is widely believed to be caused by low levels of germinant receptors, negatively influencing the rate of germination (Wei et al., 2010).

2.3. Resistance of spores

Bacterial spores are highly resistant to a variety of factors including heat, electromagnetic radiation, mechanical disruption and chemicals (Chandler et al., 2001; Moeller et al., 2014). Spore resistance is identified through the determinants which include: low water content, impermeability of inner membrane, spore coat, peptidoglycan cortex, α/β type SASP and DPA (Nicholson et al., 2000). Spores of *G. stearothermophillus* suspended in skim milk are known to survive at 120 °C for 191 seconds (Huemer et al., 1998) which are likely to raise food quality concerns in dairy product and for producers of commercially sterile products.

2.3.1. Heat resistance

Spores of thermophilic bacteria can be inactivated through severe heat treatments, but such methods are not welcomed in the dairy industry considering significant loss of organoleptic and nutritional effects at high temperatures. It is widely believed that spores are released from within biofilms attached to walls of evaporators in dairy environment where temperatures might reach 60-70°C (Hinton et al., 2002).

Heat resistance of spores is widely believed to be attributed to the level of dehydration of the spore cortex (Setlow, 2014). A common observation among highly heat resistant spores is the presence of a well-dehydrated spore cortex. The determining factor in the level of cortex dehydration is believed to be environmental conditions. There are several other factors determining heat resistance of spores including divalent metal ions, DPA and SASP (Setlow, 2006). DPA is associated with dehydration of the spore cortex and is found chelated with divalent metal ion(s) predominantly calcium within the spore cortex (Setlow, 2014). During germination, binding of germinant receptors to germinants causes the activation of Ca-DPA channels and excretion of Ca-DPA thus causing the loss of heat

resistance. The α/β-type SASP are nonspecific DNA binding proteins which protect the spore DNA from high temperature, UV and peroxides (Hayes & Setlow, 2001).

2.3.2. Role of pH and temperature of sporulation media on heat resistance

High sporulation temperatures result in spores with high wet heat resistance(Condon et al., 1992). Murray and Headlee (1931), showed that heightened heat resistance of spores produced at high temperatures is possibly due to the high levels of Ca^{2+}, Mg^{2+}, DPA and DAP within these spores. Recent studies have confirmed the role of high sporulation temperature with mineralization and DPA accumulation(Beaman & Gerhardt, 1986; Den Besten et al., 2010; Palop et al., 1999). Thermophilic spores, although highly heat resistant, are sensitive to the pH of the sporulation medium. Sporulation in acidic environments results in the production of heat sensitive spores (Marquis & Bender, 1985) whereas sporulation in alkaline conditions results in spores that are resistant to higher temperatures (Guizelini et al., 2012). Alderton *et al.* (1963), proposed the reason for the reduction of heat resistance of spores produced in acidic environments is likely due to the displacement of divalent cations at low pH. Marquis *et al.* (1985a), concluded that acidic pH results in de-mineralization and loss of cations from the spore protoplast, contributing towards the decreased heat resistance of spores produced at low pH. Palop *et al.* (1999a), determined that spores formed at high temperatures were more resistant to acidic pH in comparison with spores that were formed at low temperatures. Hayrapetyan *et al.* (2016), determined that spores isolated from wet stainless steel (SS) surfaces were more heat resistant than spores obtained from planktonic culture. It is widely agreed by most authors that heat resistance is highest at pH's close to neutrality and decreases with acidification (Cameron et al., 1980; Ramaswamy et al., 2019; Löwik and Anema, 1972; Marquis and Bender, 1985b).

To the authors knowledge, no previous study has discused the role of pH and temperature of the sporulation media on the heat resistance of spores of dairy isolates of *Geobacillus* sp.

2.4. Biofilm formation

Biofilms as defined by Flint et al. (1997), are an aggregation of microbial cells and their associated extracellular polymeric substances, actively attached to, growing and multiplying on a surface. Dairy processing surfaces are generally made of SS surfaces as it has no chemical, bacteriological and organoleptic effect on food (Bremer et al., 2009). Bacterial spores and vegetative cells attach to stainless steel surfaces and form biofilms upon attachment (Iñiguez-Moreno et al., 2019; Parkar et al., 2001). In the dairy industry, thermophilic streptococci and bacilli are the most prevalent bacterial species capable of forming biofilms. Flint et al. (2001), identified through 16S rDNA sequencing that thermophilic bacilli present in New Zealand milk powder are dominated by two bacterial species namely *Anoxybacillus* and *Geobacillus* sp. A similar finding was reported by Zhao et al. (2013), wherein *G. thermoglucosidans*, *G. stearothermophillus* and *A. flavithermus* were the three most prevalent biofilm forming strains in samples isolated from a milk powder manufacturing facility in The Netherlands. Flint et al. (1997), determined that these biofilms generally possess a low cell density (10^6 – 10^7 cells cm^{-2}) and are dominated by a single species. The single species biofilm that are unique to dairy environments are termed as process biofilms. Process biofilms are commonly observed at various sites within the dairy industry however in particular, they are well known to develop rapidly on the surface of heating equipment maintained at temperatures of 40-65°C (Flint et al., 1997).

2.4.1. Steps involved in biofilm formation

Bacterial biofilm formation occurs in several steps which are conserved between species (Stoodley et al., 2002). However, three basic steps involved in biofilm formation are; adhesion, proliferation and growth phase followed by the detachment phase (Figure 2.5).

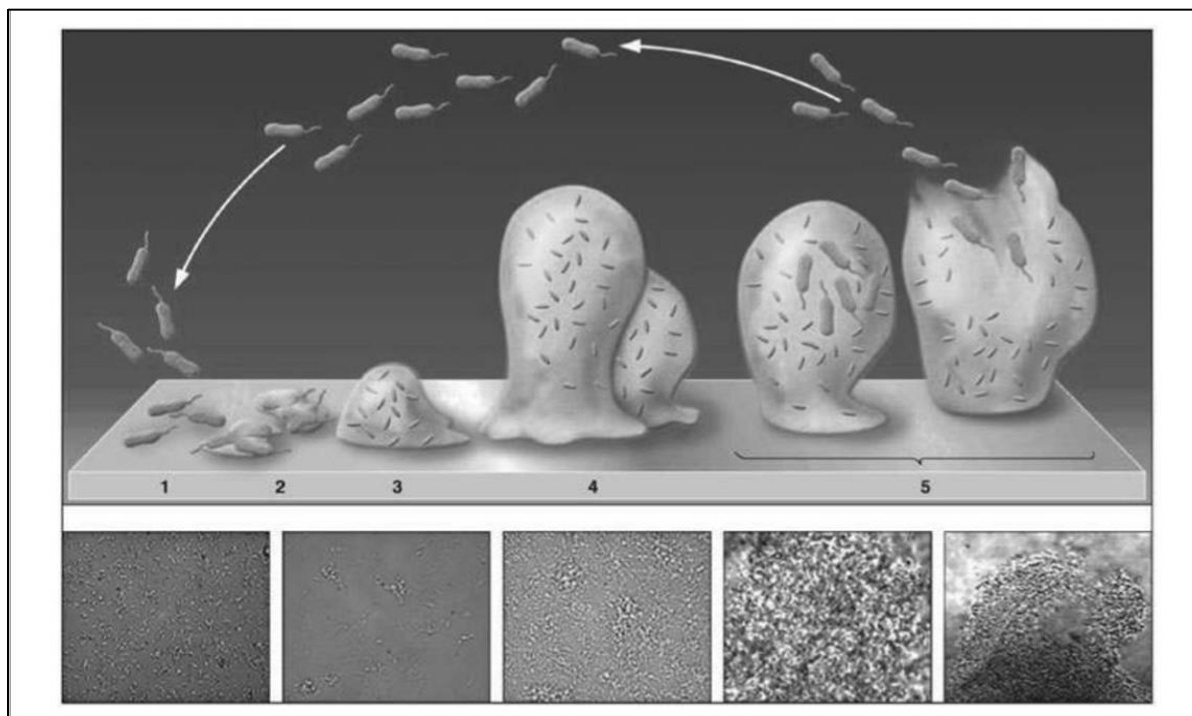


Figure 2.5. Stages of bacterial biofilm development taken from Stoodley et al. 2002

2.4.1.1. Steps involved in the adhesion phase

Adhesion of spores and vegetative cells on processing surfaces occurs in two key steps - reversible and irreversible adhesion. During the initial phase of adhesion, spores and vegetative cells from the bulk fluid are propelled towards the solid surface through physical forces or by bacterial appendages i.e. flagella (Garrett et al., 2008). Attachment of spores and vegetative cells is mediated through weak forces of attraction including Van der Waals forces and electrostatic interaction (Bos et al., 1999). During this phase of attachment, bacteria still exhibit Brownian motion and can be easily dislodged from the surface through shear forces (Marshall et al., 1971). Hence, this stage is termed as reversible attachment.

The reversible step is followed by an irreversible step where the bacterium attaches to the solid surface through the secretion of adhesive polymeric substances. During this stage, vegetative cells and spores interact with the conditioning layer that is formed on the solid surface. In the dairy industry, the conditioning layer is comprised of proteins, fats and salts present in milk. The adsorption of organic molecules such as proteins on the solid surface might play a vital role in the attachment of vegetative cells and spores. The transition from the reversible to the irreversible adhesion involves several short range forces including covalent, hydrogen bonding and hydrophobic interactions (Kumar & Anand, 1998).

Stainless steel, which is the most common food contact material in a food processing plant, is prone to surface roughness, crevices and cracks on the microscopic level, which can harbour microbial cells. Increased surface roughness was believed to enhance bacterial adhesion on these surfaces (Leclercq-Perlat & Lalande, 1994; Pedersen, 1990), but more recent research contradicts these findings (Riedewald, 2006).

2.4.1.2. Role of surface conditioning on bacterial attachment

Preconditioning of the substratum plays a vital but conflicting (positive and negative) role in the attachment of spores and vegetative cells onto processing surfaces (Palmer et al., 2007). Conditioning of a surface leads to a higher concentration of nutrients at the surface in comparison with the liquid phase (Kumar & Anand, 1998). Dickson et al. (1989), determined that the adsorption of organic molecules on the surface could alter the physical-chemical properties (i.e. surface charge, hydrophobicity, free energy), which may enhance bacterial attachment onto these surfaces.

In the dairy context, conditioning is often referred to as fouling, which occurs during the contact of milk with the stainless steel surfaces (Rosmaninho et al., 2007). Jeurnink et al. (1996), determined that the fouling layer is formed by the build -up of proteins and calcium phosphate salts in milk(Figure 2.6). Notably, during heat treatments above 65°C, denaturation of whey proteins occurs which adhere faster to stainless steel surfaces than proteins in their native state. De Jong (1997),

suggested that the protein layer may increase the likelihood of bacterial adherence to the stainless-steel surfaces. In one study, attachment of *G. stearothermophilus* to stainless steel increased 10-100 fold in the presence of milk foulant on stainless steel (Flint et al., 2001). Hinton et al. (2002), demonstrated that thermophiles that were attached to fouled surfaces were more difficult to remove than those attached to clean stainless-steel surface. One cause of foulant formation is the decrease in solubility of calcium phosphate salts upon heating (Jeurnink et al., 1996). The presence of cations i.e. calcium within the fouling layer, may increase the ability of vegetative cells of *A. flavithermus* and *G. stearothermophilus* to attach to stainless steel surfaces (Somerton et al., 2012). Fouling has been identified as a problem in heat exchangers and evaporators where high temperatures (>70 °C) prevail (Jeurnink et al., 1996).

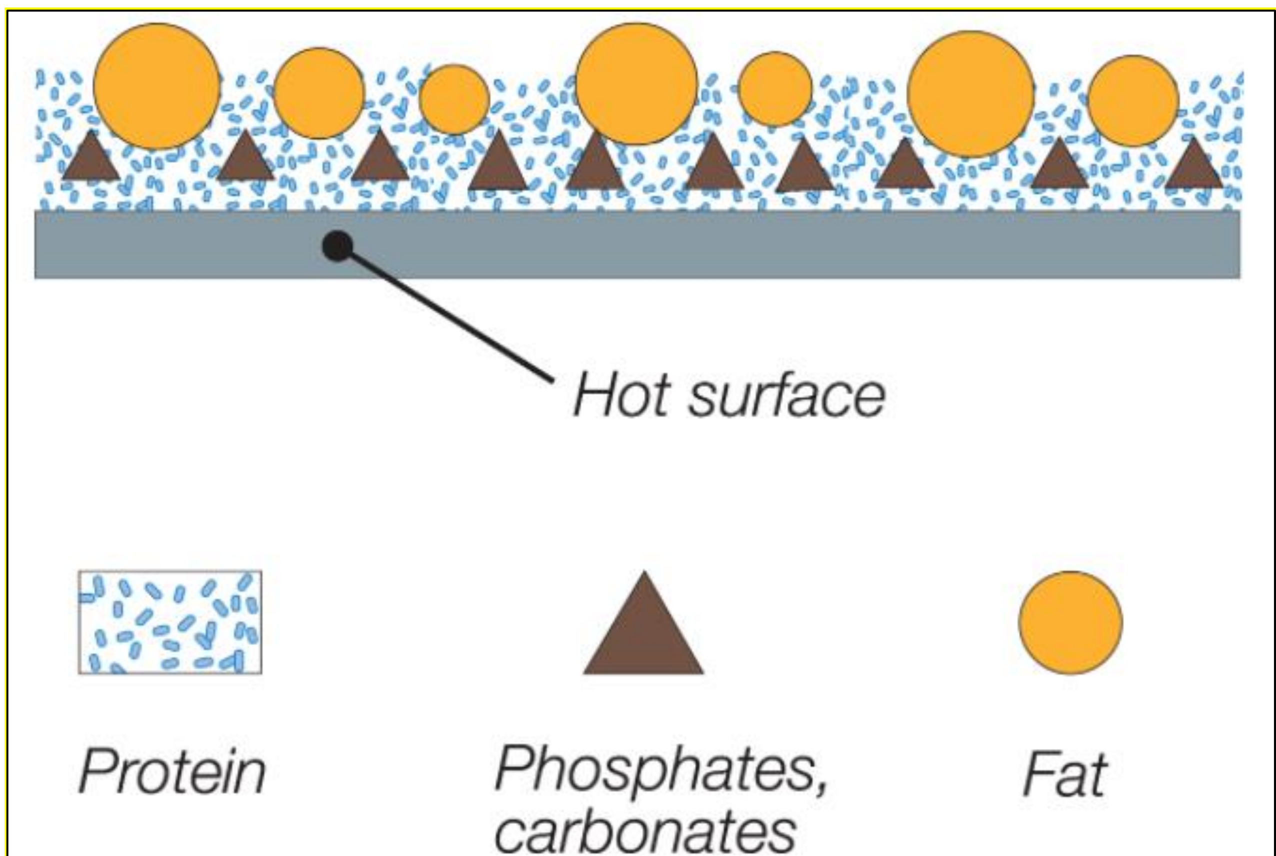


Figure 2.6. Milk component deposition on heated surface taken from Dairy Processing Handbook, Tetrapak.

2.4.1.3. Milk fouling and factors influencing milk fouling

Milk fouling can be classified into two categories : type A and type B (Visser & Jeurnink, 1997). Type A fouling occurs at temperatures between 75 and 110°C. The foulant is composed of 70% proteins, 30-40% minerals and 4-8% fat. Type B fouling occurs at temperatures exceeding 110°C. The composition of a type B foulant is 70-80% minerals, 15-20% proteins and 4-8% fat. Whey proteins constitute 50% of the type A fouling. Amongst the two types of whey protein in milk – β lactoglobulin and α lactalbumin, β lactoglobulin is the most heat sensitive protein and as a result it is the dominant protein in heat induced fouling (Gotham et al., 1992; Lyster, 1970). Caseins are resistant to thermal processing as opposed to whey proteins (Visser & Jeurnink, 1997). Upon heating of milk, the β lactoglobulin unfolds and denatures thus exposing the core containing sulfhydryl groups which reacts with similar or other protein molecules forming aggregates (Jeurnink & De Kruif, 1993) (Figure 2.7). Within the heat exchanger, denaturation of proteins starts at temperatures above 70-74°C (Fryer & Belmar-Beiny, 1991). The steps controlling fouling depend on the bulk (milk) and surface (e.g. stainless steel) processes. The first stage involves denaturation and aggregation of proteins in the bulk followed by transport of the aggregated proteins to the stainless steel surface. It is then followed by surface reactions wherein proteins are incorporated into the foulant layer (Belmar-Beiny & Fryer, 1993). The role of fouling on bacterial attachment is critical for the present study and has been discussed in the following section.

The role of temperature within the heat exchanger on fouling has been previously discussed (Belmar-Beiny & Fryer, 1993; Fryer & Belmar-Beiny, 1991; Jeurnink & Brinkman, 1994).Within the heat exchanger, increasing the temperature beyond 110°C shifts the nature of fouling from type A to B (Burton, 1968). The role of surface temperature on milk fouling was determined to be the most important factor initiating fouling (Aouanouk et al., 2018). In the present study, temperature ranges of 55-65°C were used which is lower than the temperature that is previously mentioned in

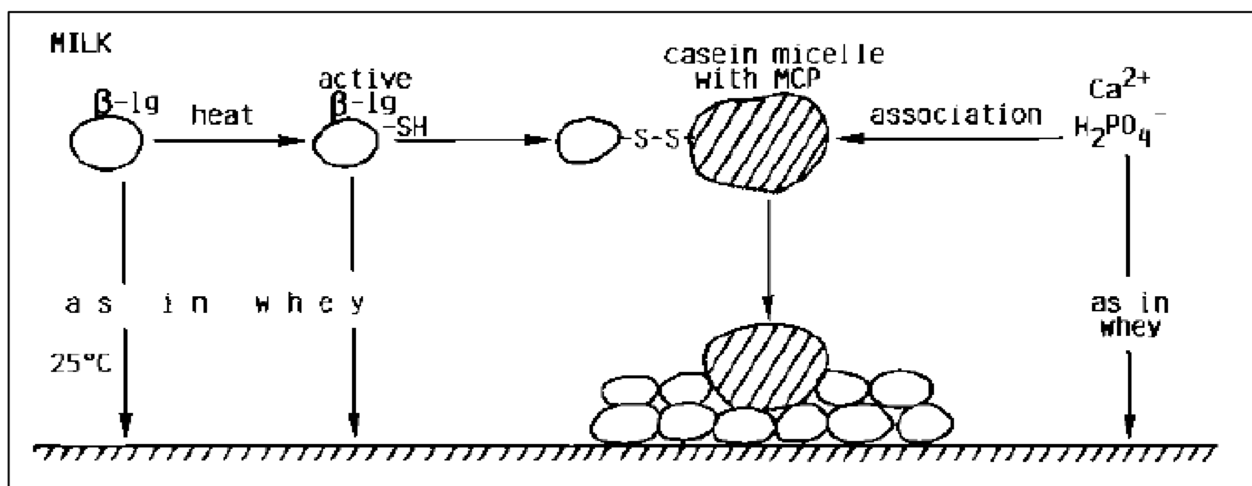


Figure 2.7. Schematic representation of the fouling mechanisms during the heating of milk taken from Juernink et al. 1996.

literature. However, in addition to temperature the role of the protein concentration of milk on fouling has been discussed previously (Changani et al., 1997). Increasing the whey protein concentration of milk has been previously shown to increase fouling deposit on the wall of plate heat exchangers (Alharthi, 2014). Roefs & De Kruif (1994), who studied the effect of β-lactoglobulin concentration on the denaturation and aggregation behaviour concluded that the size of the protein polymer particles (fouling layer) is proportional to the initial β-lactoglobulin concentration.

2.4.1.4. Role of temperature and pH on casein particles

Orlien et al. (2010), demonstrated that increasing the temperature from 4 to 37°C results in enhanced hydrophobic interactions which contributes to the stabilisation of the casein micelle. Ono et al. (1990), demonstrated the increase in turbidity of milk when changing the temperature of milk from 4 to 37°C due to increase in the content of large sized casein micelles of the size 65 nm. An increase in the holding temperature of milk has been demonstrated in increasing the solubility of colloidal calcium phosphate in milk and vice versa thereby demonstrating that reversibility of the changes in colloidal calcium phosphate concentration in milk is temperature dependent (Pouliot et al., 1989). Heat treatment of milk has been shown to change the serum phase of the environment around the

casein micelle (e.g. change in soluble minerals, in particular calcium ions) as well as the casein micelle themselves (e.g. association of whey proteins and changes in colloidal calcium phosphate dephosphorylation) (Singh, 2004). The role of pH on the casein micelle structure has been previously discussed by Sinaga et al. (2017), who concluded that increasing milk pH gives rise to more negatively charged micelles which results in strengthening of the repulsive forces leading to expanded structures in the micelles. McMahon et al. (2009), concluded that acidification of milk decreases the net negative charge of caseins and leads to the solubilization of the colloidal calcium phosphate from the micelles into the solution. The effect of pH on the casein micelle is of less significance to this study where the pH was maintained constant during the experiments discussed in chapters 3 and 5. In this study, UHT skim milk treated at 142°C 8s was used as the working fluid. The heat treatment may impact the casein micelles which may influence the biofilm and spore formation and needs further investigation.

2.4.1.5. Effect of milk proteins on bacterial attachment and biofilm formation on stainless steel surface

The effect of milk proteins on the attachment of bacteria to stainless steel surface has been discussed previously. Barnes et al. (1999), demonstrated that the α-casein, β-casein and κ-casein decreased the attachment of *S. aureus* and *L. monocytogenes* to the stainless steel surface and suggested that the protein might prevent bacterial attachment due to its hydrophilic regions. Speers & Gilmour (1985), observed that the presence of whole milk, fat and casein did not cause any significant increase ($p \leq 0.05$) in bacterial attachment in the presence of milk components. Fletcher (1976), meanwhile demonstrated that casein protein after adsorption to polystyrene surface had little effect on the attachment of *Pseudomonas* sp. Friedlander et al. (2019), demonstrated that the pre conditioning of stainless steel surface with milk peptide lowered biofilm formation of *P. aeruginosa* and *B. licheniformis*. Duanis-Assaf et al. (2020), concluded that *B. subtillis* enabled biofilm formation of *S. mutans* in co culture by the degradation of caseins adsorbed on hydroxyapatite discs. Though the attachment of bacteria has been known to increase on fouled surfaces (section 2.4.1.4),

the effect of individual milk proteins on the attachment of bacteria to the stainless steel surface is contrary and needs further investigation.

2.4.1.6. Proliferation and growth of attached population

Upon successful attachment to stainless steel surfaces, thermophilic spores have been speculated to undergo germination and the formation of biofilm (Burgess et al., 2009). Burgess *et al.*, demonstrated that vegetative cells of *A. flavithermus* also have the potential to form biofilm. Once attached to surfaces, these cells undergo a metabolic shift from planktonic to biofilm mode of growth, which changes the expression of genes and proteins (Beloin & Ghigo, 2005). Generally, genes associated with biosynthesis of EPS and QS molecules are up regulated during biofilm formation among *E. coli*. Likewise, genes responsible for motility and flagella biosynthesis are repressed in biofilm cells (Donlan, 2002). Growth rates of *Geobacillus* sp. and *A. flavithermus* sp. within biofilms generated from vegetative cells indicated that maximum cell densities of 10^6- 10^7 cells cm^{-2} are reached within six hours (Burgess et al. 2009, 2010). This growth rate is similar to the growth rate of thermophilic bacteria observed in manufacturing plants. Burgess et al. (2009), demonstrated that spore formation occurs in parallel to the growth of vegetative cells of *A. flavithermus* within a biofilm. Burgess et al*.,* also observed that spores constituted 10-50% of an eight-hour *A. flavithermus* biofilm.

2.4.1.7. Detachment phase

The final stage of biofilm development is the detachment of cells from the biofilm and dispersal into the surrounding environment. This phase of the biofilm life cycle plays a vital role in biological dispersal and bacterial survival.

Biofilm dispersal is categorized as active and passive based on the mechanism of dispersion. Active dispersion refers to the mechanisms that are initiated by the bacteria whereas passive dispersion is caused by external forces such as shear forces, abrasion and high process temperatures (Choi & Morgenroth, 2003; Ymele-Leki & Ross, 2007). In a dairy context, it is highly likely that dispersion

occurs through both active and passive mechanisms. Passive dispersion can occur either due to high shear stress caused by high flow rates of milk within pipelines or due to higher process temperatures, which causes loosening of biofilms (Karatan & Watnick, 2009). Depending on the mode of dispersal, biofilm dispersion is classified into three modes: erosion, sloughing and seeding. Erosion is defined as the continuous release of single cells or small clusters of cells from within a biofilm at low levels over the course of biofilm formation (Kaplan, 2010). Among the three modes of dispersal, sloughing of bacterial biofilms has been observed and filmed by Stoodley et al., (2001). Sloughing is defined as a sudden detachment of large portions of the biofilm, usually occurring at the later stages of biofilm formation (Stoodley et al., 2001). Sloughing, like erosion, can be an active or passive process (Figure 2.8). Seeding is defined as the rapid release of a large number of single cells or small clusters of cells from hollow cavities that form inside the biofilm colony (Boles et al., 2005; Lappin-Scott & Bass, 2001; Ma et al., 2009; Marshall, 1988; Stoodley et al., 2001). While sloughing and erosion can be either an active or passive process, seeding is exclusively an active process.

Applegate et al. (1991), investigated the sloughing of biofilms under turbulent flow conditions under oxygen and carbon substrate limitation and varying aqueous phase calcium concentration. They concluded that oxygen limited biofilms experience catastrophic sloughing events in comparison with carbon-limited biofilms. Sloughing of the biofilm may release bacteria into the process stream, which may result in the compromise of product quality. Microbial contamination caused by sloughing of biofilms has been reported in the manufacture of milk powder, cheese and whey

products (Flint, Bremer & Brooks, 1997).

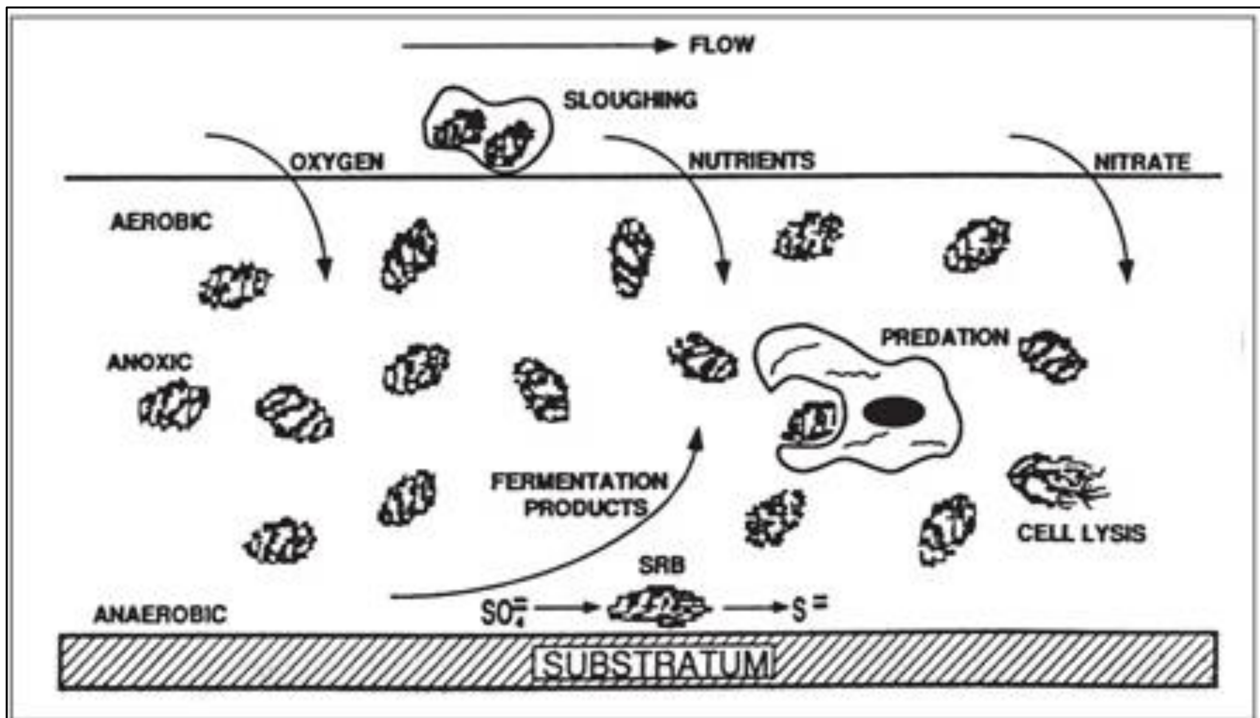


Figure 2.8. Diagrammatic representation showing the sloughing of a mature biofilm taken from ASM News, 1992, 58:202-207

2.5. Factors impacting biofilm formation

Biofilm formation enables single microorganisms to assume a multicellular form, which enables them to adopt and/or prolong their survival in diverse environmental niches. Within a biofilm, bacteria undergo a transition from planktonic to sessile state thereby altering gene expression, which results in spatial and temporal reorganization of the bacterial cell (Pratt & Kolter, 1998; O'Toole et al., 2000; Monds and O'Toole, 2009; Parsek & Singh, 2003). The reorganization helps the bacteria to modify critical metabolic functions in response to unfavourable conditions such as nutrient limitation, which assists in its survival during unfavourable conditions (Stanley et al., 2003). It was proposed that there exists a "division of labour" among bacterial communities within a biofilm, wherein communities alter gene expression based on oxygen and nutrient availability (Lewis, 2005; Domka et al., 2007). Formation of biofilms among Bacillus sp. is considered to be a stress mediated

response to several physiological stresses (Vlamakis et al., 2013) though biofilm formation can be a mere survival mechanism employed by bacteria (Johnson, 2008).

In the dairy industry, biofilm formation is influenced by several factors viz. nutritional and oxygen availability, processing temperature, composition of the processing fluid and flow conditions (Flint & Hartley, 1996; Friedlander et al., 2019; Jeurnink & de Kruif, 1995; Klausen et al., 2003; Stoodley et al., 2002, 2010). The above-mentioned factors may serve as a physiological stress to which the bacteria respond by producing biofilm. Understanding how these factors impact biofilm formation is important as this may help in the design of control strategies aimed towards minimising thermophilic contamination in the dairy industry.

2.5.1.Role of nutrient limitation on biofilm formation

Nutrient limitation is one of the contributing factors towards biofilm formation (Chandy & Angles, 2001). Previous research suggests that lack of carbon source in water could contribute towards accelerating biofilm formation in wastewater pipelines (Chandy & Angles, 2001). EPS production among *B. subtilis* was triggered by nutrient limitation in liquid culture (Zhang et al., 2014). Nutrient limitation among *B. subtillis* causes antagonization of SinR activity, responsible for flagellar production and motility machinery and in parallel expression of matrix, components are de-repressed (Lemon et al., 2008). *B. cereus* is capable of forming biofilm from spores on glass wool only under a nutrient rich condition (Lindsay et al., 2006). It was previously demonstrated that starvation results in detachment of cells in search for a nutrient rich habitat (O'Toole et al., 2000). A similar phenomenon was observed among *P. aeruginosa*- cells detaching from within biofilms due to starvation were similar to planktonic cells rather than their biofilm counterparts. These results demonstrate that *P. aeruginosa* exhibits multiple phenotypes during biofilm formation which might be helpful in understanding and controlling the biofilm formation of *P. aeruginosa*. Stoodley et al. (1999), concluded that *Pseudomonas* biofilms will grow on surfaces in very diluent nutrient solutions where planktonic growth is not favoured. They also suggested that biofilms grown under

high nutrient concentrations are thicker, denser and more mushroom like than biofilms grown under low nutrient concentrations.

To the contrary, during milk powder manufacture fresh milk gets concentrated as it passes through multiple stages of the evaporator and the nutrient concentration of milk increases. The effect of milk components on the biofilm formation has been discussed by several authors (Atulya et al., 2014; Barnes et al., 1999; Karaca et al., 2019; Xue et al., 2014), however, previous studies have not discussed the role of elevated milk solute (lactose, proteins and minerals) concentration on the biofilm and spore formation. The present study aimed to address this research gap by studying the biofilm and spore formation of A1, D1, P3 and ATCC 12980 in the presence of 10 and 20% (w/v) skim milk and discuss the role of whey and casein proteins in detail.

2.5.2. Role of oxygen on biofilm formation

Oxygen limitation plays a critical role in inducing biofilm formation among bacteria. Currently there is limited information in this area. Impaired respiration among *Bacillus subtillis* induces activation of a matrix production gene (Kolodkin-Gal et al., 2013). Oxygen depletion among *B. subtillis* causes production of γ- Polyglutamate A; a major extracellular polymeric substance which plays a major role in biofilm formation at the air liquid interface (Morikawa et al., 2006).

In the dairy industry, the combination of heat and vacuum within the evaporator stages is used to remove the water from the milk during milk powder manufacture (Bouman et al., 1993; Murphy et al., 2007). Bacteria belonging to the *Geobacillus* sp. have been known previously to grow in the absence of oxygen (Zeigler, 2014). Zhao et al. (2013), concluded that *G. thermoglucosidans* TNO-09.020, a dairy isolate, tended to form an air-liquid interface biofilm rather than a submerged biofilm. However, *G. stearothermophilus* A1, D1, P3 were previously isolated from the surface of pipelines filled with milk. This is contrary to previous findings and the observed difference may be due to the ability of *G. stearothermophilus* to grow under both an aerobic and anaerobic environment (Jensen et al., 2017). The study of the role of oxygen on the biofilm and spore

formation is important in the design strategies aimed at minimising thermophilic related product spoilage.

2.5.3. Role of temperature on biofilm formation

The role of temperature on biofilm formation has been studied previously by several authors. Previously conducted research suggests that temperature impacts the biofilm formation with lower temperature impacting the biofilm formation negatively (Berenjian et al., 2013). It was previously demonstrated that incubation temperature was an important determinant in *B. cereus* biofilm formation (Wijman et al., 2007). Thermal shock kills cells within the biofilm, makes the biofilm more vulnerable and reduces biovolume thereby inhibiting biofilm formation (Chang et al., 2017). Total cells count within the biofilm of *B. cereus* increased significantly ($p \leq 0.05$) during the first two days and remained constant for another four days when incubated at 22 °C whereas the total cells count decreased after two days when incubated at 12 °C (Ryu & Beuchat, 2005).

In a milk powder manufacturing plant, certain processes involve temperatures ranging between 45-65 °C (Hill & Smythe, 2012). These temperatures are conducive for the biofilm and spore formation of thermophilic bacteria including members of the *Geobacillus* spp. Understanding the effect of temperature ranges on the biofilm and spore formation will aid in the design of thermal processes aimed towards minimising thermophilic bacterial contamination during milk powder manufacture.

2.6. Conclusion

The thermophilic *bacillus*, *G. stearothermophilus* is a major bacterial contaminant in the dairy industry capable of forming biofilms on industrial processing surfaces and producing heat resistant spores which end up in the final product. It is necessary to determine the role of abiotic factors including temperature and total dissolved solids on the biofilm and spore forming capacity to design operating procedures aimed towards minimising biological contamination. In addition, steps aimed

towards lowering the heat resistance of spores of *G. stearothermophilus* are necessary to reduce the risk of thermophilic bacterial contamination in the dairy industry.

2.7. References

Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R., & Voort, M. V. D. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: diversity and role of germinant receptors. *Food Microbiology,* 28(2), 199–208.

Ahn, S. J., & Burne, R. A. (2007). Effects of oxygen on biofilm formation and the AtlA autolysin of *Streptococcus mutans*. *Journal of Bacteriology*, 189(17), 6293–6302.

Alderton, G., Ito, K. A., & Chen, J. K. (1976). Chemical manipulation of the heat resistance of *Clostridium botulinum* spores. *Applied and Environmental Microbiology*, 31(4), 492–498.

Alfa Laval/Tetra Pak. (1995). Dairy Processing Handbook. In *Tetra Pak Processing Systems AB*. Sweeden.

Alharthi, M. (2014). Fouling and cleaning studies of protein fouling at pasteurisation temperatures. PhD thesis. University of Birmingham.

Amaha, M., & Ordal, Z. J. (1956). The effect of certain sporulation conditions on the thermal death rate of *Bacillus coagulans* var. thermoacidurans. *Journal of Bacteriology*, 71(1), 1–9.

Amaha, M., & Ordal, Z. J. (1957). Effect of divalent cations in the sporulation medium on the thermal death rate of *Bacillus coagulans* var. thermoacidurans. *Journal of Bacteriology*, 74(5), 596–604.

André, S., Zuber, F., & Remize, F. (2013). Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. *International Journal of Food Microbiology*, 165(2), 134–143.

Aouanouk, A. S., Absi, R., Mouheb, A., & Tounsi, Z. (2018). Milk heat treatments: temperature

effect on fouling. *Chemical Engineering Transactions*, 71(1), 1267–1272.

Applegate, D. H., & Bryers, J. D. (1991). Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes. *Biotechnology and Bioengineering*, 37(1), 17–25.

Atulya, M., Jesil Mathew, A., Venkata Rao, J., & Mallikarjuna Rao, C. (2014). Influence of milk components in establishing biofilm mediated bacterial mastitis infections in cattle: A fractional factorial approach. *Research in Veterinary Science*, 96(1), 25–27.

Baril, E., Coroller, L., Couvert, O., El Jabri, M., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., & Mafart, P. (2012). Sporulation boundaries and spore formation kinetics of *Bacillus* spp. as a function of temperature, pH and aw. *Food Microbiology*, 32(1), 79–86.

Barnes, L. M., Lo, M. F., Adams, M. R., & Chamberlain, A. H. L. (1999). Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology*, 65(10), 4543–4548.

Baweja, R. B., Zaman, M. S., Mattoo, A. R., Sharma, K., Tripathi, V., Aggarwal, A., Dubey, G. P., Kurupati, R. K., Ganguli, M., Chaudhury, N. K., Sen, S., Das, T. K., Gade, W. N., & Singh, Y. (2008). Properties of Bacillus anthracis spores prepared under various environmental conditions. *Archives of Microbiology*, 189(1), 71–79.

Beaman, T. C., & Gerhardt, P. (1986). Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. *Applied and Environmental Microbiology*, 52(6), 1242–1246.

Belmar-Beiny, M. T., & Fryer, P. J. (1993). Preliminary stages of fouling from whey protein solutions. *Journal of Dairy Research*, 60(4), 467–483.

Beloin, C., & Ghigo, J. M. (2005). Finding gene-expression patterns in bacterial biofilms. *Trends in Microbiology*, 13(1), 16–19.

Ben-Ishay, N., Oknin, H., Steinberg, D., Berkovich, Z., Reifen, R., & Shemesh, M. (2017). Enrichment of milk with magnesium provides healthier and safer dairy products. *Npj Biofilms and Microbiomes*, 3(1), 1–7.

Berendsen, E. M., Boekhorst, J., Kuipers, O. P., & Wells-Bennik, M. H. J. (2016). A mobile genetic element profoundly increases heat resistance of bacterial spores. *ISME Journal*, 10(11), 2633–2642.

Berendsen, E. M., Zwietering, M. H., Kuipers, O. P., & Wells-Bennik, M. H. J. (2015). Two distinct groups within the *Bacillus* subtilis group display significantly different spore heat resistance properties. *Food Microbiology*, 45(5), 18–25.

Berenjian, A., Chan, N. L. C., Mahanama, R., Talbot, A., Regtop, H., Kavanagh, J., & Dehghani, F. (2013). Effect of biofilm formation by *Bacillus subtilis* natto on menaquinone-7 biosynthesis. *Molecular Biotechnology*, 54(2), 371–378.

Bergey, D. H. (2007). Thermophillic bacteria. *Journal of Bacteriology*, 4(4), 301-306.

Boles, B. R., Thoendel, M., & Singh, P. K. (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Molecular Microbiology*, 57(5), 1210–1223.

Bos, R., Van Der Mei, H. C., & Busscher, H. J. (1999). Physico-chemistry of initial microbial adhesive interactions - Its mechanisms and methods for study. *FEMS Microbiology Reviews*, 23(2), 179–229.

Bouman, S., Waalewijn, R., Jong, P. DE, & Linden, H. J. L. J. V. der. (1993). Design of falling-film evaporators in the dairy industry. *International Journal of Dairy Technology*, 46(3), 100–106.

Bremer, P., Seale, B., Flint, S., & Palmer, J. (2009). Biofilms in dairy processing. *Biofilms in the Food and Beverage Industries*, 396–431. Woodhead Publishing Series. United Kingdom

Bulgarelli, M. A., & Shelef, L. A. (1985). Effect of Ethylenediaminetetraacetic Acid (EDTA) on growth from spores of *Bacillus cereus*. *Journal of Food Science*, 50(661), 1–4.

Burgess, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2009). The formation of spores in biofilms of *Anoxybacillus flavithermus*. *Journal of Applied Microbiology*, 107(3), 1012–1018.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophillic bacilli from a milk powder processing plant. *Journal of Applied Microbiology*, 116(2), 350–359.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215–225.

Burton, H. (1968). Reviews of the progress of dairy science. *Journal of Dairy Research*, 35(1), 317.

Butler, R. R., Schill, K. M., Wang, Y., & Pombert, J. F. (2017). Genetic characterization of the exceptionally high heat resistance of the non-toxic surrogate Clostridium sporogenes PA 3679. *Frontiers in Microbiology*, 8(4), 1–11.

Cameron, M. S., Leonard, S. J., & Barrett, E. L. (1980). Effect of moderately acidic pH on heat restistance of *Clostridium sporogenes* spores in phosphate buffer and in buffered pea puree. *Applied and Environmental Microbiology*, 39(5), 943–949.

Cappello, S., & Guglielmino, S. P. P. (2006). Effects of growth temperature on the adhesion of *Pseudomonas aeruginosa* ATCC 27853 to polystyrene. *Annals of Microbiology*, 56(4), 383–385.

Cempírková, R. (2007). Contamination of cow's raw milk by psychrotrophic and mesophilic microflora in relation to selected factors. *Czech Journal of Animal Science*, 52(11), 387–393.

Chandler, D. P., Brown, J., Bruckner-Lea, C. J., Olson, L., Posakony, G. J., Stults, J. R., Valentine, N. B., & Bond, L. J. (2001). Continuous spore disruption using radially focused, high-

frequency ultrasound. *Analytical Chemistry*, 73(15), 3784–3789.

Chandy, J. P., & Angles, M. L. (2001). Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Research*, 35(11), 2677–2682.

Chang, S., Chen, J., & Shi, L. (2017). Using thermal shock to inhibit biofilm formation in the treated sewage source heat pump systems. *Applied Sciences (Switzerland)*, 7(4). 343.

Changani, S. D., Belmar-Beiny, M. T., & Fryer, P. J. (1997). Engineering and chemical factors associated with fouling and cleaning in milk processing. *Experimental Thermal and Fluid Science*, 14(4), 392–406.

Choi, Y. C., & Morgenroth, E. (2003). Monitoring biofilm detachment under dynamic changes in shear stress using laser-based particle size analysis and mass fractionation. *Water Science and Technology*, 47(5), 69–76.

Condon, S., Bayarte, M., & Sala, F. J. (1992). Influence of the sporulation temperature upon the heat resistance of *Bacillus subtilis*. *The Journal of Applied Bacteriology*, 73(3), 251–256.

Cook, A. M., & Gilbert, R. J. (1968). Factors affecting the heat resistance of *Bacillus stearothermophilus* spores. *International Journal of Food Science & Technology*, 3(3), 295–302.

Cortezzo, D. E., & Setlow, P. (2005). Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *Journal of Applied Microbiology*, 98(3), 606–617.

De Jonghe, V., Coorevits, A., De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., De Vos, P., & Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *International Journal of Food Microbiology*, 136(3), 318–325.

Delaunay, L., Cozien, E., Gehannin, P., Mouhali, N., Mace, S., Postollec, F., Leguerinel, I., &

Mathot, A. G. (2021). Occurrence and diversity of thermophilic sporeformers in French dairy powders. *International Dairy Journal*, 113(2), 104889.

Den Besten, H. M. W., Van Mark, E. J. D., Hensen, L., Abee, T., & Zwietering, M. H. (2010). Quantification of the effect of culturing temperature on salt-induced heat resistance of *Bacillus* species. *Applied and Environmental Microbiology*, 76(13), 4286–4292.

Dettling, A., Wedel, C., Huptas, C., Hinrichs, J., Scherer, S., & Wenning, M. (2020). High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines. *International Journal of Food Microbiology*, 335(1), 108888.

Dickson, J. S., & Koohmaraie, M. (1989). Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology*, 55(4), 832–836.

Domka, J., Lee, J., Bansal, T., & Wood, T. K. (2007). Temporal gene-expression in *Escherichia coli* K-12 biofilms. *Environmental Microbiology*, 9(2), 332–346.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. In *Emerging Infectious Diseases*, 8(9), 881–890.

Duanis-Assaf, D., Kenan, E., Sionov, R., Steinberg, D., & Shemesh, M. (2020). Proteolytic activity of *Bacillus subtilis* upon κ-casein undermines its “caries-safe” effect. *Microorganisms*, 8(2), 221.

Eijlander, R. T., van Hekezen, R., Bienvenue, A., Girard, V., Hoornstra, E., Johnson, N. B., Meyer, R., Wagendorp, A., Walker, D. C., & Wells-Bennik, M. H. J. (2019). Spores in dairy – new insights in detection, enumeration and risk assessment. *International Journal of Dairy Technology*, 72(2), 303–315.

El-bisi, H. M., & Ordal, Z. J. (1956). The effect of sporulation temperature on the thermal resistance of *Bacillus coagulans* var. thermoacidurans. *Journal of Bacteriology*, 71(1), 10–16.

Fernandez, P. S., Ocio, M. J., Sanchez, T., & Martinez, A. (1994). Thermal resistance of *Bacillus stearothermophilus* spores heated in acidified mushroom extract. *Journal of Food Protection*, 57(1), 37–41.

Fletcher, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology*, 94(2), 400–404.

Flint, S., Palmer, J., Bloemen, K., Brooks, J., Crawford, R., (2001). Growth of *Bacillus stearothermophilus* on stainless steel. Journal of Applied Microbiology, 90(2), 151-157.

Flint, S., Bremer, P., Brooks, J., Palmer, J., Sadiq, F. A., Seale, B., Teh, K. H., Wu, S., & Md Zain, S. N. (2020). Bacterial fouling in dairy processing. *International Dairy Journal*, 101(2), 104593.

Flint, S. H., Bremer, P. J., Brooks, J. D., (1997). Biofilms in dairy manufacturing plant - description, current concerns and methods of control. *Biofouling*, 11(1), 81–97.

Flint, S., & Hartley, N. (1996). A modified selective medium for the detection of *Pseudomonas* species that cause spoilage of milk and dairy products. *International Dairy Journal*, 6(2), 223–230.

Flint, S. H., Ward, L. J. H., & Walker, K. M. R. (2001). Functional grouping of thermophilic *Bacillus* strains using amplification profiles of the 16S-23S internal spacer region. *Systematic and Applied Microbiology*, 24(4), 539–548.

Friedlander, A., Nir, S., Reches, M., & Shemesh, M. (2019). Preventing biofilm formation by dairy-associated bacteria using peptide-coated surfaces. *Frontiers in Microbiology*, 10(5).1405

Fryer, P. J., & Belmar-Beiny, M. T. (1991). Fouling of heat exchangers in the food industry: a chemical engineering prespective. *Trends in Food Science and Technology*, 2(4), 33–37.

Garrett, T. R., Bhakoo, M., & Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces.

Progress in Natural Science, 18(9), 1049–1056.

Gaucheron, F. (2005). The minerals of milk. *Reproduction, Nutrition, Development*, 45(4), 473–483.

Giaouris, E., Chorianopoulos, N., & Nychas, G.-J. E. (2005). Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection*, 68(10), 2149–2154.

Gonzalez, I., Lopez, M., Mazas, M., Gonzalez, J., & Bernardo, A. (1995). The effect of recovery conditions on the apparent heat resistance of *Bacillus stearothermophilus* spores. *Journal of Applied Bacteriology*, 78(5), 548–554.

Gopal, N., Hill, C., Ross, P. R., Beresford, T. P., Fenelon, M. A., & Cotter, P. D. (2015). The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. *Frontiers in Microbiology*,6(308), 1418.

Gotham, S. M., Fryer, P. J., & Pritchard, A. M. (1992). β-lactoglobulin denaturation and aggregation reactions and fouling deposit formation: a DSC study. *International Journal of Food Science & Technology*, 27(3), 313–327.

Grossman, A. D., & Losick, R. (1988). Extracellular control of spore formation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, 85(12), 4369-4373

Guizelini, B. P., Vandenberghe, L. P. S., Sella, S. R. B. R., & Soccol, C. R. (2012). Study of the influence of sporulation conditions on heat resistance of *Geobacillus stearothermophilus* used in the development of biological indicators for steam sterilization. *Archives of Microbiology*, 194(12), 991–999.

Hayes, C. S., & Setlow, P. (2001). An α/β-type, small, acid-soluble spore protein which has very

high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. *Journal of Bacteriology*, 183(8), 2662–2666.

Hayrapetyan, H., Abee, T., & Nierop Groot, M. (2016). Sporulation dynamics and spore heat resistance in wet and dry biofilms of *Bacillus cereu*s. *Food Control*, 60(2), 493–499.

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., & Nierop Groot, M. (2015). Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology*, 200(5), 72–79.

Helke, D. M., Somers, E. B., & Wong, A. C. L. (1993). Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and Buna-N in the presence of milk and individual milk components. *Journal of Food Protection*, 56(6), 479–484.

Heyndrickx, M., & Scheldeman, P. (2008). Bacilli associated with spoilage in dairy products and other food. *Applications and Systematics of Bacillus and Relatives*. Blackwell Science. United Kingdom

Hill, B. M., & Smythe, B. W. (2012). Endospores of thermophilic bacteria in ingredient milk powders and their significance to the manufacture of sterilized milk products: an industrial perspective. *Food Reviews International,* 28(3), 299–312.

Hinton, A. R., Trinh, K. T., Brooks, J. D., & Manderson, G. J. (2002). Thermophile survival in milk fouling and on stainless steel during cleaning. *Food and Bioproducts Processing: Transactions of the Institution of of Chemical Engineers*, 80(4), 299–304.

Hoštacká, A., Čižnár, I., & Štefkovičová, M. (2010). Temperature and pH affect the production of bacterial biofilm. *Folia Microbiologica*, 55(1), 75–78.

Huang, S. S., Chen, D., Pelczar, P. L., Vepachedu, V. R., Setlow, P., & Li, Y. Q. (2007). Levels of Ca2+-dipicolinic acid in individual *Bacillus* spores determined using microfluidic Raman

tweezers. *Journal of Bacteriology*, 189(13), 4681–4687.

Huemer, I. A., Klijn, N., Vogelsang, H. W. J., & Langeveld, L. P. M. (1998). Thermal death kinetics of spores of *Bacillus sporothermodurans* isolated from UHT milk. *International Dairy Journal*, 8(10), 851–855.

Iñiguez-Moreno, M., Gutiérrez-Lomelí, M., & Avila-Novoa, M. G. (2019). Kinetics of biofilm formation by pathogenic and spoilage microorganisms under conditions that mimic the poultry, meat, and egg processing industries. *International Journal of Food Microbiology*, 303(8), 32–41.

Jamroskovic, J., Chromikova, Z., List, C., Bartova, B., Barak, I., & Bernier-Latmani, R. (2016). Variability in DPA and calcium content in the spores of *Clostridium* species. *Frontiers in Microbiology*, 7(11), 1–11.

Jensen, T. Ø., Pogrebnyakov, I., Falkenberg, K. B., Redl, S., & Nielsen, A. T. (2017). Application of the thermostable β-galactosidase, BgaB, from *Geobacillus stearothermophilus* as a versatile reporter under anaerobic and aerobic conditions. *AMB Express*, 7(2017), 169.

Jeurnink, T. J .M, & de Kruif, K. G. (1995). Calcium concentration in milk in relation to heat stability and fouling. *Netherlands Milk Dairy Journal*, 49(3), 151–165.

Jeurnink, Th JM, Walstra, P., & De Kruif, C. G. (1996). Mechanisms of fouling in dairy processing. *Netherlands Milk Dairy Journal*, 50(5), 407–426.

Jeurnink, Theo J., & De Kruif, K. G. (1993). Changes in milk on heating: viscosity measurements. *Journal of Dairy Research*, 60(2), 139–150.

Jeurnink, Theo J.M., & Brinkman, D. W. (1994). The cleaning of heat exchangers and evaporators after processing milk or whey. *International Dairy Journal*, 4(4), 347–368.

Johnson, L. R. (2008). Microcolony and biofilm formation as a survival strategy for bacteria.

Journal of Theoretical Biology, 251(1), 24–34.

Kaplan, J. B. (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of Dental Research*, 89(3), 205–218.

Karaca, B., Buzrul, S., & Coleri Cihan, A. (2019). *Anoxybacillus* and *Geobacillus* biofilms in the dairy industry: effects of surface material, incubation temperature and milk type. *Biofouling*, 35(5), 551–560.

Karatan, E., & Watnick, P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews*, 73(2), 310–347.

Kent, D. J., Chauhan, K., Boor, K. J., Wiedmann, M., & Martin, N. H. (2016). Spore test parameters matter: mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. *Journal of Dairy Science*, 99(7), 5180–5191.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., & Tolker-Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Molecular Microbiology*, 48(6), 1511–1524.

Knight, G. C., Nicol, R. S., & McMeekin, T. A. (2004). Temperature step changes: A novel approach to control biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plant. *International Journal of Food Microbiology*, 93(3), 305–318.

Kokare, C. R., Chakraborty, S., Khopade, A. N., & Mahadik, K. R. (2009). Biofilm: Importance and applications. *Indian Journal of Biotechnology*, 8(2), 159–168.

Kolodkin-Gal, I., Elsholz, A. K. W., Muth, C., Girguis, P. R., Kolter, R., & Losick, R. (2013). Respiration control of multicellularity in *Bacillus subtilis* by a complex of the cytochrome chain with a membrane-embedded histidine kinase. *Genes and Development*, 27(8), 887–899.

Kroukamp, O., Dumitrache, R. G., & Wolfaardt, G. M. (2010). Pronounced effect of the nature of

the inoculum on biofilm development in flow systems. *Applied and Environmental Microbiology*, 76(18), 6025–6031.

Kumar, C. G., & Anand, S. . (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*, 42(1), 9–27.

Lappin-Scott, H. M., & Bass, C. (2001). Biofilm formation: attachment, growth, and detachment of microbes from surfaces. *American Journal of Infection Control*, 29(4), 250–251.

Leclercq-Perlat, M.-N., & Lalande, M. (1994). Cleanability in relation to surface chemical composition and surface finishing of some materials commonly used in food industries. *Journal of Food Engineering*, 23(4), 501–517.

Ledenbach, L. H., & Marshall, R. T. (2010). Microbiological Spoilage of Dairy Products. *Compendium of the Microbiological Spoilage of Foods and Beverages*. Springer. New York

Lee Wong, A. C. (1998). Biofilms in Food Processing Environments. *Journal of Dairy Science*, 81(10), 2765–2770.

Lemon, K. P., Earl, A. M., Vlamakis, H. C., Aguilar, C., & Kolter, R. (2008). Biofilm development with an emphasis on *Bacillus subtilis*. *Current Topics in Microbiology and Immunology*, 322(1), 1–16.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry,*70(2), 267–274.

Li, F., Hunt, K., Van Hoorde, K., Butler, F., Jordan, K., & Tobin, J. T. (2019). Occurrence and identification of spore-forming bacteria in skim-milk powders. *International Dairy Journal*, 97(10), 176–184.

Lindsay, D., Brözel, V. S., & Von Holy, A., (2006). Biofilm-spore response in *Bacillus cereus* and *Bacillus subtilis* during nutrient limitation. *Journal of Food Protection*, 69(5), 1168–1172.

Lindsay, D., & Flint, S. (2009). Biofilm formation by spore-forming bacteria in food processing

environments. *Biofilms in the food and beverage industries*. Woodhead Publishing Limited. United Kingdom.

López, M., González, I., Condón, S., & Bernardo, A. (1996). Effect of pH heating medium on the thermal resistance of *Bacillus stearothermophilus* spores. *International Journal of Food Microbiology*, 28(3), 405–410.

López, M., Mazas, M., González, I., González, J., & Bernardo, A. (1996). Thermal resistance of *Bacillus stearothermophilus* spores in different heating systems containing some approved food additives. *Letters in Applied Microbiology*, 23(3), 187–191.

Löwik, J. A. M., & Anema, P. J. (1972). Effect of pH on the heat resistance of *Clostridium sporogenes* spores in minced meat. *Journal of Applied Bacteriology*, 35(1), 119–121.

Lyster, J. (1970). The denaturation of -α lactalbumin and - β lactoglobulin in heated milk. *Journal of Dairy Research*, 37(2), 233–243.

Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., & Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathogens*, 5(3).1000354

Magge, A., Granger, A. C., Wahome, P. G., Setlow, B., Vepachedu, V. R., Loshon, C. A., Peng, L., Chen, D., Li, Y.-Q., & Setlow, P. (2008). Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*. *Journal of Bacteriology*, 190(14), 4798–4807.

Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L. (2012). Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 133–147.

Marquis, R. E., & Bender, G. R. (1985). Mineralization and heat resistance of bacterial spores. *Journal of Bacteriology*, 161(2), 789–791.

Marshall, K. C. (1988). Adhesion and growth of bacteria at surfaces in oligotrophic habitats.

Canadian Journal of Microbiology , 34(4), 503–506.

Marshall, K. C., Stout, R., & Mitchell, R. (1971). Mechanism of the initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology*, 68(3), 337–348.

Mazas, M., López, M., González, I., Bernardo, a, & Martín, R. (1997). Effects of sporulation pH on the heat resistance and the sporulation of *Bacillus cereus*. *Letters in Applied Microbiology*, 25(5), 331–334.

Mazas, M., Lopez, M., Martinez, S., Bernardo, A., & Martin, R. (1999). Heat resistance of *Bacillus* cereus spores : effects of milk constituents and stabilizing additives. *Journal of Food Protection*, 62(4), 410-413.

McGuiggan, J. T. M., McCleery, D. R., Hannan, A., & Gilmour, A. (2002). Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. *International Journal of Dairy Technology*, 55(2), 100–107.

McIlvaine T.C. (1921). A buffer solution for colorimetric comparison. *Journal of Biological Chemistry*, 49(1), 183-186.

McMahon, D. J., Du, H., McManus, R., & Larsen, K. M. (2009). Microstructural changes in casein supramolecules during acidification of skim milk. *Journal of Dairy Science*, 92(12), 5854–5867.

Meesilp, N., & Mesil, N. (2019). Effect of microbial sanitizers for reducing biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on stainless steel by cultivation with UHT milk. *Food Science and Biotechnology*, 28(1), 289–296.

Miller, R. A., Kent, D. J., Boor, K. J., Martin, N. H., & Wiedmann, M. (2015). Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. *Journal of Dairy Science*, 98(7), 4338–4351.

Mittelman, M. W. (1998). Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science*, 81(10), 2760–2764.

Moeller, R., Raguse, M., Reitz, G., Okayasu, R., Li, Z., Klein, S., Setlow, P., & Nicholson, W. L. (2014). Resistance of *Bacillus subtilis* spore DNA to lethal ionizing radiation damage relies primarily on spore core components and DNA repair, with minor effects of oxygen radical detoxification. *Applied and Environmental Microbiology*, 80(1), 104–109.

Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, 101(3), 526–530.

Monds, R. D., & O'Toole, G. A. (2009). The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends in Microbiology*,17(2), 73–87.

Morikawa, M., Kagihiro, S., Haruki, M., Takano, K., Branda, S., Kolter, R., & Kanaya, S. (2006). Biofilm formation by a *Bacillus subtilis* strain that produces γ-polyglutamate. *Microbiology*, 152(9), 2801–2807.

Mtimet, N., Trunet, C., Mathot, A. G., Venaille, L., Leguérinel, I., Coroller, L., & Couvert, O. (2015). Modeling the behavior of *Geobacillus stearothermophilus* ATCC 12980 throughout its life cycle as vegetative cells or spores using growth boundaries. *Food Microbiology*, 48(6), 153–162.

Murphy, P. M., Lynch, D., & Kelly, P. M. (2007). Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. *International Journal of Dairy Technology*, 52(2), 45–50.

Murray, T. J., & Headlee, M. R. (1931). Thermal death point. *Journal of Infectious Diseases*, 48(5), 436–456.

Naghili, H., Tajik, H., Mardani, K., Razavi Rouhani, S. M., Ehsani, A., & Zare, P. (2013). Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Veterinary Research Forum*, 4(3), 179–183.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A., Belyaev, S. S., & Ivanov, M. V. (2001). Taxonomic study of aerobic thermophilic bacilli: Descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *International Journal of Systematic and Evolutionary Microbiology*, 51(2), 433–446

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews : MMBR*, 64(3), 548–572.

Nilsson, R. E., Ross, T., & Bowman, J. P. (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *International Journal of Food Microbiology*, 150(1), 14–24.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Review of Microbiology*, 54(1), 49–79.

Ono, T., Murayama, T., Kaketa, S., & Odagiri, S. (1990). Changes in the protein composition and size distribution of bovine casein micelles induced by cooling. *Agricultural and Biological Chemistry*, 54(6), 1385–1392.

Oomes, S. J. C. M., Jonker, M. J., Wittink, F. R. A., Hehenkamp, J. O., Breit, T. M., & Brul, S. (2009). The effect of calcium on the transcriptome of sporulating B. subtilis cells. *International Journal of Food Microbiology*, 133(3), 234–242.

Orlien, V., Boserup, L., & Olsen, K. (2010). Casein micelle dissociation in skim milk during high-

pressure treatment: Effects of pressure, pH, and temperature. *Journal of Dairy Science*, 93(1), 12–18.

Ostrov, I., Paz, T., & Shemesh, M. (2019). Robust biofilm-forming *Bacillus* isolates from the dairy environment demonstrate an enhanced resistance to cleaning-in-place procedures. *Foods*, 8(4), 134.

Paidhungat, M., Setlow, B., & Driks, A. (2000). Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *Journal of Bacteriology*, 182(19), 5505–5512.

Palmer J., Flint, S. & Brooks, J. (2007). Bacterial cell attachment, the beginning of a biofilm, *Journal of Industrial Microbiology and Biotechnology*, 34(9), 577-588.

Palop, A., Mañas, P., & Condón, S. (1999). Sporulation temperature and heat resistance of *Bacillus* spores: A review. *Journal of Food Safety*, 19(1), 57–72.

Parkar, S. G., Flint, S. H., Palmer, J. S., & Brooks, J. D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology*, 90(6), 901–908.

Parsek, M. R., & Singh, P. K. (2003). Bacterial Biofilms: An emerging link to disease pathogenesis. *Annual Review of Microbiology*, 57(1), 677–701.

Pedersen, K. (1990). Biofilm development on stainless steel and pvc surfaces in drinking water. *Water Research*, 24(2), 239–243.

Pouliot, Y., Boulet, M., & Paquin, P. (1989). Observations on the heat-induced salt balance changes in milk II. reversibility on cooling. *Journal of Dairy Research*, 56(2), 193–199.

Pratt, L. A., & Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology*, 30(2), 285–293.

Ramaswamy, H. S., Xu, M., & Vatankhah, H. (2019). Investigating the influence of pH and selected

heating media on thermal destruction kinetics of *Geobacillus stearothermophilus* (ATCC 10149). *Journal of Food Measurement and Characterization*, 13(2), 1310–1322.

Reder, A., Albrecht, D., Gerth, U., & Hecker, M. (2012). Integration of σB activity into the decision-making process of sporulation initiation in *Bacillus subtilis*. *Journal of Bacteriology*, 194(5), 1065–1074.

Rey, C. R., Walker, H. W., & Rohrbaugh, P. L. (1975). The influence of temperature on growth, sporulation, and heat Resistance of spores of six strains of *Clostridium perfringens*. *Journal of Milk and Food Technology*, 38(8), 461–465.

Rice, A. R., Hamilton, M. A., & Camper, A. K. (2000). Apparent surface associated lag time in growth of primary biofilm cells. *Microbial Ecology*, 40(1), 8–15.

Riedewald, F. (2006). Bacterial adhesion to surfaces: the influence of surface roughness. *PDA Journal of Pharmaceutical Science and Technology*, 60(3), 164–171.

Robitaille, G., Choinière, S., Ells, T., Deschènes, L., & Mafu, A. A. (2014). Attachment of *Listeria innocua* to polystyrene: Effects of ionic strength and conditioning films from culture media and milk proteins. *Journal of Food Protection*, 77(3), 427–434.

Roefs, S. P. F. M., & De Kruif, K. G. (1994). A model for the denaturation and aggregation of β-lactoglobulin. *European Journal of Biochemistry*, 226(3), 883–889.

Rosmaninho, R., Santos, O., Nylander, T., Paulsson, M., Beuf, M., Benezech, T., Yiantsios, S., Andritsos, N., Karabelas, A., Rizzo, G., Müller-Steinhagen, H., & Melo, L. F. (2007). Modified stainless steel surfaces targeted to reduce fouling - evaluation of fouling by milk components. *Journal of Food Engineering*, 80(4), 1176–1187.

Rotman, Y., & Fields, M. L. (1969). A study on the sporulation of rough and smooth variants of *Bacillus stearothermophilus*. *Journal of Food Science*, 34(4), 346–349.

Ryu, J. H., & Beuchat, L. R. (2005). Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *Journal of Food Protection*, 68(12), 2614–2622.

Sadiq, F. A., Li, Y., Liu, T. J., Flint, S., Zhang, G., Yuan, L., Pei, Z., & He, G. Q. (2016). The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *International Journal of Food Microbiology*, 238(12), 193–201.

Sala, F. J., Ibarz, P., Palop, A., Raso, J., & Santiago, C. (1995). Sporulation temperature and heat resistance of *Bacillus subtilis* at different pH values. *Journal of Food Protection*, 58(3), 239–243.

Santos, M., & Zarzo, J. (1997). The effect of ethylenediaminetetraacetic acid on heat resistance and recovery of *Clostridium sporogenes* PA 3679 spores treated in HTST conditions. *International Journal of Food Microbiology*, 34(3), 293–305.

Scheldeman, P., Herman, L., Foster, S., & Heyndrickx, M. (2006). *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology*, 101(3), 542–555.

Scherrer, R., & Gerhardt, P. (1972). Location of calcium within *Bacillus* spores by electron probe x-ray microanalysis. *Journal of Bacteriology*, 112(1), 559–568.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, 60(2), 109–117.

Seale, R. B., Bremer, P. J., Flint, S. H., & McQuillan, A. J. (2010). Characterization of spore surfaces from a *Geobacillus* sp. isolate by pH dependence of surface charge and infrared

spectra. *Journal of Applied Microbiology*, 109(4), 1339–1348.

Seale, R. B., Flint, S. H., McQuillan, A. J., & Bremer, P. J. (2008). Recovery of spores from thermophilic dairy bacilli and effects of their surface characteristics on attachment to different surfaces. *Applied and Environmental Microbiology*, 74(3), 731–737.

Sella, S. R. B. R., Vandenberghe, L. P. S., & Soccol, C. R. (2014). Life cycle and spore resistance of spore-forming *Bacillus atrophaeus*. *Microbiological Research*, 169(12), 931–939.

Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., & Setlow, P. (2006). Role of dipicolinic acid in resistance and stability of spores of *Bacillus subtilis* with or without DNA-protective α/β-type small acid-soluble proteins. *Journal of Bacteriology*, 188(11), 3740–3747.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, 101(3), 514–525.

Setlow, P. (1995). Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annual Review of Microbiology*, 49(1), 29–54.

Setlow, Peter. (2003). Spore germination. *Current Opinion in Microbiology*, 6(6), 550–556.

Setlow, Peter. (2014). Germination of spores of *Bacillus* species: What we know and do not know. *Journal of Bacteriology*, 196(7), 1297–1305.

Sinaga, H., Bansal, N., & Bhandari, B. (2017). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties*, 20(1), 179–197.

Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology*, 57(2), 111–119.

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Lindsay, D., & Flint, S. (2012). Influence of cations on growth of thermophilic *Geobacillus* spp. and *Anoxybacillus flavithermus* in planktonic culture. *Applied and Environmental Microbiology*, 78(7), 2477–2481.

Sonenshein, A. L., Hoch, J. A., & Losick, R. (2002). *Bacillus subtilis* and its closest relatives. From genes to cells. ASM press. United States of America.

Speers, J. G. S., & Gilmour, A. (1985). The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of Applied Bacteriology*, 59(4), 325–332.

Stanley, N. R., Britton, R. a, Grossman, A. D., & Lazazzera, B. a. (2003). Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *Journal of Bacteriology*, 185(6), 1951–1957.

Stoeckel, M., Lücking, G., Ehling-Schulz, M., Atamer, Z., & Hinrichs, J. (2016). Bacterial spores isolated from ingredients, intermediate and final products obtained from dairies: thermal resistance in milk. *Dairy Science and Technology*, 96(4), 569–577.

Stoodley, P., Dodds, I., Boyle, J. D. D., & Lappin-Scott, H. M. M. (2010). Influence of hydrodynamics and nutrients on biofilm structure. *Journal of Applied Microbiology*, 85(1), 19-28.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology*, 56(1), 187–209.

Stoodley, Paul, Wilson, S., Hall-Stoodley, L., Boyle, J. D., Lappin-Scott, H. M., & Costerton, J. W. (2001). Growth and detachment of cell clusters from mature mixed-species biofilms. *Applied and Environmental Microbiology*, 67(12), 5608–5613.

Tango, C. N., Akkermans, S., Hussain, M. S., Khan, I., Van Impe, J., Jin, Y. G., & Oh, D. H. (2018). Modeling the effect of pH, water activity, and ethanol concentration on biofilm formation of *Staphylococcus aureus*. *Food Microbiology*, 76(12), 287–295.

Thomas, A., & Sathian, C. T. (2014). Cleaning-In-Place (CIP) system in dairy plant- review. *Journal of Environmental Science, Toxicology and Food Technology*, 8(6), 41–44.

Udoh, A. P. (2000). Atomic absorption spectrometric determination of calcium and other metallic elements in some animal protein sources. *Talanta*, 52(4), 749–754.

Vasantha, N., & Freese, E. (1979). The role of manganese in growth and sporulation of *Bacillus subtilis*. *Journal of General Microbiology*, 112(2), 329–336.

Visser, J., & Jeurnink, T. J. M. (1997). Fouling of heat exchangers in the dairy industry. *Experimental Thermal and Fluid Science*, 14(4), 407–424.

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., & Kolter, R. (2013). Sticking together: building a biofilm the *Bacillus subtilis* way. *Nature Reviews Microbiology*, 11(3), 157–168.

Vlková, H., Babák, V., Seydlová, R., Pavlík, I., & Schlegelova, J. (2008). Biofilms and hygiene on dairy farms and in the dairy industry: sanitation chemical products and their effectiveness on biofilms - A review. In *Czech Journal of Food Sciences*, 26(5), 309–323.

Vries, Y. P. De. (2004). Minireview the role of calcium in bacterial spore germination. *Microbes and Environments*, 19(3), 199–202.

Wei, J., Shah, I. M., Ghosh, S., Dworkin, J., Hoover, D. G., & Setlow, P. (2010). Superdormant spores of *Bacillus* species germinate normally with high pressure, peptidoglycan fragments, and bryostatin. *Journal of Bacteriology*, 192(5), 1455–1458.

Wells-Bennik, M. H. J., Janssen, P. W. M., Klaus, V., Yang, C., Zwietering, M. H., & den Besten, H. M. W. (2019). Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. *International Journal of Food Microbiology*, 291(2), 161–172.

Wijman, J. G. E., De Leeuw, P. P. L. A., Moezelaar, R., Zwietering, M. H., & Abee, T. (2007). Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Applied and Environmental Microbiology*, 73(5), 1481–1488.

Williams, O. B., & Hennessee, A. D. (2006). Studies on heat resistance.VII. The effect of phosphate on the apparent heat resistance of spores of *Bacillus stearothermophilus*. *Journal of Food Science*, 21(1), 112–116.

Xue, T., Chen, X., & Shang, F. (2014). Short communication: Effects of lactose and milk on the expression of biofilm-associated genes in *Staphylococcus aureus* strains isolated from a dairy cow with mastitis. *Journal of Dairy Science*, 97(10), 6129–6134.

Yao, M., & Walker, H. W. (1967). Liquid medium for production of spores by *Bacillus stearothermophilus*. *Applied Microbiology*, 15(2), 455.

Yazdany, S., & Lashkari, K. B. (1975). Effect of pH on sporulation of *Bacillus stearothermophilus*. *Applied Microbiology*, 30(1), 1–3.

Ymele-Leki, P., & Ross, J. M. (2007). Erosion from Staphylococcus aureus biofilms grown under physiologically relevant fluid shear forces yields bacterial cells with reduced avidity to collagen. *Applied and Environmental Microbiology*, 73(6), 1834–1841.

Yuan, D. D., Liu, G. C., Ren, D. Y., Zhang, D., Zhao, L., Kan, C. P., Yang, Y. Z., Ma, W., Li, Y., & Zhang, L. B. (2012). A survey on occurrence of thermophilic bacilli in commercial milk powders in China. *Food Control*, 25(2), 752–757.

Yuan, L., Sadiq, F. A., Burmølle, M., Liu, T., & He, G. (2018). Insights into bacterial milk spoilage with particular emphasis on the roles of heat-stable enzymes, biofilms, and quorum sensing. *Journal of Food Protection*, 81(10), 1651–1660.

Zeigler, D. R. (2014). The Geobacillus paradox: why is a thermophilic bacterial genus so prevalent on a mesophilic planet? *Microbiology*, 160(1), 1–11.

Zhang, W., Seminara, A., Suaris, M., Brenner, M. P., Weitz, D. A., & Angelini, T. E. (2014). Nutrient depletion in *Bacillus subtilis* biofilms triggers matrix production. *New Journal of Physics*, 16(1), 015028.

Zhang, Y., Munir, M. T., Udugama, I., Yu, W., & Young, B. R. (2018). Modelling of a milk powder falling film evaporator for predicting process trends and comparison of energy consumption. *Journal of Food Engineering*, 225(5), 26–33.

Zhao, Y., Caspers, M. P. M., Metselaar, K. I., De Boer, P., Roeselers, G., Moezelaar, R., Groot, M. N., Montijn, R. C., Abee, T., & Kort, R. (2013). Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. *Applied and Environmental Microbiology*, 79(18), 5652–5660.

Objectives of this study

The purpose of this study was to investigate the effect of abiotic factors on the biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980. This was achieved by monitoring spore and biofilm formation in a continuous flow laboratory reactor.

In addition, the heat resistance of spores of A1, D1, P3 and ATCC 12980 was studied and the effect of phosphate on the heat resistance was investigated.

The specific objectives of this study are as follows:

1. Determine the role of abiotic factors, temperature and total dissolved solids on the spore and biofilm formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 using a CDC biofilm reactor

2. Determine the heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 and investigate the effect of pH of the heating medium on heat resistance

3. Investigate the effect of phosphate on the heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 and the underlying mechanism of action of phosphate

4. Compare the heat resistance of spores obtained from a milk biofilm system under continuous flow condition and obtained by a sporulation medium

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Murali Kumar
Name/title of Primary Supervisor:	Steve Flint
Name of Research Output and full reference:	
Murali Kumar, Steve Flint, Jon Palmer, Sawatdeenaruenat Chanapha, Chris Hall. Effect of the incubation temperature and total dissolved solids concentration on the biofilm and spore formation of dairy isolates of Geobacillus stearothermophilus	
In which Chapter is the Manuscript /Published work:	2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
This paper was the work of the candidate who researched and wrote the paper under the guidance of the four co-authors	
For manuscripts intended for publication please indicate target journal:	
Applied and Environmental Microbiology (AEM)	
Candidate's Signature:	Murali Kumar Digitally signed by Murali Kumar Date: 2020.12.16 13:55:17 +13'00'
Date:	16 Dec 2020
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2020.12.16 17:32:42 +13'00'
Date:	16th December 2020

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019

Preface to Chapter 3

The literature review identified abiotic factors such as temperature and total dissolved solids influence the biofilm and spore formation. However, *G. stearothermophilus* was not included in previous studies. In this chapter, the role of temperature and total dissolved solids on the biofilm and spore formation of A1, D1, P3 and ATCC 12980 was investigated. In addition, the role of milk proteins on the biofilm and spore formation was determined.

Chapter 3

3 Effect of incubation temperature and the total dissolved solids on biofilm and spore formation

Abstract

Geobacillus species are a contaminant in the dairy industry and their presence is often considered as an indicator of poor plant hygiene with the potential to cause spoilage. They can form heat resistant spores that adhere to surfaces of processing equipment and germinate to form biofilms. Therefore, strategies aimed towards preventing or controlling biofilm formation in the dairy industry are desirable. In this study the preferred temperature for biofilm and spore formation among *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 was 65 °C. Increasing the total dissolved milk solids to 20 % (w/v) caused an apparent delay in the onset of biofilm and spore formation to detectable concentrations among all the strains at 55 °C. Compared to the onset time for biofilm formation of A1 in 10 % (w/v) reconstituted skim milk, the addition of milk protein (whey protein and sodium caseinate) caused an apparent delay in the onset of biofilm formation to detectable concentrations by an average of 10 h at 55 °C. This study proposes that temperature and total dissolved solids have a cumulative effect on the biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980. In addition, the findings from this study may indicate that preconditioning of stainless-steel surface with adsorbed milk proteins may delay the onset of biofilm and spore formation of thermophilic bacteria during milk powder manufacture.

3.1. Introduction

G. stearothermophilus (formerly known as *Bacillus stearothermophilus*) is a Gram positive, rod shaped, spore forming bacteria that is capable of growing at temperatures of 55-65 ºC (Nazina et al., 2001). *G. stearothermophilus* is a common contaminant in the food industry, particularly in milk powder plants where temperatures that are suitable for their growth prevail (Scott et al., 2007). Although *G. stearothermophilus* is not pathogenic, the presence of their spores in the dairy industry is an indicator of poor plant hygiene with the potential for spoilage through acid and enzyme production (Lindsay & Flint, 2009). The major thermophilic spore contaminants in whole milk power manufacture are *Anoxybacillus flavithermus* and *G. stearothermophilus* (Scott et al., 2007). The sporulation of *G. stearothermophilus* is confined to the evaporator during the production of whole milk powder (Scott et al., 2007). The growth of *G. stearothermophilus* during milk powder manufacturing is believed to occur as biofilms within the sections of the evaporator (Burgess et al., 2010).The microorganism is capable of forming vegetative cells and heat resistant spores within the biofilm (Burgess et al., 2010). Upon maturation of the biofilm, the spores are released into the milk and contaminate the end product (Marchand et al., 2012).

In milk powder manufacture, fresh milk flows through a plate heat exchanger which is maintained at a temperature between 50-65 °C before passing through an evaporator (Scott et al., 2007). As pre-heated milk passes through the stages of the evaporator, physical parameters such as temperature and total dissolved solids vary. Biofilm and spore formation are affected by several factors (Ahn & Burne, 2007; Burgess et al., 2009; Giaouris et al., 2005; Tango et al., 2018). The temperature gradient within the evaporator can vary between 40 and 70 °C (Zhang et al., 2018). Fresh milk during milk powder manufacture is usually concentrated from an initial solids content of 9-13 % to a final concentration of 40-50 % during evaporation before being pumped into the drier (Alfa Laval/Tetra Pak, 1995). A previous study discussing the effect of incubation temperature on the formation of spores in *A. flavithermus* biofilms indicated that spores were formed at 55 and 60°

C, but not at 48 °C (Burgess et al., 2009). Scott et al. (2007), surveyed a whole milk powder manufacturing plant and observed that thermophilic growth was absent in the later stages of the evaporator and related it to the increased milk solute concentration. The development of *G. stearothermophilus* biofilm on a stainless-steel surface in the presence of milk was studied previously (Flint et al., 2001) however, the role of temperature and the total dissolved solids on spore formation has not been studied.

The objective of this study was to determine the effect of two key variables - incubation temperature and the total dissolved solids on the biofilm and spore formation of dairy isolates of *G. stearothermophilus*. The outcome of this study will be useful in the design of thermal processing steps in milk powder manufacturing plants aimed towards controlling biofilm and spore formation of *G. stearothermophilus* in the dairy industry.

3.2. Materials and Methods

3.2.1. Culture preparation

G. stearothermophilus A1, D1 and P3, isolated from the evaporator section of a New Zealand milk powder manufacturing plant were specifically chosen due to the variation in their ability to form biofilms and spores in the milk environment with A1 and D1 forming a mono layer biofilm and P3 forming a three dimensional biofilm (Burgess et al., 2014). In this study, *G. stearothermophilus* ATCC 12980 was used as a reference strain. Cultures of A1, D1, P3 and ATCC 12980, were prepared by batch culturing in trypticase soy broth (TSB; BD Biosciences, USA) for 12 h at 55 °C, washed twice and re-suspended in phosphate buffer saline (PBS; Oxoid, UK).

3.2.2. Design and assembly of the CDC biofilm reactor

The CDC biofilm reactors (CBR 90) (Biosurface technologies, USA) were used for these experiments. Stainless steel coupons (10 mm dia, 3 mm thick, 304 grade with a 2B finish) were used. The coupons were prepared by soaking in 99.5 % acetone (Merck, USA) for 12 h, rinsed with

distilled water, suspended in 5 % (w/v) Pyroneg solution (Thermofisher scientific, USA) and cleaned using an ultrasonic cleaner (Agar scientific, UK) for 60 min. Following the cleaning steps, coupons were then rinsed in distilled water and sterilized by autoclaving at 121 °C for 15 min. This treatment was repeated after every experiment. The reactor was then assembled, and all components were autoclaved before use.

3.2.3. Inoculation of the CDC biofilm reactor

Sterile reconstituted skim milk (RSM) was prepared by dissolving 10 or 20 % (w/v) of instant skim milk powder (Fonterra Ltd., New Zealand) in water then UHT treating at 142 °C for 8 s (see Appendix section 7.1 & 7.2). The schematic diagram of the CDC reactor setup is shown in Figure S.2.1 in the supplement. 20 L of sterile RSM was inoculated with 200 mL of the 12 h culture at the start of the reactor run. The milk container was kept at 4 °C throughout the experiment. The inoculated RSM was delivered at a constant flow rate of 25 mL/min which was selected to achieve a mean residence time of 14 min, lower than the doubling time of all the strains (Table S.2.1 in the supplement) to ensure the washing out of suspended cells represented those only released from the biofilm.

3.2.4. CDC reactor runs

The incubation temperature was maintained at either 55, 60 or 65 °C during an entire reactor run of 16 h. The temperature within the reactor was continuously monitored using a thermometer connected to a digital stir hot plate (Biosurface Technologies, USA). The stirrer speed in the reactor was maintained at 100 rpm throughout the experiment. Two coupon rods holding three coupons each were carefully removed at 0, 4, 8, 12 and 16 h. The first samples, taken as soon as the desired heating temperature was reached were referred to as 0 h. Coupon rods were replaced with dummy rods to maintain a constant turbulence throughout the experiment.

3.2.5. Enumeration of bacterial cells and spores

The number of culturable cells in the biofilm was enumerated using a bead vortex mixing method as described previously (Hayrapetyan et al., 2015). In short, the coupons were washed by dipping in PBS and placing in 25 mL sterile containers filled with 5 mL of PBS and 5 g glass beads (D =100 µm, Sigma Aldrich). The containers were mixed by vortex at maximum speed for 1 min to dislodge the cells from the coupon and to obtain individual cells in the sample. Serial 10-fold dilutions were made using PBS and spread plated on tryptic soy agar (TSA) and colony forming units (CFU) were counted after 18 h incubation at 55 °C. To determine the spore count, the 25 mL containers were heat treated at 90 °C for 30 min prior to the CFU enumeration. CFU enumeration of total viable cells and total spores per coupon were determined through drop and spread plate method respectively (Naghili et al., 2013).

3.2.6. Statistical analysis

Regression analysis was performed using Minitab 19. Based on the regression analysis, the pareto chart was constructed. Combase, USA was used to construct the kinetic model.

3.3. Results and Discussion

3.3.1. The effect of temperature on the biofilm and spore formation

To determine the spore forming capacity of *G. stearothermophilus* A1, D1, P3 and ATCC 12980, a continuous flow CDC reactor was operated over a period of 16 h at three different temperatures of 55, 60 and 65 °C. These temperatures were specifically chosen since they lie within the growth temperature range of these bacteria (Table S.2.2 in the supplement). Biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 on stainless steel coupons is shown in Figure. 3.1.

In comparison with the minimum detectable concentration of total viable cells and spores per coupon an apparent delay in the onset of biofilm formation of A1, P3 and ATCC12980 was observed

at 55 °C. At 60 °C, an apparent delay in the onset of biofilm formation among P3 and ATCC12980 was observed, whereas, such a delay was not observed for A1 and D1. All strains showed no delay in the onset of biofilm formation at 65 °C in comparison with the minimum detectable concentration. Spore formation for A1, D1, P3 and ATCC12980 was influenced by the incubation temperature. An apparent delay in the spore formation for A1, P3 and ATCC12980 was observed at 55 °C whereas, no delay was observed in D1. At 60 °C, an apparent delay in the spore formation for A1, P3 and ATCC12980 was observed. At 65 °C, no delay in the spore formation for the three dairy strains was observed, however, the spore formation in ATCC 12980 showed an apparent delay of 0-4 h. The number of vegetative cells and spores released into the milk is shown in Figure S.3.3.

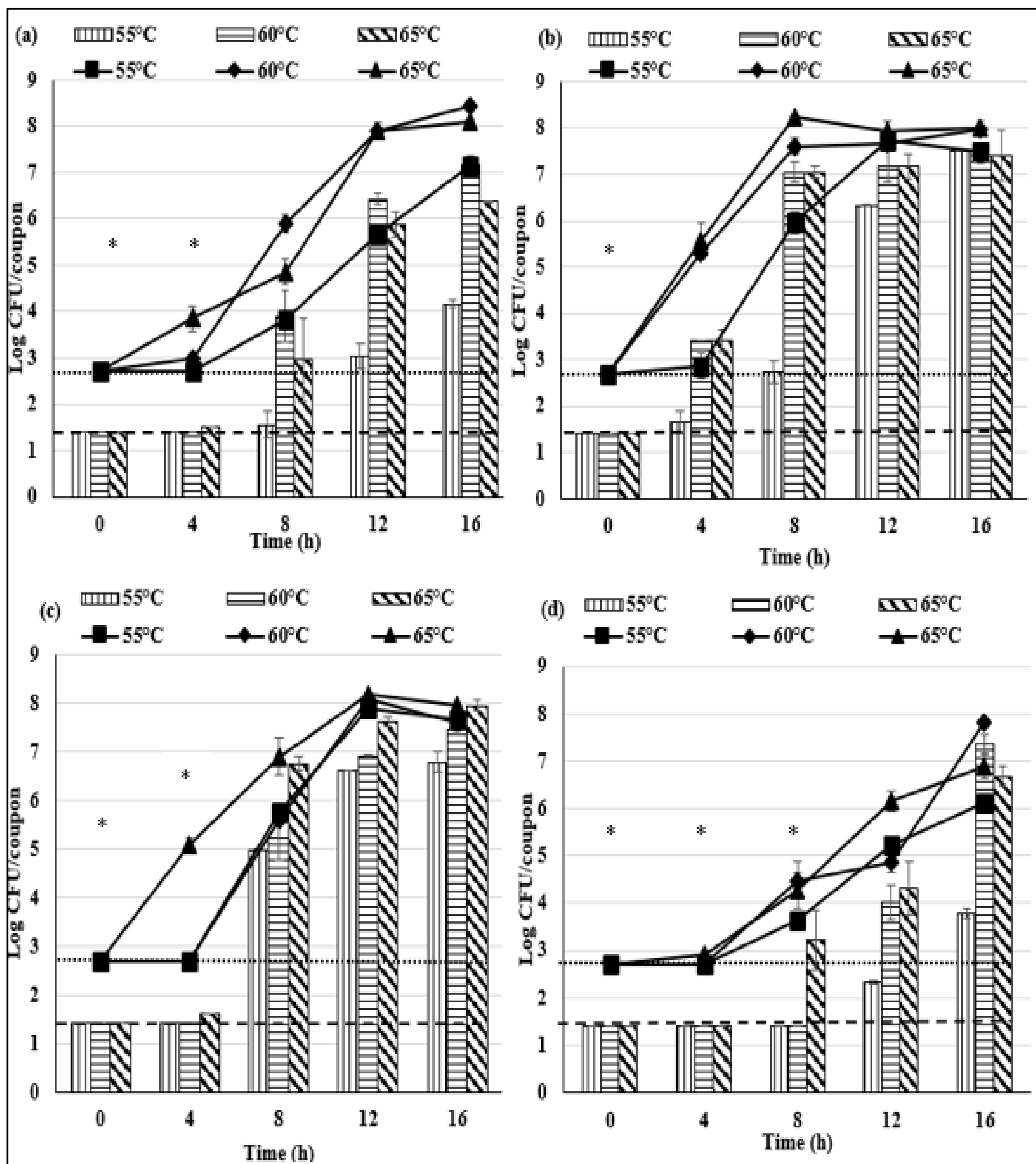


Figure 3.1. Total viable cells and spores obtained from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM. The bar graph represents the total spores and line graph represents total viable cells attached to the stainless-steel coupon. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon for total spores and total viable cells respectively. * represents one or more observations below the detection limit

In comparison with the minimum detectable concentrations of total viable cells and spores released into the milk, release of spores for A1, P3 and ATCC12980 showed an apparent delay at 55 and 60 °C. At 65 °C, the release of spores of P3 and ATCC 12980 showed an apparent delay however, no such delay was observed for A1. D1 showed no delay in the release of spores at 55, 60 and 65 °C.

There was a wide variation in the biofilm and spore forming capabilities of *G. stearothermophilus* A1, D1, P3 and ATCC12980 at 55, 60 and 65 °C in 10 % (w/v) RSM. Incubation temperature effects on biofilm and spore formation in various bacterial species have been previously shown (Baril et al., 2012; Hoštacká et al., 2010; Nilsson et al., 2011). Biofilm of *A. flavithermus* develops at 48, 55 and 60 °C, however, spores develop only at 55 and 60 °C, not at 48 °C (Burgess et al., 2009). The effect of temperature on the sporulation of *Bacillus* spp. has been studied showing low temperatures delay sporulation compared with optimum temperature for growth (Baril et al., 2012). In the present study, the delay in the biofilm and spore formation of the three dairy isolates was unobserved at 65 °C in comparison with 55 and 60 °C, indicating that 65 °C is the preferred temperature for biofilm and spore formation for A1, D1 and P3 in 10 % RSM. *G. stearothermophilus* D1 showed no delay in biofilm and spore formation at 55 and 60 °C in comparison with 65 °C in 10 % RSM (Figure. 3.1). This indicates that strain variation among A1, D1, P3 and ATCC12980 in the biofilm and spore formation that has been previously reported for this species (Burgess et al., 2014).

Spore formation can occur within 11 h in a milk powder manufacturing facility (Scott et al., 2007), however in the present study spore formation among the dairy isolates occurred within 4 h at 65 °C in 10 % RSM. It is difficult to compare a laboratory biofilm system with a commercial milk powder manufacturing facility. Two key factors may influence this difference in spore formation – the numbers of cells in the inoculum and the physiological state of those cells. In a milk powder manufacturing industry, the initial contamination arises due to the colonisation of processing surfaces by spores present in raw milk that survive pasteurisation. The numbers are often undetectable by normal laboratory tests so believed to be very low. Upon attachment, spores

undergo germination and biofilm formation before endospores are formed within the biofilm. In this study, vegetative cells in their logarithmic phase of growth were used as inocula which may shorten the time required for biofilm and spore formation.

In the present study, on average spores comprised more than 90 % of a 16 h biofilm of A1, D1 and P3 at 65 °C in 10 % RSM. This is an important observation, given that an average length of a manufacturing cycle in a milk powder manufacturing plant is 16 h (Burgess et al., 2014). In a previous study, it was observed that *G. stearothermophilus* A1 does not produce spores within the 16 h time period (Burgess et al., 2014). In the present study A1 produced spores as early as 0-8 and 0-4 h at 60 and 65 °C respectively. This difference in the results may be due to the continuous flow milk biofilm system employed in this study as opposed to the static biofilm system employed in the study conducted by Burgess et al. (2014). Yazdany & Lashkari (1975), demonstrated that sporulation of *G. stearothermophilus* was not induced at a low pH whereas abundant sporulation was noticed at pH 7.7 to 8.7. Burgess et al. 2014, had employed a static biofilm system during which the drop in pH was recorded as opposed to the continuous flow system used in the present work where the pH was maintained at 6.5-6.7 throughout the experimental run of 16h.

3.3.2. The effect of total dissolved solids on the biofilm and spore formation

To study the effect of total dissolved solids on the biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980, the total viable cells and spores within / released from the biofilm in the presence of 20 % w/v RSM milk were determined and compared with the results from 10 % w/v RSM milk. Biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 on stainless steel coupons is shown in Figure 3.2

In comparison with the minimum detectable concentration of total viable cells and spores per coupon, the onset of biofilm formation for A1, D1, P3 was apparently delayed at 55 °C. At 60 °C the biofilm formation was apparently delayed for A1 and P3 whereas at 65 °C the biofilm formation of P3 was delayed and no such delay was observed for A1 and D1. The onset of biofilm formation

in ATCC12980 was apparently delayed at 55, 60 and 65 °C. Spore formation for A1, D1, P3 and ATCC12980 was influenced by the incubation temperature. The onset of spore formation for A1, D1 and P3 was apparently delayed at 55 and 60 °C. At 65 °C, spore formation for A1, D1 and P3 was delayed until 0-4 h. Spore formation in ATCC12980 was apparently delayed at 55, 60 and 65 °C. The number of vegetative cells and spores released from within the biofilm into the milk is shown in Figure S.3.4

In comparison with the minimum detectable concentrations of total viable cells and spores released into the milk, the release of spores into the milk for A1, D1, P3 and ATCC12980 was apparently delayed at 55, 60 and 65 °C.

Regression analysis on the effect of incubation temperature, incubation time and total solids concentration on the biofilm and spore formation yielded a regression equation and a regression coefficient (R^2) which is shown in supplementary Table S.3.3 and S.3.4. Based on the regression analysis, the Pareto chart indicating the standardized effect of individual parameter on the biofilm formation was determined (supplementary Figures S.3.5 and S.3.6). It was observed that incubation time is the most influential parameter impacting the biofilm formation of A1, P3 and ATCC 12980 whereas the total dissolved solids is the most influential parameter impacting the biofilm formation of D1. Spore formation of A1 and ATCC 12980 is most influenced by the incubation time whereas the total dissolved solids is the most influential parameter influencing spore formation of D1 and P3. The variation in the parameters influencing the biofilm and spore formation may reflect the location within the evaporator from where these strains were isolated. A1 and P3 were isolated from the top and D1 was isolated from the bottom of the evaporator (Burgess et al., 2014). That physiological conditions that exist at the top and bottom of the evaporator may reflect the variation in the growth of these strains. In addition, the strain variation in the biofilm and spore formation that has been reported previously in this species should also be considered (Burgess et al., 2014).

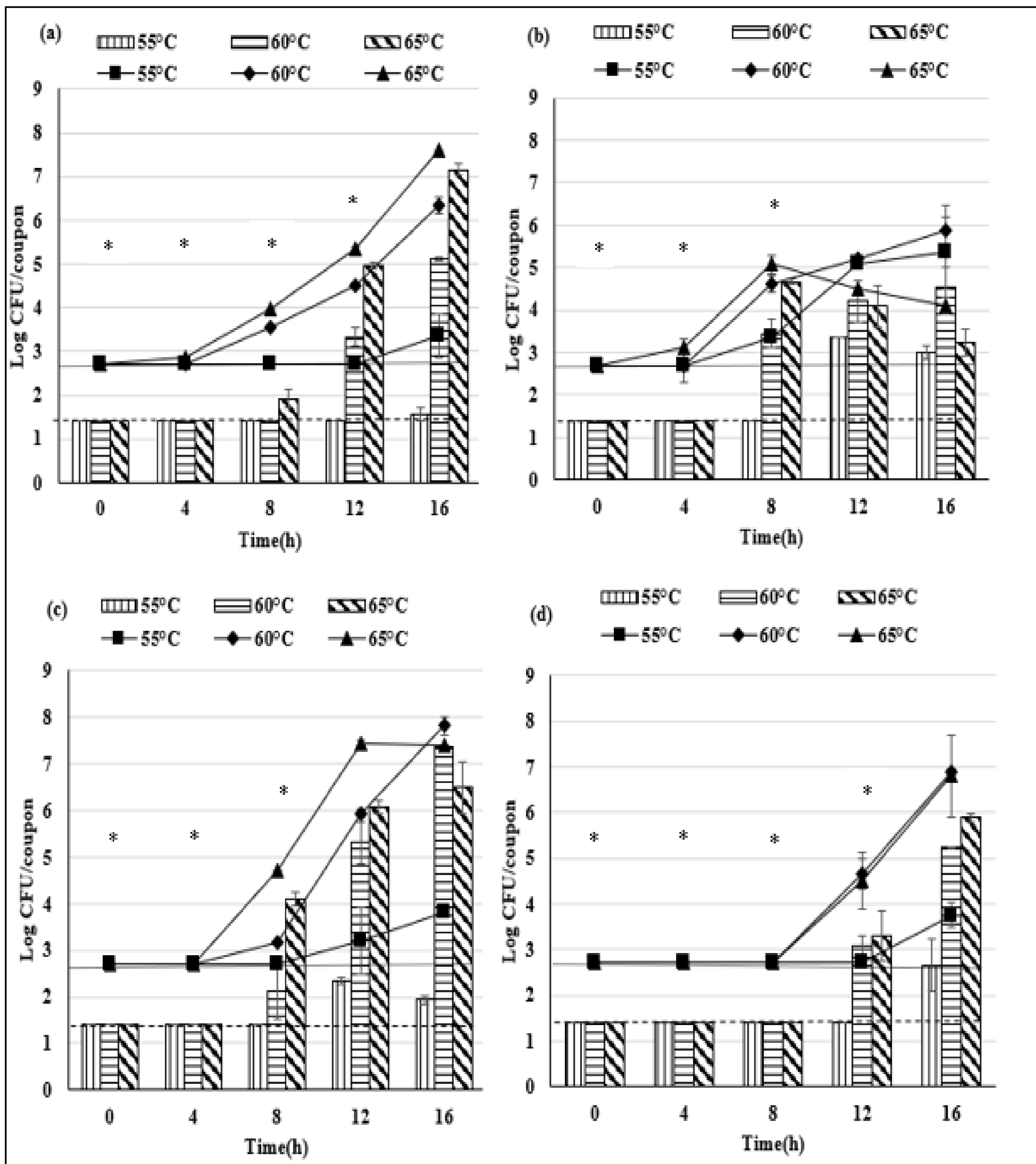


Figure 3.2. Total viable cells and spores obtained from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 20% (w/v) RSM. The bar graph represents the total spores and line graph represents total viable cells attached to the stainless-steel coupon. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon for total spore and total viable cells respectively. * represents one or more observations below the detection limit

In addition to the nonlinear regression analysis, a kinetic model depicting the biofilm and spore formation in the CDC reactor was constructed using Combase software. Based on the model, fit parameter values were calculated (see Appendix section 7.5). The Baranyi - Roberts model was used to demonstrate the stages of biofilm and spore formation. This model was specifically chosen as it is widely employed in previous studies involving bacterial growth in food (Baranyi & Roberts, 1994; McKellar, 2001). Curve fitting parameters are given in Appendix section 7.6.

Based on the parameter values, the rate of biofilm formation of A1 in 10% solids was lowest at 55°C whereas there was no significant difference ($p \leq 0.05$) in the rate of biofilm formation at 60 and 65°C. In 20% solids concentration, the rate of biofilm formation of A1 was the highest and not significantly different ($p \leq 0.05$) at 60 and 65°C whereas the lowest at 55°C. The rate of biofilm formation for D1 in 10% solids was not significantly different ($p \leq 0.05$) among 55, 60 and 65°C. In 20% solids, the rate of biofilm formation of D1 was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C. The rate of biofilm formation of P3 in 10% solids was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C. In 20% solids, the rate of biofilm formation of P3 was lowest at 55°C whereas the rate of biofilm formation was the highest and not significantly different ($p \leq 0.05$) at 60 and 65°C. The rate of biofilm formation of ATCC 12980 in 10% solids was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C, whereas in 20 % solids, the rate of biofilm formation was lowest at 55°C and the highest at 60 and 65°C. The lag phase in the biofilm formation of A1 in 10% solids was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C. The lag phase in the biofilm formation of P3 in 10% solids at 55 and 60°C was not significantly different ($p \leq 0.05$). The lag phase in the biofilm formation of ATCC 12980 in 10% solids at 55, 60 and 65°C was not significantly different ($p \leq 0.05$). In 20% solids, the lag phase in the biofilm formation of A1 was longest at 55°C in comparison with 60 and 65°C. The lag phase in the biofilm formation of P3 at 55, 60 and 65°C was not significantly different ($p \leq 0.05$). The lag phase in the biofilm formation of ATCC 12980 was not significantly different at 60 and 65°C ($p \leq 0.05$).

The rate of spore formation of A1 in 10% solids was lowest at 55°C whereas there was no significant difference ($p \leq 0.05$) in the rate of spore formation at 60 and 65°C. In 20% solids concentration, the rate of spore formation of A1 was not significantly different ($p \leq 0.05$) at 60 and 65°C. The rate of spore formation for D1 in 10% solids was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C. The rate of spore formation of P3 in 10% solids was not significantly different ($p \leq 0.05$) at 55, 60 and 65°C. In 20% solids, the rate of spore formation of P3 was lowest at 55°C whereas the rate of spore formation was not significantly different ($p \leq 0.05$) at 60 and 65°C. The rate of spore formation of ATCC 12980 in 10% solids was the highest at 60°C and 55°C, whereas in 20 % solids, the rate of spore formation was lowest at 55°C and the highest at 65°C. The lag phase in the spore formation of A1 in 10% solids was the longest at 55°C and not significantly different ($p \leq 0.05$) at 60 and 65°C. The lag phase in the spore formation of D1 in 10% solids was the longest at 55°C and the lowest at 65°C. The lag phase in the spore formation of ATCC 12980 in 10% solids was shortest at 65°C was not significantly different ($p \leq 0.05$) at 55 and 60°C. In 20% solids, the lag phase in the spore formation of A1 was longest at 55°C in comparison with 60 and 65°C. The lag phase in the spore formation of ATCC 12980 was not significantly different at 60 and 65°C ($p \leq 0.05$).

Based on the kinetic model, I observe a strain variation in the response of A1, D1, P3 and ATCC 12980 towards the variables (temperature and solids concentration). I predict that the observed difference in the rate of the biofilm and spore formation might arise due to the strain variation in the biofilm formation that has been reported previously in this species (Burgess et al., 2014). In addition, A1 and P3 was isolated from the top and D1 was isolated from the bottom of the evaporator respectively (Burgess et al., 2014). I predict the physiological conditions that exist at the top and bottom of the evaporator might be different which may reflect the difference in the rate of biofilm formation.

At 55°C, the biofilm formation of A1 and D1and P3 was delayed in 20 % (w/v) RSM compared with 10 % (w/v) RSM. Preliminary studies conducted in this laboratory (data not shown), indicated

that the addition of lactose in excess to 10 % (w/v) RSM did not affect biofilm formation whereas the addition of milk protein in excess to 10 % (w/v) RSM negatively affected biofilm and spore formation of A1, D1, P3 and ATCC12980 at 55 °C.

3.3.3. The effect of milk proteins on biofilm and spore formation

Biofilm and spore formation were studied in the presence of 10 % (w/v) RSM with 4 % (w/v) milk protein concentrate (MPC) (Figure. 3.3).

In comparison with the minimum detectable concentrations of total viable cells and spores per coupon, the onset of biofilm formation for A1, D1, P3 and ATCC 12980 was apparently delayed at 50 °C. At 60 °C, the onset of biofilm formation for A1, P3 and ATCC12980 was apparently delayed however, there was no such delay in the biofilm formation of D1 at 60 °C. At 65 °C, there was no delay in the biofilm formation observed for A1, D1, P3 and ATCC 12980. The onset of spore formation for A1, D1, P3 and ATCC 12980 was apparently delayed at 55, 60 and 65 °C. The number of vegetative cells and spores released from the biofilm into the milk is shown in supplementary Figure S.3.7.

In comparison with the minimum detectable concentrations of total viable cells and spores released into the milk, the release of spores from *G. stearothermophilus* A1, D1, P3 and ATCC12980 was apparently delayed at 55, 60 and 65 °C (Figure. 3.2).

The addition of 4 % (w/v) MPC to the 10 % (w/v) RSM delayed the biofilm formation for A1, D1, P3 and ATCC 12980 at 55 °C in comparison with the results obtained in the presence of 10 % (w/v) RSM (Fig. 3.1). In order to determine the effect of individual milk protein (casein and whey) on the biofilm formation, strain A1 was selected as this showed the longest apparent delay in the biofilm formation of 0-12 h in the presence of 4 % MPC in 10 % RSM at 55 °C. Biofilm and spore formation of A1 was determined in the presence of 3.2 % (w/v) Na-Caseinate and 0.8 % (w/v) whey protein concentrate (WPC) added in 10 % (w/v) RSM at 55 °C (Fig. 3.4). The concentration of casein and whey protein were chosen based on their ratio in milk .

Based on the minimum detectable bacterial concentration, the addition of 0.8 % (w/v) WPC and 3.2 % (w/v) Na- Caseinate to 10 % (w/v) RSM apparently delayed the onset of the biofilm and spore formation at 55 °C. The results obtained by the addition of 3.2 % (w/v) Na-Caseinate to 10 % (w/v) RSM were similar to the results previously obtained by the addition of 4 % (w/v) MPC (Figure 3.3).

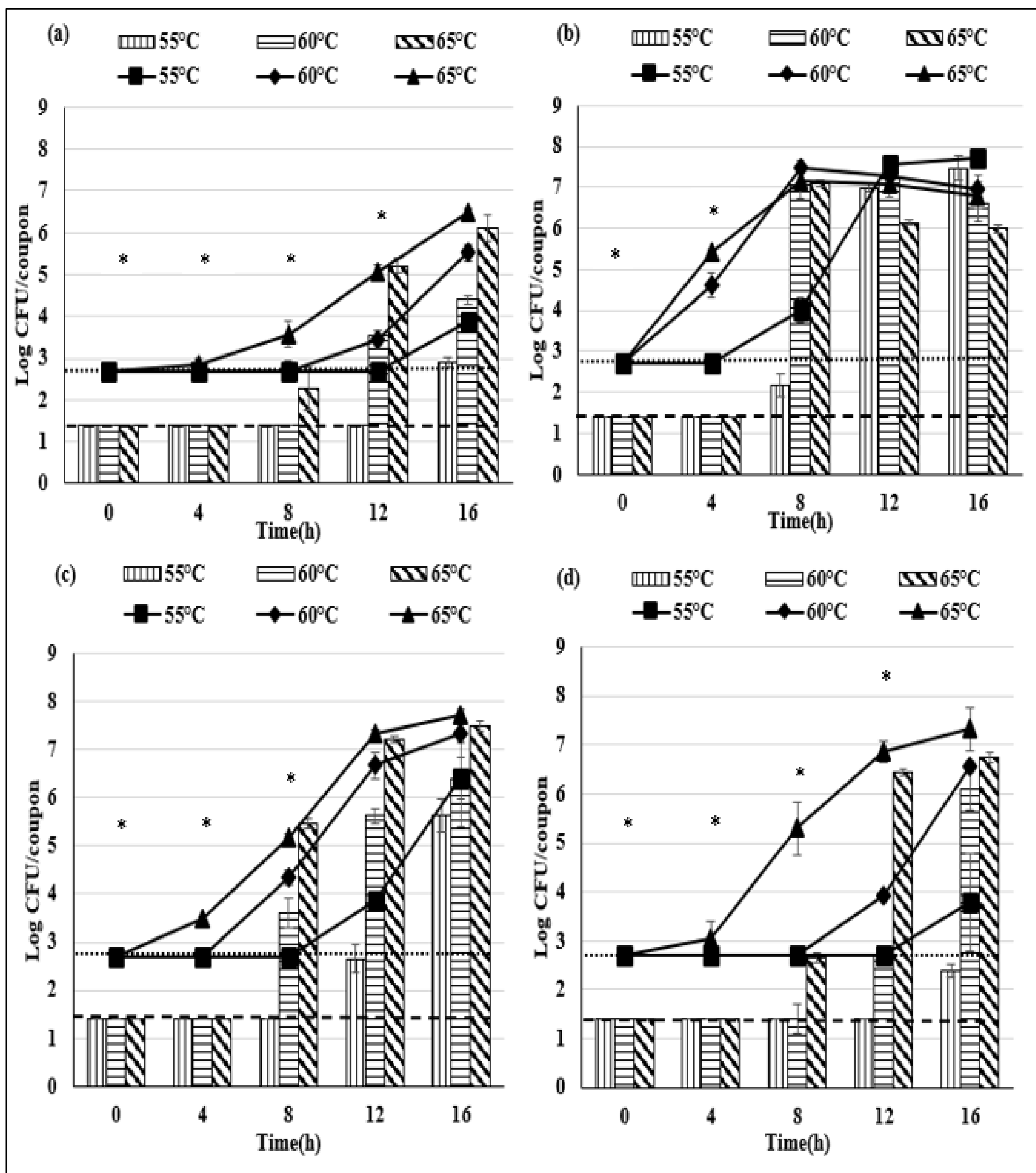


Figure 3.3. Total viable cells and spore released into the milk from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM with 4% (w/v) MPC. The bar graph represents the total spores and line graph represents total viable cells released into the flowing milk respectively. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/mL for total spore numbers and total viable cells respectively. * represents one or more observations below the detection limit

.

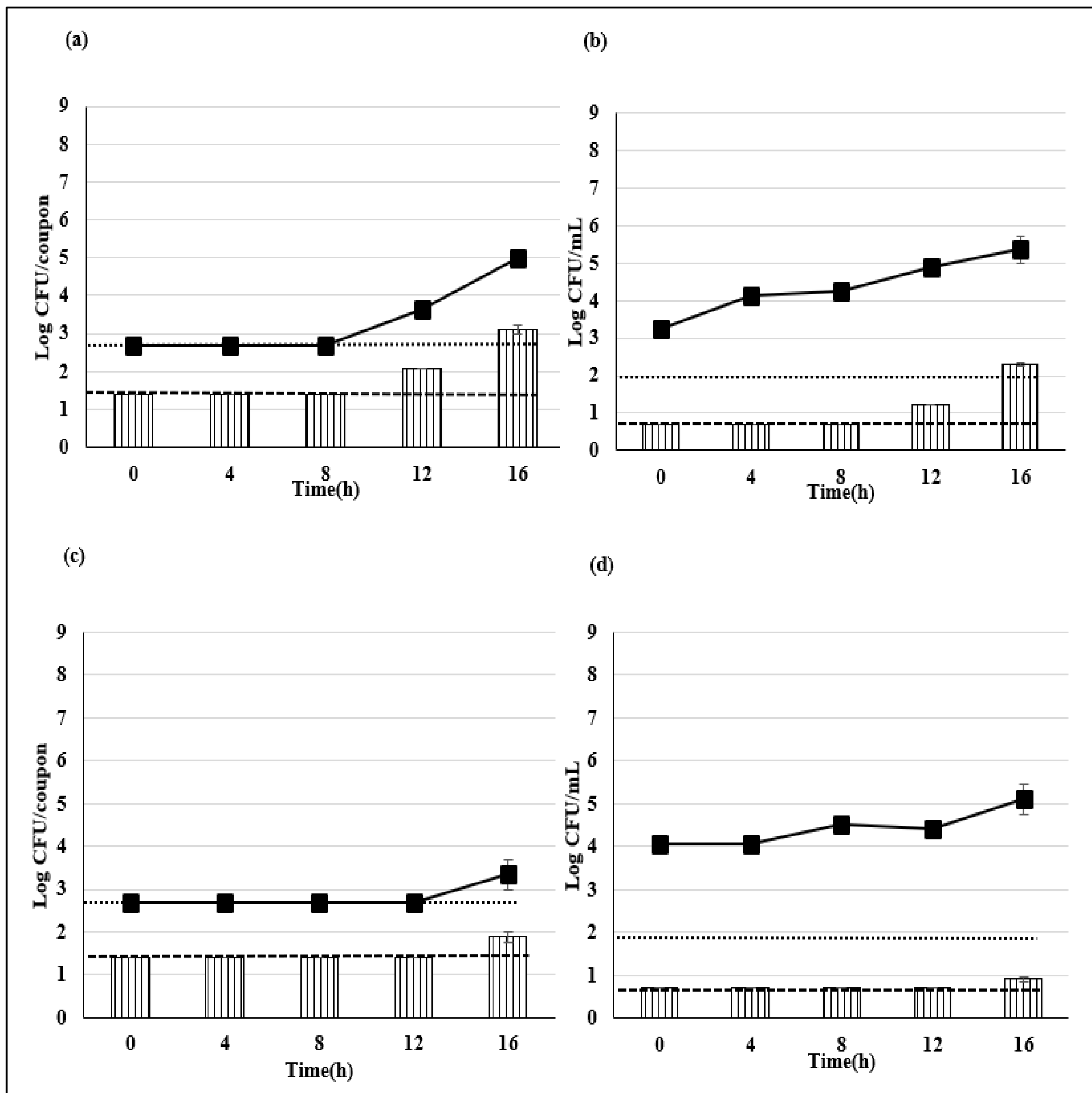


Figure 3.4. The total viable cells and spores of *G. stearothermophilus* A1 in 10% (w/v) RSM with 0.8% (w/v) WPC(a and b) and 4.2% (w/v) Na- Caseinate(c and d) added at 55°C obtained from the biofilms(a and c) and released into the milk (b and d). The bar graph represents the total spores and line graph represents total viable cells respectively. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon (a and c) and 0.7 and 2.0 log CFU/mL (b and d) for total spore numbers and total viable cells respectively. * represents one or more observations below the detection limit

In 1993, Helke, Somers & Wong (Helke et al., 1993) concluded that casein, α-lactalbumin and β-lactoglobulin inhibited the attachment of *Listeria monocytogenes* and *Salmonella Typhimurium* to stainless steel and Buna-N surfaces when present in the attachment menstruum or when the surfaces are pre-treated with these proteins. Conditioning films made of whey protein isolate (WPI), β-lactoglobulin and bovine serum albumin (BSA) have been shown to have a transient effect by slowing down the adhesion of *Listeria innocua* to polystyrene surfaces (Robitaille et al., 2014). Individual milk proteins α- casein, β-casein, κ-casein and α-lactalbumin have been known to reduce the attachment of *Staphylococcus aureus* and *L. monocytogenes* to stainless steel surfaces (Barnes et al., 1999). However, the extent of bacterial attachment depends on the type of surface and the bacterial species involved (Fletcher, 1976). Competition between various components present in milk might be the reason behind the reduced bacterial attachment (Fletcher, 1976; Speers & Gilmour, 1985). The conditioning film like layer can be formed in <5 h on clean surfaces (Mittelman, 1998). This layer can affect surface-properties i.e. free surface energy, hydrophobicity and electrostatic charge used in food processing environments (Dickson & Koohmaraie, 1989). Reversible attachment starts between the electrically charged surface of the bacteria and the conditioning film. Following reversible attachment, irreversible attachment occurs by aid of fimbra, pili and secreted extracellular components (Kokare et al., 2009). One of the major factors affecting the tight binding of the bacteria to the pre-conditioned surface is incubation temperature. Elevated temperatures of 30 and 47 °C have been shown to increase the surface hydrophobicity of *Pseudomonas aeruginosa* ATCC 27853 thereby aiding their attachment to polystyrene surfaces (Cappello & Guglielmino, 2006). This proposes the hypothesis that the preconditioning layers of adsorbed milk protein can play a vital role in the attachment of dairy isolates of *Geobacillus* spp. to stainless steel surfaces and this needs further investigation. To test this hypothesis, we determined the role of individual milk proteins on the attachment of *G. stearothermophilus* A1 on preconditioned stainless-steel surface (Figure S.3.8)(see Appendix section 7.3). We observed that 10% RSM had the highest attachment on the preconditioned stainless-steel surface whereas the

attachment was lowest on the untreated surface. In comparison with the untreated surface, preconditioning of stainless-steel surface in the presence of 3.2% (w/v) Na-Caseinate and 0.8% (w/v) whey protein lowered the attachment of A1 with Na-caseinate exhibiting the strongest effect. Based on the above observation, we suggest that the type of protein does significantly influence the attachment of A1 on a pre - conditioned stainless-steel surface ($p \leq 0.05$).

In this study, planktonic cells undergo transition to a sessile state upon attachment to the stainless steel surface. Primary biofilm cells of *Pseudomonas aeruginosa* PA01 have been known to exhibit a lag phase attributed to their adaptation to a biofilm environment (Rice et al., 2000). The delay in the biofilm formation that is observed in this study may be due to the adaptation of planktonic cells to the biofilm environment and further investigation is required. Nutrient concentration of the growth medium and the phenotypic origin of the inoculum have been known to influence the lag phase of biofilms of *Pseudomonas aeruginosa* PA01and *Pseudomonas fluorescens* CT07 grown using a continuous flow system (Kroukamp et al., 2010). An in-depth study focusing on the phenotypic and genotypic changes in the cells from a planktonic to a sessile state of growth may help further my understanding on the effect of different abiotic factors on the lag phase during biofilm formation of *G. stearothermophilus*.

3.4. Conclusion

In this study, the preferred temperature for biofilm and spore formation of *G. stearothermophilus* A1, D1 and P3 was 65 °C. Increasing the total dissolved solids extended the apparent delay in the onset of biofilm and spore formation to detectable concentrations of all the strains at 55 °C. Similarly, the addition of milk proteins showed an apparent delay in the onset of the biofilm and spore formation to detectable concentration of *G. stearothermophilus* A1 at 55 °C with sodium-caseinate having the most effect. These findings indicate that preconditioning of stainless-steel surfaces using adsorbed milk proteins may play a vital role in delaying the biofilm and spore formation of thermophilic bacteria during the manufacture of dairy products.

3.5. Supplementary information

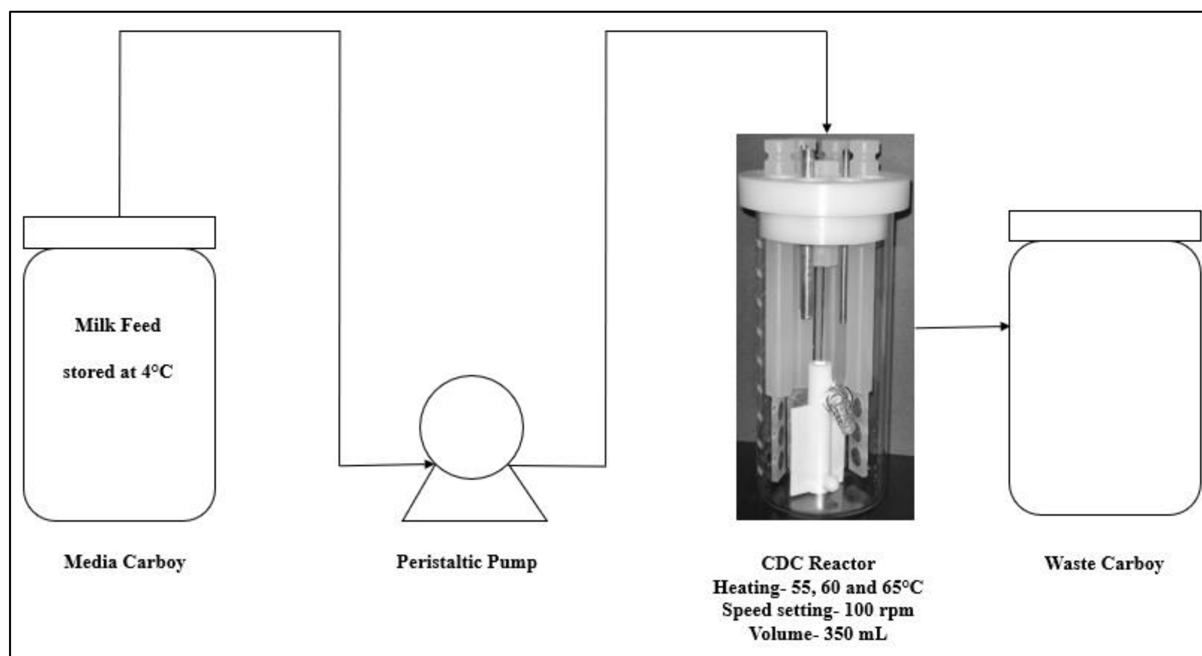


Figure S.3.1. Schematic diagram of the CDC reactor setup

Table S.3.1. Doubling time of *G. stearothermophillus* A1, D1, P3 and ATCC 12980 in 10% (w/v) UHT skim milk.

Strain	Doubling time (min)		
	55°C	60°C	65°C
A1	25.85	24.07	24.73
D1	26.33	22.88	24.42
P3	28.96	24.70	25.79
ATCC 12980	31.11	28.21	28.96

Doubling time was calculated based on the growth curve (Figure S.2)

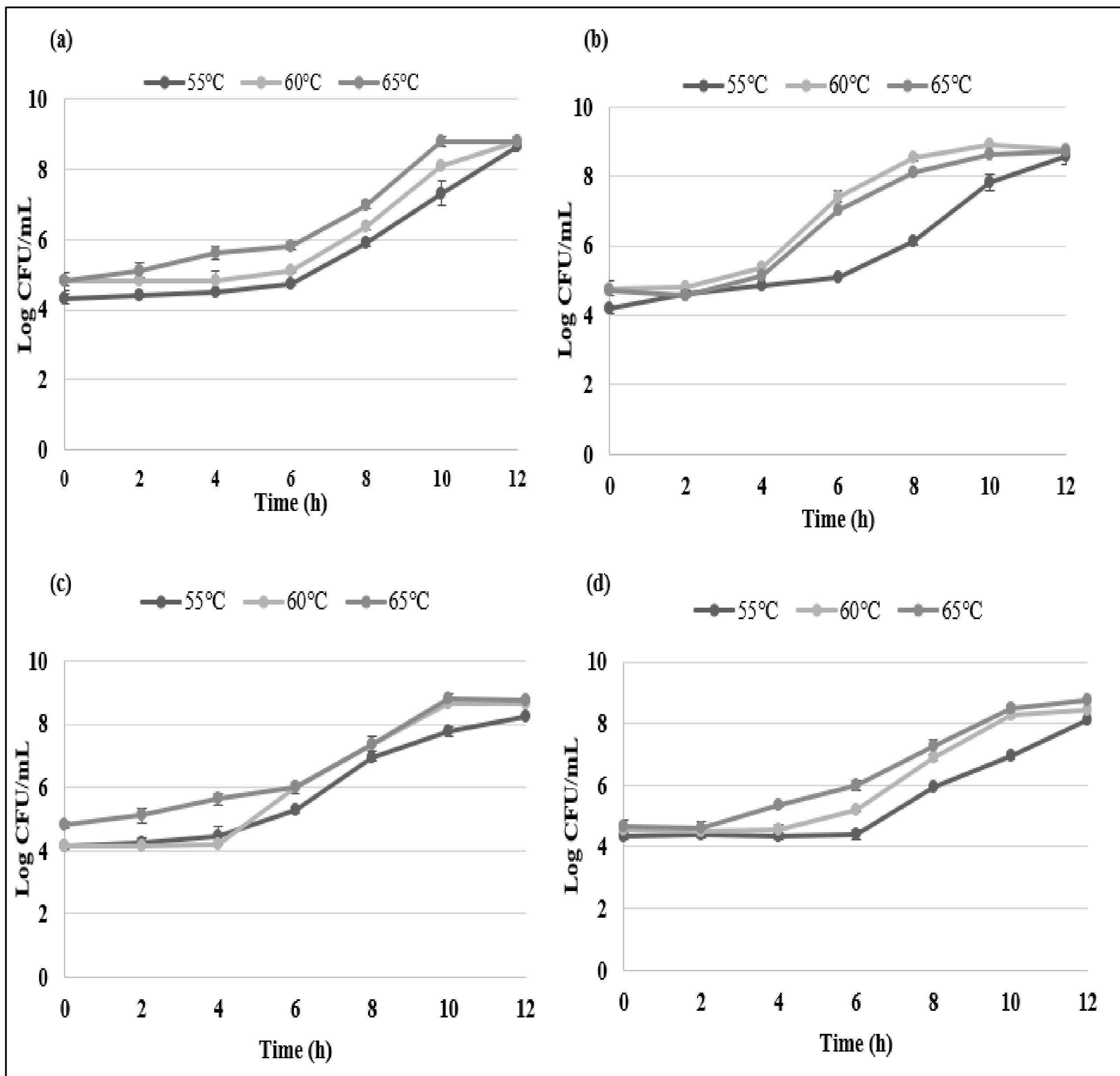


Figure S.3.2. Effect of temperature on the growth of *G. stearothermophilus* A1 (a), D1 (b), P3 (c) and ATCC 12980 (d) in 10% (w/v) UHT skim milk. Error bars represent standard deviation of triplicates

Table S.3.2. Effect of temperature on the maximum specific growth rate of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in 10% (w/v) UHT skim milk.

Strain	Maximum specific growth rate (/h)		
	55°C	60°C	65°C
A1	1.61	1.73	1.68
D1	1.58	1.82	1.70
P3	1.44	1.68	1.61
ATCC 12980	1.34	1.47	1.44

Maximum specific growth rate was calculated based on the growth curve (figure S.2)

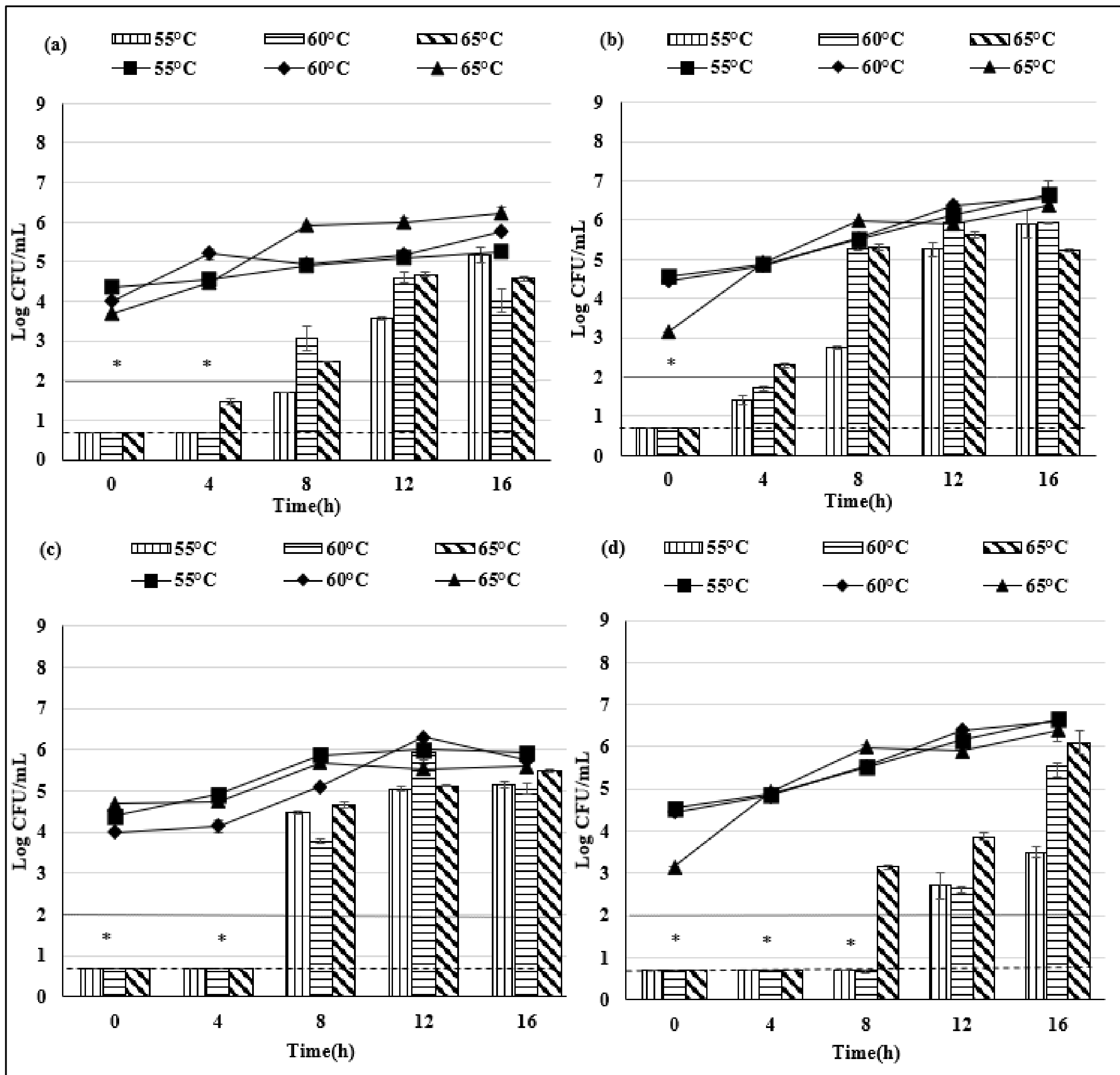


Figure S.3.3. Total viable cells and spores released into the milk from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM. The bar graph represents the total spores and line graph represents total viable cells released into the milk. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 0.7 and 2.0 log CFU/mL for total spore and total viable cells respectively. * represents one or more observations below the detection limit

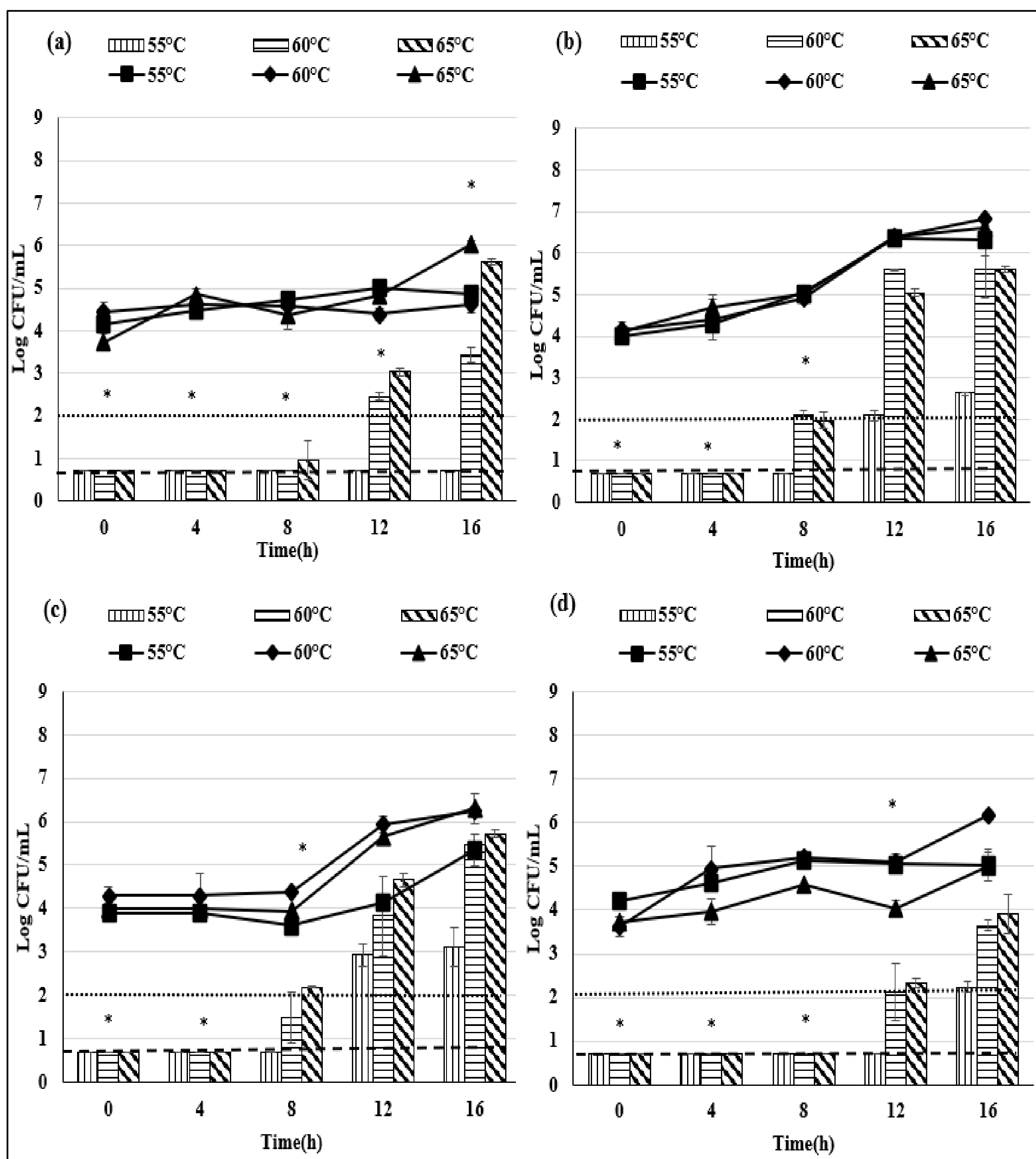


Figure S.3.4. Total viable cells and spore released into the milk from the biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 20% (w/v) RSM. The bar graph represents the total spores and the line graph represents total viable cells released into the milk. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 0.7 and 2.0 log CFU/mL for total spore and total viable cells respectively. * represents one or more observations below the detection limit

Table S.3.3. Regression analysis of the effect of incubation temperature(°C), total dissolved solids (% w/v) and incubation time (h) on the biofilm formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980. The reported parameters are significant with $p \leq 0.05$

Strain	Regression equation of the best fitting model	Adjusted R^2 value (%)
A1	Total viable cells / coupon = -8.13 + 0.3809 incubation time – 201.14 total dissolved solids + 0.2015 incubation temperature	82.4
D1	Total viable cells / coupon = -1.16 + 0.932 incubation time -27.01 total solids + 0.0926 incubation temperature -0.0345 incubation time * incubation time	80.38
P3	Total viable cells / coupon = -85.2 + 3.665 incubation time -2.314 total dissolved solids + 2.858 incubation temperature	63.77
ATCC 12980	Total viable cells /coupon = 4.97 + 0.3707 incubation time – 11.60 total dissolved solids + 0.1192 incubation temperature	74.01

Table S.3.4. Regression analysis of the effect of incubation temperature (°C), total dissolved solids (% w/v) and incubation time (h) on the spore formation of G. stearothermophilus A1, D1, P3 and ATCC 12980. The reported parameters are significant with $p \leq 0.05$

Strain	Regression equation of the best fitting model	Adjusted R^2 value (%)
A1	Total spores / coupon = -16.84 + 0.426 incubation time - 16.52 total dissolved solids + 0.2995 incubation temperature	58.81
D1	Total spores / coupon = -5.66 + 1.162 incubation time - 27.30 total solids + 0.1270 incubation temperature - 0.0432 incubation time * incubation time	70.24
P3	Total spores / coupon = -16.41 + 1.844 incubation time - 24 total dissolved solids + 0.2223 incubation temperature – 0.0647 incubation time * incubation time	75.76
ATCC 12980	Total spores / coupon = -172.6 -1.924 incubation time - 12.45 total dissolved solids + 6.05 incubation temperature + 0.0893 incubation time * incubation time - 0.0485 incubation temperature * incubation temperature	86.92

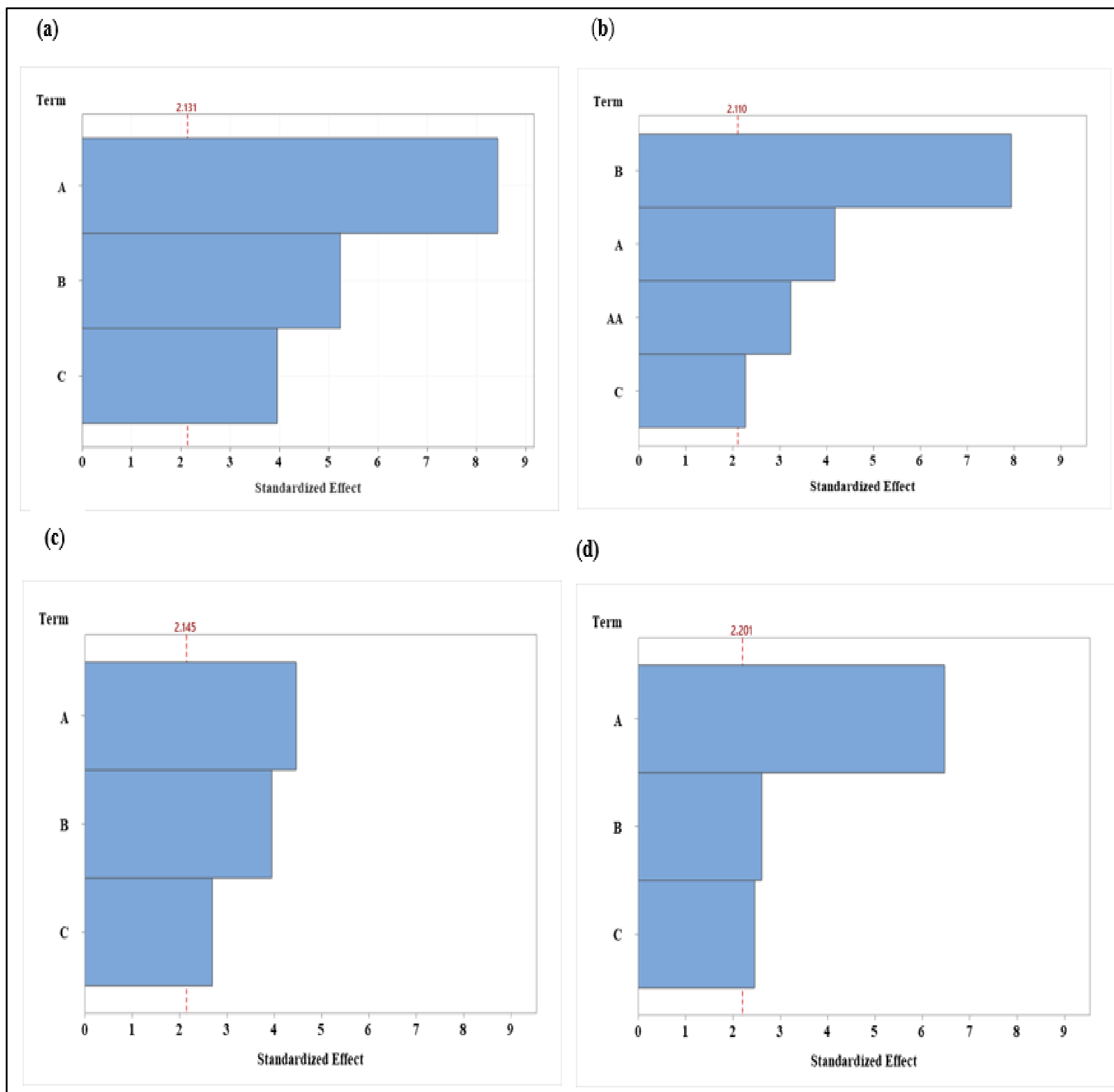


Figure S.3.5. Pareto analysis of the effect of incubation time (A), total dissolved solids (B) and incubation temperature (C) on the biofilm formation of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC 12980 (d)

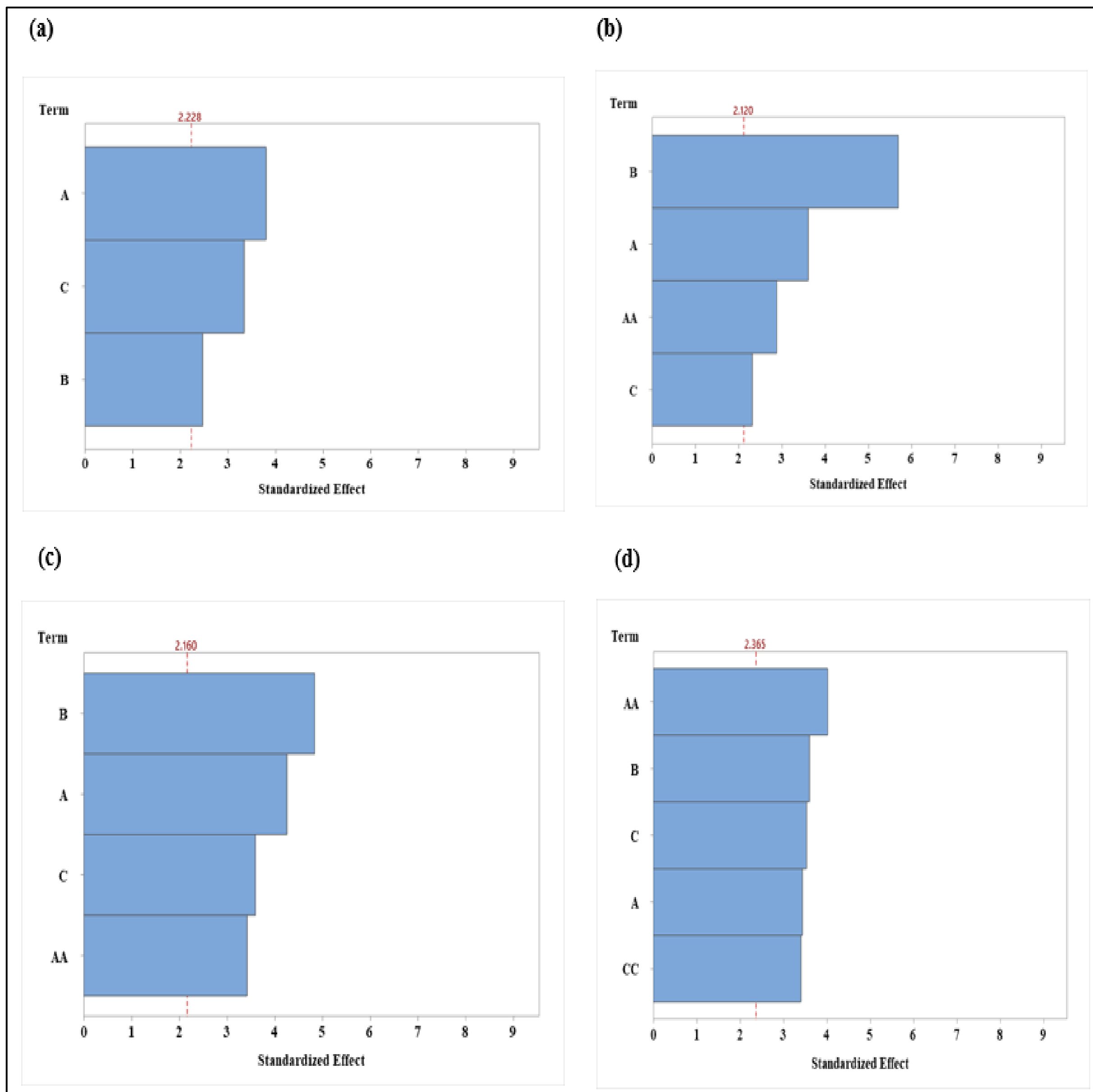


Figure S.3.6. Pareto analysis of the effect of incubation time (A), total dissolved solids (B) and incubation temperature (C) on the spore formation of *G. stearothermophilus* A1 (a), D1 (b), P3 (c) and ATCC 12980 (d)

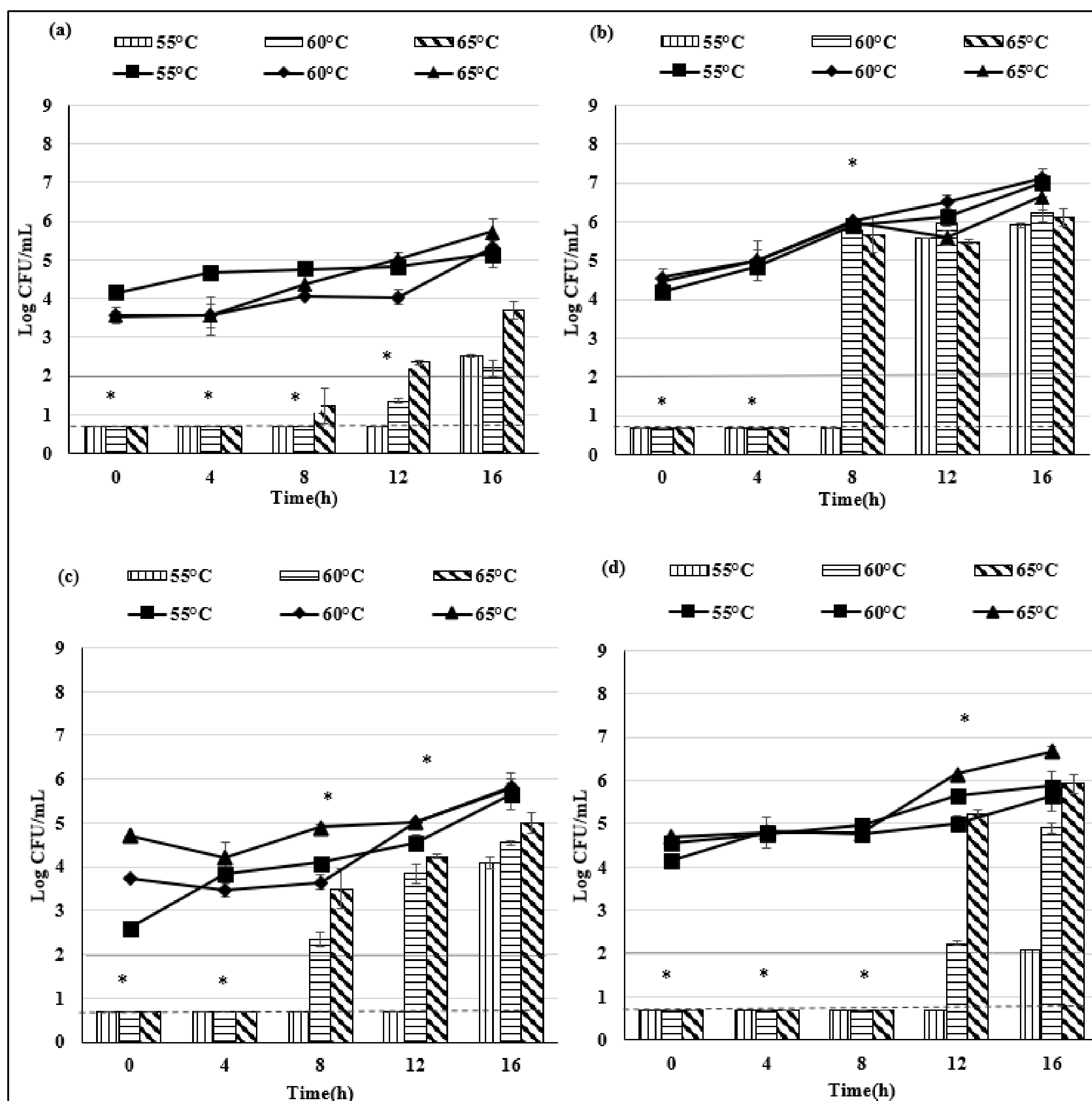


Figure S.3.7. Total viable cells and spores obtained from biofilms of *G. stearothermophilus* A1(a), D1(b), P3(c) and ATCC12980(d) in 10% (w/v) RSM with 4% (w/v) MPC added in excess. The bar graph represents the total spores and the line graph represents total viable cells attached to the stainless-steel coupon. Error bars represent standard deviation of triplicates. Dotted lines represent minimum detection limit of 1.4 and 2.7 log CFU/coupon for total spore numbers and total viable cells respectively. * represents one or more observations below the detection limit.

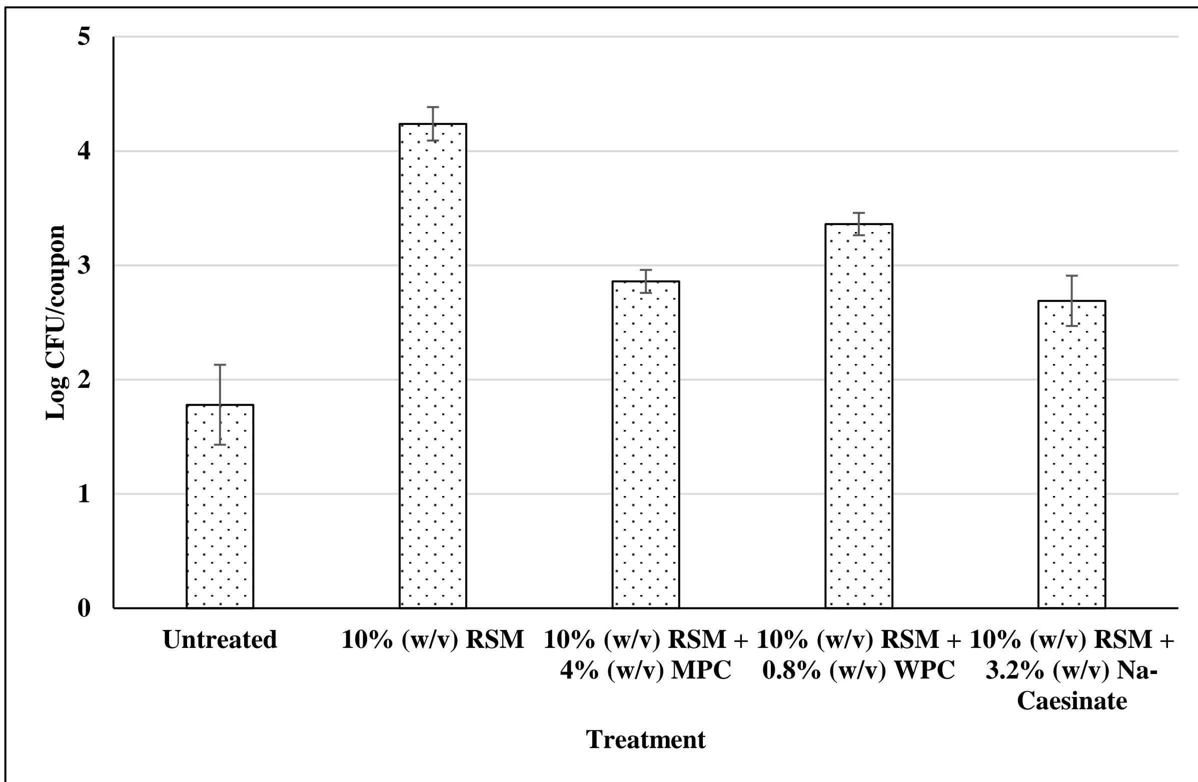


Figure S.3.8. Effect of pre-conditioned stainless steel surface on the attachment of *G. stearothermophilus* A1 in the presence of 10% (w/v) RSM, 10% (w/v) RSM + 4% (w/v) MPC, 10% (w/v) RSM + 0.8% (w/v) WPC, 10% (w/v) RSM + 3.2% (w/v) Na-Caseinate . Error bars represent standard deviation of triplicates. See Appendix section 7.3 for the experiment design.

3.6. References

Ahn, S. J., & Burne, R. A. (2007). Effects of oxygen on biofilm formation and the AtlA autolysin of *Streptococcus mutans*. *Journal of Bacteriology*, 189(17), 6293–6302.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology, 23(3–4), 277–294.

Baril, E., Coroller, L., Couvert, O., El Jabri, M., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., & Mafart, P. (2012). Sporulation boundaries and spore formation kinetics of *Bacillus* spp. as a function of temperature, pH and aw. *Food Microbiology*, 32(1), 79–86.

Barnes, L. M., Lo, M. F., Adams, M. R., & Chamberlain, A. H. L. (1999). Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology*, 65(10), 4543–4548.

Burgess, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2009). The formation of spores in biofilms of *Anoxybacillus flavithermus*. *Journal of Applied Microbiology*, 107(3), 1012–1018.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophillic bacilli from a milk powder processing plant. *Journal of Applied Microbiology*, 116(2), 350–359.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215–225.

Cappello, S., & Guglielmino, S. P. P. (2006). Effects of growth temperature on the adhesion of *Pseudomonas aeruginosa* ATCC 27853 to polystyrene. *Annals of Microbiology*, 56(4), 383–385.

Dickson, J. S., & Koohmaraie, M. (1989). Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology*, 55(4), 832–836.

Fletcher, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology*, 94(2), 400–404.

Giaouris, E., Chorianopoulos, N., & Nychas, G.-J. E. (2005). Effect of temperature, pH, and water Activity on biofilm formation by *Salmonella enterica* Enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection*, 68(10), 2149–2154.

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., & Nierop Groot, M. (2015). Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology*, 200(4), 72–79.

Helke, D. M., Somers, E. B., & Wong, A. C. L. (1993). Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and Buna-N in the presence of milk and individual milk components. *Journal of Food Protection*, 56(6), 479–484.

Hoštacká, A., Čižnár, I., & Štefkovičová, M. (2010). Temperature and pH affect the production of bacterial biofilm. *Folia Microbiologica*, 55(1), 75–78.

Kokare, C. R., Chakraborty, S., Khopade, A. N., & Mahadik, K. R. (2009). Biofilm: importance and applications. In *Indian Journal of Biotechnology*,8(2), 159–168.

Kroukamp, O., Dumitrache, R. G., & Wolfaardt, G. M. (2010). Pronounced effect of the nature of the inoculum on biofilm development in flow systems. *Applied and Environmental Microbiology*, 76(18), 6025–6031.

Lindsay, D., & Flint, S. (2009). Biofilm formation by spore-forming bacteria in food processing environments. In *Biofilms in the food and beverage industries*. Woodhead Publishing Limited. United Kingdom.

Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L. (2012). Biofilm Formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 133–147.

Mittelman, M. W. (1998). Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science*, 81(10), 2760–2764.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, 23(3–4), 277–294.

Grijspeerdt, K., & Vanrolleghem, P. (1999). Estimating the parameters of the Baranyi model for bacterial growth. *Food Microbiology*, 16(6), 593–605.

McKellar, R. C. (2001). Development of a dynamic continuous-discrete-continuous model describing the lag phase of individual bacterial cells. *Journal of Applied Microbiology*, 90(3), 407–413

Naghili, H., Tajik, H., Mardani, K., Razavi Rouhani, S. M., Ehsani, A., & Zare, P. (2013). Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Veterinary Research Forum*, 4(3), 179–183.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A., Belyaev, S. S., & Ivanov, M. V.(2001). Taxonomic study of aerobic thermophilic bacilli: Descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*,

Bacillus *thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans. International Journal of Systematic and Evolutionary Microbiology*, 51(2), 433–446.

Nilsson, R. E., Ross, T., & Bowman, J. P. (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *International Journal of Food Microbiology*, 150(1), 14–24.

Rice, A. R., Hamilton, M. A., & Camper, A. K. (2000). Apparent surface associated lag time in growth of primary biofilm cells. *Microbial Ecology*, 40(1), 8–15.

Robitaille, G., Choinière, S., Ells, T., Deschênes, L., & Mafu, A. A. (2014). Attachment of *Listeria innocua* to polystyrene: Effects of ionic strength and conditioning films from culture media and milk proteins. *Journal of Food Protection*, 77(3), 427–434.

Flint, S, Palmer, J, Bloemen, K, Brooks, J. (2001). Growth of *Bacillus stearothermophillus* on stainless steel. *Journal of Applied Microbiology*, 90(2), 151–157.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, 60(2), 109–117.

Speers, J. G. S., & Gilmour, A. (1985). The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of Applied Bacteriology*, 59(4), 325–332.

Tango, C. N., Akkermans, S., Hussain, M. S., Khan, I., Van Impe, J., Jin, Y. G., & Oh, D. H. (2018). Modeling the effect of pH, water activity, and ethanol concentration on biofilm formation of *Staphylococcus aureus*. *Food Microbiology*, 76(2), 287–295.

Yazdany, S., & Lashkari, K. B. (1975). Effect of pH on sporulation of *Bacillus stearothermophilus*. *Applied Microbiology*, 30(1), 1–3

Zhang, Y., Munir, M. T., Udugama, I., Yu, W., & Young, B. R. (2018). Modelling of a milk powder falling film evaporator for predicting process trends and comparison of energy consumption. *Journal of Food Engineering*, 225(6), 26–33.

DRC 16

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Murali Kumar
Name/title of Primary Supervisor:	Steve Flint
Name of Research Output and full reference:	
M. Kumar, S.H. Flint, J. Palmer, P.G. Plieger, M. Waterland (2019). The effect of phosphate on the heat resistance of spores of dairy isolates of Geobacillus stearothermophilus . 309: 108334	
In which Chapter is the Manuscript /Published work:	3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
This paper was the work of the candidate who researched and wrote the paper under the guidance of the four co-authors	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Murali Kumar — Digitally signed by Murali Kumar Date: 2020.04.16 16:46:26 +12'00'
Date:	16/04/2020
Primary Supervisor's Signature:	Steve Flint — Digitally signed by Steve Flint Date: 2020.04.16 17:07:16 +12'00'
Date:	16th April 2020

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019

Preface to Chapter 4

In chapter 3, the role of abiotic factors, temperature and the total dissolved solids on the biofilm and spore forming capacity of *G. stearothermophilus* A1, D1, P3 and ATCC12980 was investigated. These abiotic factors influenced the biofilm and spore forming capacity of A1, D1, P3 and ATCC12980. In chapter 4, the heat resistance of spores of A1, D1, P3 and ATCC12980 and the effect of phosphate on the heat resistance of these spores is determined. In addition, the effect of pH of the heating medium on the heat resistance of spores and the DPA content of spores of A1, D1, P3 and ATCC12980 is determined.

Chapter 4

4 Heat Resistance and the Effect of Phosphate

Abstract

Phosphate decreases the spore heat resistance by accelerating the rate of loss of cations from spores. Heat resistance of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 was determined in distilled water containing varying concentrations (0.1, 1 and 2% w/v) of di-sodium phosphate. The average decimal reduction times (D value) for strains A1, D1, P3 and ATCC 12980 in distilled water were 5.8, 6.8, 5.7 and 9 min at 110 °C respectively. On the addition of 0.1, 1 and 2% w/v of di-sodium phosphate, the average D_{110} values of all the strains in distilled water were lowered by 50, 61 and 70% respectively. Addition of 0.05% w/v of Na-EDTA to distilled water lowered the average D_{110} value of all the strains by 55%. Heat resistance of spores of A1, D1, P3 and ATCC 12980 was associated with the Dipicolinic Acid (DPA) content with concentrations of 0.25, 0.30, 0.27 and 1.6 pg per spore respectively. Analysis by atomic absorption spectroscopy revealed that the phosphate present in the heating medium causes release of calcium from spores with 2% w/v phosphate causing the highest release of calcium, thus confirming the chelating effect of phosphate. This study provides insight into the heat resistance and the increased heat sensitivity of spores of *G. stearothermophilus* A1, D1 and P3 in the presence of phosphate, which can be used in the design of Cleaning in Place (CIP) systems involving phosphate based cleaning agents to control spores in the dairy industry.

4.1. Introduction

Thermophilic bacteria are prevalent during dairy powder manufacture where elevated temperatures (40-65 ºC) prevail (Cempírková, 2007). These bacteria are capable of forming heat resistant spores in dairy processing facilities and their presence is an indication of poor plant hygiene (Burgess et al., 2010). Germination of spores upon attachment to stainless steel surfaces leads to biofilm formation (Scott et al., 2007). Upon maturation of the biofilm, vegetative cells and spores can slough off and contaminate the processing line and end product (Seale et al., 2010).

Heat resistance of bacterial spores is attributed to a variety of factors including the outer coat of the spore and the relatively impermeable inner membrane (Cortezzo and Setlow, 2005; Nicholson et al., 2000; Sonenshein et al., 2002). In addition, the spore's central region of the core contains DNA bound with soluble acid soluble proteins (SASP) which protects the DNA from heat, desiccation and chemicals (Setlow, 1995; Sonenshein et al., 2002). The core of the spore apart from hosting the DNA contains high levels (5 to 15% of dry weight) of DPA, which is found as a 1:1 chelate with metal cations predominantly calcium (Setlow, 2006). DPA, present within the core is responsible for maintaining the low core water content which plays a vital role in the heat resistance of spores to wet heat (Paidhungat et al., 2000). During initial stage of spore germination, DPA is lost and is replaced with water which leads to rehydration of the core and revival of spore metabolism (Peter Setlow, 2003).

In dried milk powder samples, the three commonly isolated spore formers are *G stearothermophilus*, *A. flavithermus* and *Bacillus* species (Scheldeman et al., 2006). Owing to their high heat resistance, spores of *G. stearothermophilus* are commonly used as a biological indicator to validate the efficiency of sterilization processes (Gonzalez et al., 1995). The heat resistance of spores is influenced by several factors including sporulation temperature, pH of the heating medium and spore recovery conditions (Cook & Gilbert, 1968; Fernandez et al., 1994; Gonzalez et al., 1995). According to Cook and Gilbert (1968), the heat resistance of spores of *G. stearothermophilus* is

reduced when the pH of the heating medium is lowered. In addition, they concluded that phosphate present in the heating medium might lower the heat resistance of spores. Although, phosphate is known to lower the heat resistance of spores of *G. stearothermophilus* (López et al., 1996b), only a few studies have reported on the possible mechanism of action of phosphate. Amaha and Ordal (1957), suggested that the effect of phosphate might be that of a chelating agent, wherein phosphate may accelerate the rate of loss of cations from within the spore which might contribute towards the heat resistance of the spore. The role of phosphate as a chelating agent was hypothesised but was not studied further.

In this study, the key aim is to validate the previously suggested hypothesis on the role of phosphate as a chelating agent in lowering the heat resistance of spores of *G. stearothermophilus* A1, D1 and P3 isolated from a milk powder manufacturing plant.

4.2. Materials and Methods

4.2.1. Production of spores

G. stearothermophilus A1, D1 and P3 were previously isolated from the evaporator section of a New Zealand milk powder manufacturing plant and the genotypic, phenotypic analyses revealed that these three strains varied in their ability to produce biofilm and spores (Burgess et al., 2014). In this study, *G. stearothermophilus* ATCC 12980 was used as a reference strain. The strains used in this study were obtained from -80 ºC stocks. Tryptic soy agar (plates) and tryptic soy broth (BD diagnostic systems, Germany) were used as growth media unless mentioned otherwise. Serial dilutions were performed using 0.1% (w/v) buffered peptone water (Difco, Germany). *G. stearothermophilus* A1, D1, P3 and ATCC 12980 were first grown in 250 mL conical flasks containing 25 mL tryptic soy broth supplemented with 0.2% soluble starch (hBARSCI, USA). The mixture was shaken (150 rpm) for 24 h at 55 ºC. 200 µL of the 24 hour culture was spread onto sporulation agar (final pH 7.2) consisting of 40 g/L tryptic soy agar supplemented with the following

minerals (/L) $MgSO_4.7H_2O$, 0.51 g; KCl, 0.97 g; $CaCl_2$. $2H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.003 g; $FeSO_4$. $7H_2O$, 0.55 mg. The plates were then incubated at 55 ºC for 5 days. Presence of spores was visually confirmed through staining with malachite green and observed through a light microscope (Olympus Bx41, Japan). Spores were harvested with cold sterile distilled water (5 mL) by scraping the entire surface of the sporulation agar using sterile hockey spreaders and were then subjected to three centrifugation washings (9000 xg, 10 min, 23 ºC). After washing, spore suspensions were stored at 4 ºC in sterile water until experimental use. Spore numbers were enumerated using heat treatment at 90 ºC for 30 min in a water bath to eliminate vegetative cells then subsequently diluted using buffered peptone water and plated on tryptic soy agar plates and incubated for 24 h at 55 ºC.

4.2.2 Estimation of DPA content of spores

DPA concentration of spores was measured using a terbium complexation method (Jamroskovic et al., 2016). Known concentrations of DPA (0.005, 0.05, 0.5 µM) (Sigma-Aldrich, USA) were suspended in sterile distilled water containing terbium chloride ($TbCl_3$) (Sigma-Aldrich, USA) at a final concentration of 30 µM. Photo luminescence was measured using a spectro fluorimeter (Perkin Elmer LS55, USA) with the following settings: excitation wavelength: 276 nm, emission wavelength: 546 nm, slit width: 15 nm and scan speed: 500 nm/min. A 420 nm long pass filter was used in front of the emission monochromator throughout the analysis to eliminate second order diffraction effects. A sample containing 30 µM $TbCl_3$ was used as a blank.

Spore suspensions of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 containing 10^8 spores/mL were autoclaved at 121 °C for 30 min to ensure the complete release of DPA. After autoclaving, the spore suspension was centrifuged, and the resulting supernatant was diluted five times in sterile distilled de-ionized water. $TbCl_3$ was then added to each dilution at a final concentration of 30 µM and the photoluminescence was measured in triplicate. DPA concentration per millilitre of spore suspension containing 10^8 spores was determined and used to calculate the DPA content per spore.

4.2.3. Heat treatment of spores

Spores were subjected to three centrifugal washings (2500 x g, 4 min, 23 ºC) before every heat treatment. Microscopic observation of the washed spore suspensions revealed the presence of vegetative cells which were inactivated by heating at 90 ºC for 30 min in a water bath. The heat resistance of spores was estimated using the capillary tube method (Sadiq et al., 2016). 50 µL of spore suspension was aseptically transferred into a soda glass capillary tube (Globe Scientific, USA) with an inner diameter of 1 mm and the ends of the tube were heat-sealed. Heat treatments were carried out at 110 ºC in a thermostat controlled oil bath containing silicone oil. Each tube was completely submerged in the oil for a selected time interval. After heating, tubes were immediately transferred to cold water (temperature. < 4 ºC) for 30 s, then washed with 70% ethanol, cut aseptically and serial diluted using 0.1% peptone water. Serial dilutions were then plated on tryptic soy agar and incubated at 55 ºC up to 24 h. The minimum detection limit was 1.7 log CFU/mL. Decimal reduction time (D value) was calculated through the negative inverse of the slope of the regression line plotted with values of the survival curve. All heating experiments were carried out as triplicate experiments involving three individual spore suspensions.

4.2.4. Role of the heating medium

To study the effect of pH of the heating medium on the heat resistance of spores, citrate phosphate buffer of pH 4, 7 and 8 were prepared as described previously (McIlvaine, 1921) by mixing 7.7, 16.5 and 19.5 mL of 0.2 M solution of di-sodium phosphate (Sigma- Aldrich, USA) with 12.3, 3.5 and 0.5 mL of 0.1 M solution of citric acid (Sigma- Aldrich, USA) respectively.

Di-sodium phosphate solution of varying concentrations (0.1, 1 and 2%) (w/v) were prepared in sterile distilled water and were used as the heating medium for experiments as described below. The pH of the 0.1, 1 and 2% di-sodium phosphate solutions before heating were 8, 8.2 and 8.6 respectively. The effect of phosphate on the heat resistance of spores was further studied in the presence of an equimolar concentration of calcium acetate (Sigma-Aldrich, USA) and di-sodium

phosphate (0.07 M) as the heating medium. The effect of EDTA on the heat resistance of spores was studied in the presence of 0.05% of EDTA (Sigma-Aldrich, USA) in distilled water as the heating medium.

4.2.5. Effect of phosphate on the release of calcium

Atomic absorption spectroscopy was used to determine the influence of di-sodium phosphate on the release of calcium from within spores. Spore suspensions containing 10^8 spores/mL were centrifugally washed three times at 9000 x g in deionized distilled water prior to the heat treatment step to get rid of cell debris. The procedures employed were adopted from those described previously (Rotman & Fields, 1969; Bulgarelli & Shelef, 1985). In short, release of calcium from spores exposed to 0.1, 1 and 2% w/v of di-sodium phosphate was determined before and after incubation of spores at 110 °C for 5 min. After heat treatment, 1 mL of spore suspension was filtered through a 0.45 µm syringe filter to eliminate intact spores. The filtrate was then transferred to Pyrex glass tubes (Corning, USA) and were subjected to dry ashing at 500 °C overnight. The ash was then dissolved in 1 mL of 6 M ion free sulphuric acid and 5 mL of 5000 ppm lanthanum chloride solution was added (Udoh, 2000). The solution was then warmed carefully to boiling and contents were transferred quantitatively to a 10 mL volumetric flask and made to volume using deionized distilled water. An Xplor AA atomic absorption spectroscope (GBC Scientific Equipment, Australia) was used in all analyses. A nitrous oxide- acetylene flame was used to minimize chemical interference by phosphate. The amount of calcium released was determined through the differences in content of control and heat-treated samples. Total calcium content of spores after autoclaving the spores at 121 °C for 15 min was also determined through atomic absorption spectroscopy. Values obtained were used to calculate the percentage release of calcium from spores. All data in the study represents the average of triplicates.

4.3. Results and Discussion

4.3.1. Heat resistance of spores

Spore suspensions obtained by plating of cultures of each strain for 5 days were heated in distilled water at 110 ºC and plated to determine viability. Heat survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 were generated for each strain (Figure 4.1). Regression lines were established for each strain studied and the D_{110} values were obtained as the negative inverse of the slope of the regression line. D_{110} value of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water were between 5.7 and 9.0 minutes (Table 4.1).

The D_{110} value of the reference strain ATCC 12980 was the highest whereas the dairy strain P3 exhibited the lowest D_{110} value among all the strains that were studied. Variation in the spore heat resistance among different strains of *G. stearothermophilus* has been reported to be caused by strain variability and cultivation media conditions during sporulation (Wells-Bennik et al., 2019). In this study, the spore cultivation conditions were kept uniform for all strains, which suggests that the observed variability in the spore heat resistance is due to strain variability.

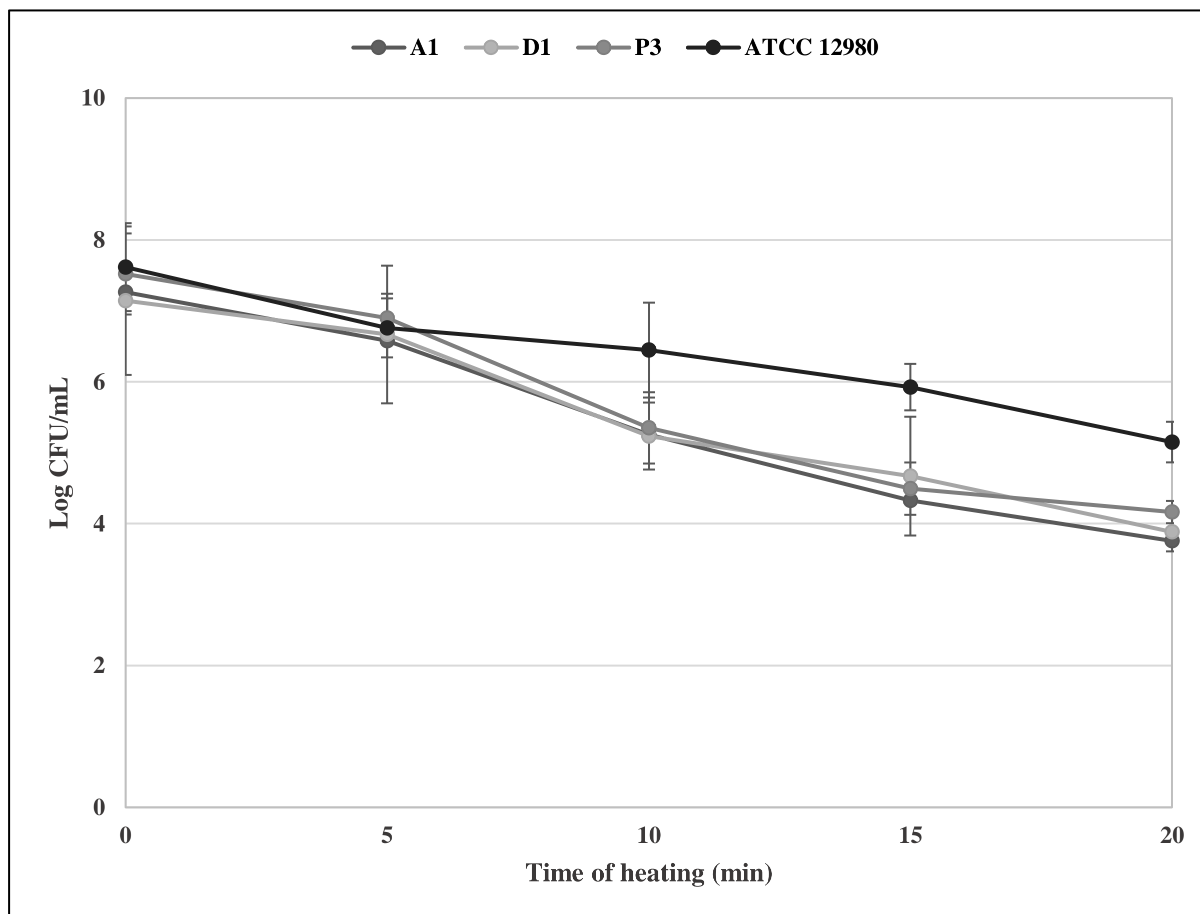


Figure 4.1. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ℃ in distilled water. Error bars represent standard deviation of triplicates.

4.3.2. DPA content of spores

DPA content per spore is not constant and varies depending on the size of the spore with a larger spore containing a higher DPA content and vice versa (Huang et al., 2007). In this study, DPA concentration per spore was calculated assuming that all spores are uniform in size. My results indicate that spores from dairy and non-dairy environments exhibit a considerable difference in their DPA content (Table. 4.1).

DPA content per spore was the highest among spores of ATCC 12980. Spores of the dairy strains exhibited lower DPA content per spore in comparison with the reference strain. On comparing the D_{110} values of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 suspended in distilled

water with their respective DPA content per spore, we suggest that the D value of the spores may be associated to their respective DPA content. The findings in this study are in accordance with previous findings, discussing the role of DPA in lowering the core water content and protecting the spore DNA against heat damage thereby influencing their wet heat resistance (Magge et al., 2008; Setlow et al., 2006)

Table 4.1. Comparison of the D_{110} value and the dipicolinic acid content of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980

Strain	DPA content per spore (pg)	Estimated D_{110} value in distilled water (min)
A1	0.25 ± 0.07	5.8 ± 1.9
D1	0.30 ± 0.1	6.7 ± 2.7
P3	0.27 ± 0.08	5.7 ± 1.5
ATCC 12980	1.6 ± 0.05	9.0 ± 2.1

4.3.3. Effect of pH of the heating medium on the heat resistance of spores

Survival curves of spores of *G. stearothermophilus* A1, D1, P3, ATCC 12980 in citrate-phosphate buffer at pH 4, 7 and 8 are shown in Figure 4.2. D_{110} values of spores of A1, D1, P3 and ATCC 12980 at pH 4, 7 and 8 are shown in supplementary Table S.4.1. Maximum heat resistance of spores of A1, D1, P3 and ATCC 12980 was obtained at pH 7. Heat resistance was lowered at pH 8 and at pH 4 in comparison with the heat resistance at pH 7 for all the strains.

In this study, the D_{110} values of spores in citrate-phosphate buffer were lower than the values obtained in distilled water. These findings are in agreement with Cook & Gilbert (1968), who found that spores of *B. stearothermophilus* (now *G. stearothermophilus*) were more heat resistant when heated in water than in citrate-phosphate buffer.

López et al. (1996a) found that the heat resistance of spores of *B. stearothermophilus* was at a maximum when the pH of the heating medium was 7 and decreased as the pH of the heating medium was altered. Variation among heat resistance of spores is known to be affected by the pH, the strain and the composition of the heating medium (Berendsen et al., 2015; López et al., 1996a). Demineralization of spores leading to the lowering of the heat resistance of spores under acidic pH conditions has also been reported previously (Alderton et al., 1976). To confirm whether the phosphate present in the heating medium impacts the heat resistance of spores, the heat resistance of spores in 3-(N-morpholino)propanesulfonic acid (MOPS), a non- phosphate based buffer, was determined. We observed that the heat resistance of spores at pH 7.0 was significantly lower ($p \leq 0.05$) in the citrate-phosphate buffer in comparison with the MOPS buffer (see Appendix section 7.4).

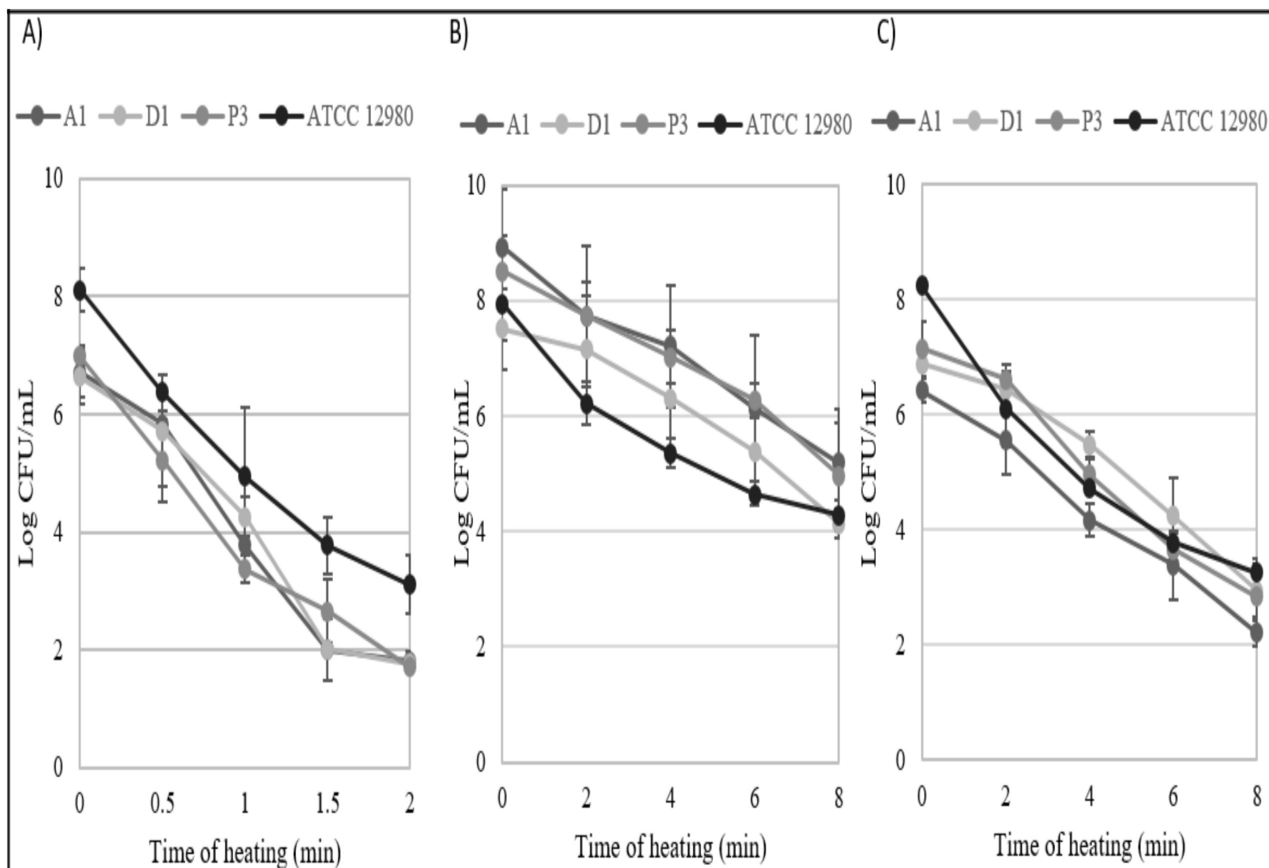


Figure 4.2. Survival curves of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in citrate-phosphate buffer at pH 4 (A), pH 7 (B) and pH 8 (C). Error bars represent standard deviation of triplicates

4.3.4 Effect of phosphate in lowering the heat resistance of spores

Based on the result from the previous experiment, the heat resistance of spores suspended in citrate phosphate buffer was lower in comparison with distilled water. The lowering of the heat resistance of spores in the presence of phosphate in the heating medium has been reported previously (Amaha & Ordal, 1956; El-bisi & Ordal, 1956; Williams & Hennessee, 2006). El-bisi and Ordal (1956) hypothesised that this was due to the carryover of phosphate into the plating medium that influenced results. Results from preliminary experiments conducted in this laboratory suggest that the reduction in the heat resistance of spores is not caused by the carryover of phosphate into the plating medium (data not shown). Amaha and Ordal (1957), offered an

alternative explanation that phosphate present in the heating medium acts as a chelating agent for divalent cations present within the spore thereby lowering the heat resistance of spores through enhancing the rate at which divalent cations are removed from the heated spores. It was of interest, therefore, to determine whether phosphate added to the heating medium would affect the heat resistance of spores. To validate this hypothesis, the heat resistance of spores in the presence of varying concentrations of di-sodium phosphate in the heating medium was determined. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 suspended in distilled water containing varying concentration of di-sodium phosphate (0.1, 1 and 2% w/v) are shown in Figure 4.3. D_{110} values of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.1, 1 and 2% w/v di-sodium phosphate are shown in supplementary Table S.4.2.

The heat resistance of spores was maximum when suspended in distilled water and decreased as the

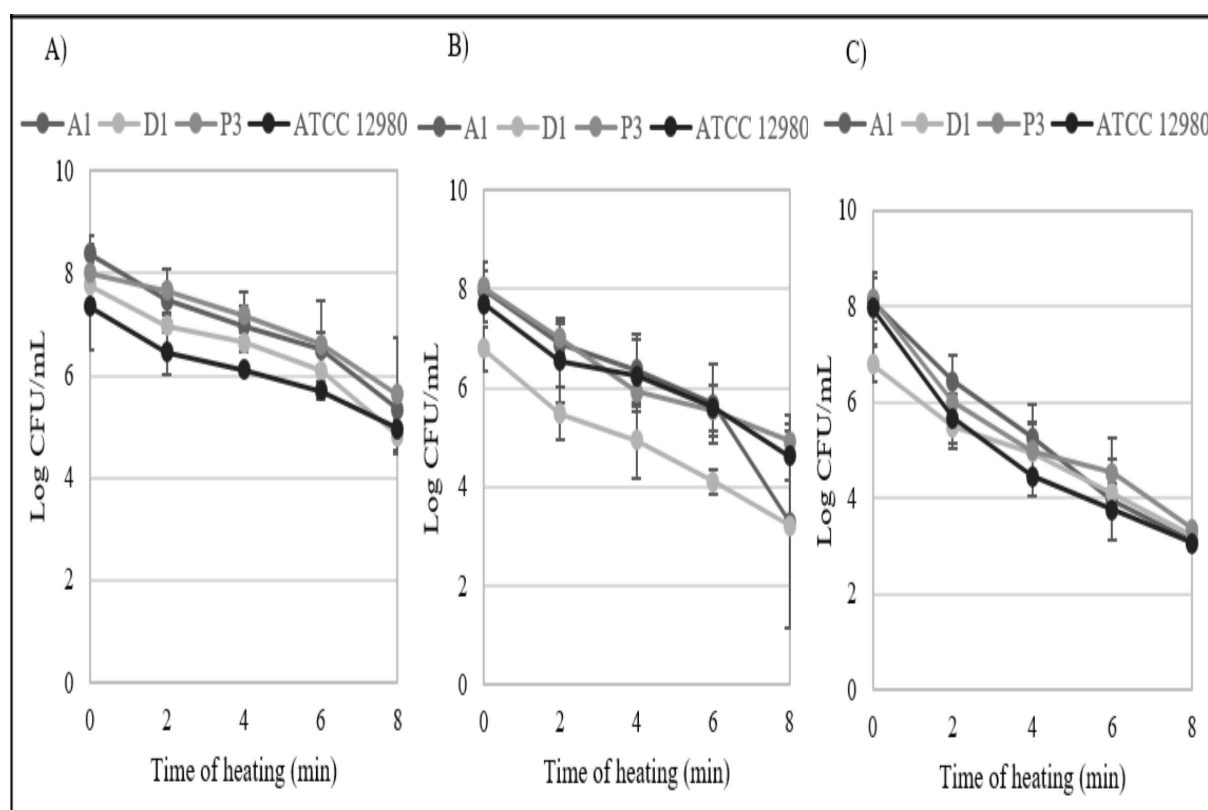


Figure 4.3. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ºC in distilled water containing 0.1% (A), 1% (B) and 2% (C) (w/v) of di-sodium phosphate. Error bars represent standard deviation of triplicates.

phosphate concentration in the heating medium increased. 2% di-sodium phosphate was highly

effective in reducing the heat resistance of spores whereas 0.1% of di-sodium phosphate had the least effect. Reduction in the heat resistance varied between strains. Amaha & Ordal (1957), suggested that the rate of loss of cations from within spores should be greater in the presence of more powerful or more specific chelating agents. To determine whether the heat resistance of spores was altered in the presence of a strong chelating agent, the heat resistance of spores suspended in 0.05% w/v of EDTA was determined (Figure 4.4). D_{110} values of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in the presence of 0.05% w/v Na-EDTA are shown in supplementary Table S.4.3. The heat resistance of spores in the presence of 0.05% w/v of EDTA in the heating medium (Figure 4.4) was lower than the heat resistance in distilled water (Figure 3.1). This finding is in accordance with Santos & Zarzo (1997), who concluded that EDTA when present in the heating medium lowers the heat resistance of spores of *C. sporogenes*.

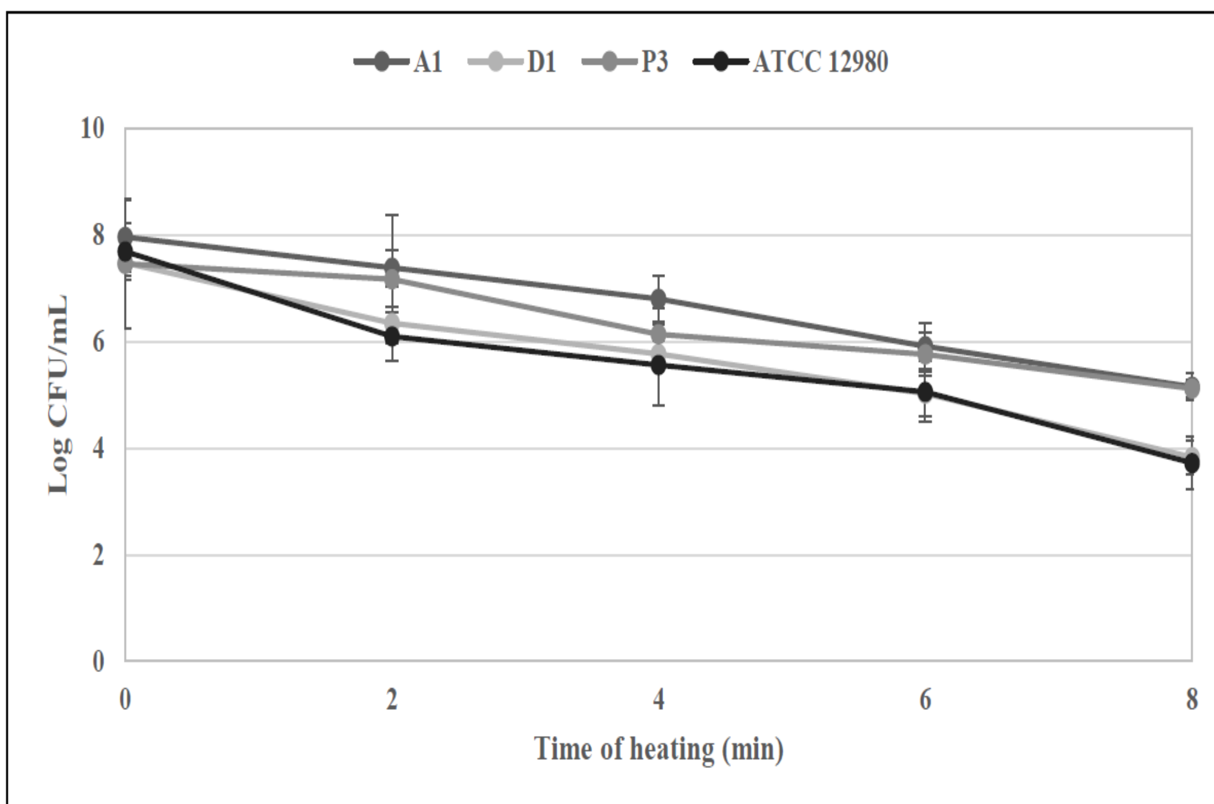


Figure 4.4. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 ℃ in distilled water containing 0.05% w/v Na-EDTA. Error bars represent standard deviation of triplicates.

4.3.5. Effect of phosphate on the release of calcium

To validate my previously mentioned hypotheses, the release of calcium from within the spore in the presence of 0.1, 1 and 2% w/v of di-sodium phosphate through atomic absorption spectroscopy was estimated. The percentage release of calcium following the heat treatment of spores at 110 °C in the presence of 0.1, 1 and 2% w/v of di-sodium phosphate in the heating medium is summarised in Table 4.2. The amounts released were calculated as the difference in concentration between the control and heat-treated samples. The release of calcium from spores increased with increasing concentration of di-sodium phosphate in the heating medium. Maximum release of calcium was obtained in the presence of 2% w/v of di-sodium phosphate whereas the minimum release of calcium was obtained in the absence of di-sodium phosphate in the heating medium.

Dairy strains exhibited a higher percentage release of calcium than the reference strain irrespective of the concentration of di-sodium phosphate in the heating medium. The reason for this is unknown and needs further investigation. The release of calcium is closely associated with the loss of spores heat resistance (Vries, 2004). Results of this study primarily point toward the effect of phosphate in accelerating the release of calcium, thus contributing towards the lowering of the heat resistance of spores as observed previously in my heat resistance studies.

Table 4.2. Effect of phosphate on the percentage release of calcium from spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980

Strain	Percentage release of Ca[a] in distilled water containing			
	Concentration of phosphate (% w/v)			
	0	0.1	1	2
A1	11 ± 0.9	30 ± 2.1	31 ± 1.4	36 ± 3.6
D1	24 ± 1.6	24 ± 3.3	30 ± 5.7	56 ± 2.6
P3	10 ± 1.9	12 ± 3.6	16 ± 1.5	44 ± 1.7
ATCC 12980	4.2 ± 0.4	8.7 ± 0.9	9.9 ± 0.8	11 ± 1.8

[a]Expressed as percent of average Ca concentration of 10^8 inactivated spores (supplementary Table S.4.4)

Incubation time for all samples was 5 minutes at 110 ºC

All values are based on average of triplicates ± standard deviation

4.4. Conclusion

In conclusion, this study indicated that phosphate lowers the heat resistance of spores of *G. stearothermophilus* by accelerating the release of calcium from within the spore. The reason behind the variation in the percentage release of calcium between the dairy isolates and the reference strains is unknown and needs further investigation. In addition, the D values of the spores were found to be associated with their respective DPA content. The outcome of this study can be beneficial in the design of thermal processing steps involved in the manufacture of commercially sterile dairy products. The cleaning in place (CIP) method of cleaning currently employed in the dairy industry to combat biofilms and spores involves the use of acid or alkali based detergents (Thomas & Sathian, 2014). The increased heat sensitivity of spores in the presence of phosphate as shown in this study suggest the use of a phosphate-based cleaning agent in the future.

4.5. Supplementary information

Table S.4.1. Heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in citrate phosphate buffer at pH 4, 7 and 8

Strain	D value (min) in citrate phosphate buffer at		
	pH 4	pH 7	pH 8
A1	0.37 ± 0.01	2.4 ± 0.48	1.9 ± 0.02
D1	0.38 ± 0.07	2.4 ± 0.29	2.0 ± 0.34
P3	0.38 ± 0.02	2.2 ± 0.2	1.7 ± 0.13
ATCC 12980	0.4 ± 0.02	2.2 ± 0.09	1.6 ± 0.14

All values are average of triplicates ± standard deviation

Table S.4.2. Heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.1, 1 and 2% w/v di-sodium phosphate

Strain	D value (min) in distilled water containing Concentration of phosphate (% w/v)		
	0.1	1	2
A1	2.9 ± 0.33	2.1 ± 0.97	1.6 ± 0.07
D1	3.0 ± 0.48	2.7 ± 0.66	2.4 ± 0.57
P3	3.7 ± 1.1	2.6 ± 0.1	2.0 ± 0.11
ATCC 12980	3.7 ± 0.75	2.8 ± 0.04	1.8 ± 0.35

All values are average of triplicates ± standard deviation

Table S.4.3. Heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.05% w/v Na-EDTA

Strain	D value (min)
A1	2.9 ± 0.47
D1	2.3 ± 0.29
P3	3.9 ± 1.7
ATCC 12980	2.3 ± 0.49

Table S.4.4. Total calcium content of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980

Strain	Total calcium content (ppm)
A1	1.8 ± 0.5
D1	1.0 ± 0.34
P3	1.9 ± 0.03
ATCC 12980	6.4 ± 1.0

All values are average of triplicates ± standard deviation

4.6. References

Alderton, G., Ito, K. A., & Chen, J. K. (1976). Chemical manipulation of the heat resistance of *Clostridium botulinum* spores. *Applied and Environmental Microbiology*, 31(4), 492–498.

Amaha, M., & Ordal, Z. J. (1956). The effect of certain sporulation conditions on the thermal death rate of *Bacillus coagulans* var. thermoacidurans. *Journal of Bacteriology*, 71(1), 1–9.

Amaha, M., & Ordal, Z. J. (1957). Effect of divalent cations in the sporulation medium on the thermal death rate of *Bacillus coagulans* var. thermoacidurans. *Journal of Bacteriology*, 74(5), 596–604.

Berendsen, E. M., Zwietering, M. H., Kuipers, O. P., & Wells-Bennik, M. H. J. (2015). Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. *Food Microbiology*, 45(2), 18–25.

Bulgarelli, M. A., & Shelef, L. A. (1985). Effect of Ethylenediaminetetraacetic Acid (EDTA) on growth from spores of *Bacillus cereus*. *Journal of Food Science*, 50(661), 1–4.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophillic bacilli from a milk powder processing plant. *Journal of Applied Microbiology*, 116(2), 350–359.

Burgess, Sara A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. In *International Journal of Food Microbiology*, 144(2), 215–225.

Cempírková, R. (2007). Contamination of cow's raw milk by psychrotrophic and mesophilic microflora in relation to selected factors. *Czech Journal of Animal Science*, 52(11), 387–393.

Cook, A. M., & Gilbert, R. J. (1968). Factors affecting the heat resistance of *Bacillus stearothermophilus* spores. *International Journal of Food Science & Technology*, 3(3),

295–302.

Cortezzo, D. E., & Setlow, P. (2005). Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *Journal of Applied Microbiology*, 98(3), 606–617.

El-bisi, H. M., & Ordal, Z. J. (1956). The effect of sporulation temperature on the thermal resistance of *Bacillus coagulans* var. Thermoacidurans. *Journal of Bacteriology*, 71(1), 10–16.

Fernandez, P. S., Ocio, M. J., Sanchez, T., & Martinez, A. (1994). Thermal resistance of *Bacillus stearothermophilus* spores heated in acidified mushroom extract. *Journal of Food Protection*, 57(1), 37–41.

Gonzalez, I., Lopez, M., Mazas, M., Gonzalez, J., & Bernardo, A. (1995). The effect of recovery conditions on the apparent heat resistance of *Bacillus stearothermophilus* spores. *Journal of Applied Bacteriology*, 78(5), 548–554.

Huang, S. S., Chen, D., Pelczar, P. L., Vepachedu, V. R., Setlow, P., & Li, Y. Q. (2007). Levels of Ca2+-dipicolinic acid in individual *Bacillus* spores determined using microfluidic Raman tweezers. *Journal of Bacteriology*, 189(13), 4681–4687.

Jamroskovic, J., Chromikova, Z., List, C., Bartova, B., Barak, I., & Bernier-Latmani, R. (2016). Variability in DPA and calcium content in the spores of *Clostridium* species. *Frontiers in Microbiology*, *7*(11), 1–11.

López, M., González, I., Condón, S., & Bernardo, A. (1996). Effect of pH heating medium on the thermal resistance of *Bacillus stearothermophilus* spores. *International Journal of Food Microbiology*, 28(3), 405–410.

López, M., Mazas, M., González, I., González, J., & Bernardo, A. (1996). Thermal resistance

of *Bacillus stearothermophilus* spores in different heating systems containing some approved food additives. *Letters in Applied Microbiology*, 23(3), 187–191.

Magge, A., Granger, A. C., Wahome, P. G., Setlow, B., Vepachedu, V. R., Loshon, C. A., Peng, L., Chen, D., Li, Y.-Q., & Setlow, P. (2008). Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*. *Journal of Bacteriology*, 190(14), 4798–4807.

McIlvaine T.C. (1921). A buffer solution for colorimetric comparison.*Journal of biological chemsitry*. 49(1), 183-186.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews : MMBR*, 64(3), 548–572.

Paidhungat, M., Setlow, B., & Driks, A. (2000). Characterization of spores of *Bacillus subtilis* which lack Dipicolinic Acid characterization of spores of *Bacillus subtilis* which lack Dipicolinic Acid. *Journal of Bacteriology*, 182(19), 5505–5512.

Rotman, Y., & Fields, M. L. (1969). A study on the sporulation of rough and smooth variants of *Bacillus stearothermophilus*. *Journal of Food Science*, 34(4), 346–349.

Sadiq, F. A., Li, Y., Liu, T. J., Flint, S., Zhang, G., Yuan, L., Pei, Z., & He, G. Q. (2016). The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *International Journal of Food Microbiology*, 238(12), 193–201.

Santos, M., & Zarzo, J. (1997). The effect of ethylenediaminetetraacetic acid on heat resistance and recovery of Clostridium Sporogenes PA 3679 spores treated in HTST conditions. *International Journal of Food Microbiology*, 34(3), 293–305.

Scheldeman, P., Herman, L., Foster, S., & Heyndrickx, M. (2006). *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology*, 101(3), 542–555.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, 60(2), 109–117.

Seale, R. B., Bremer, P. J., Flint, S. H., & McQuillan, A. J. (2010). Characterization of spore surfaces from a *Geobacillus* sp. isolate by pH dependence of surface charge and infrared spectra. *Journal of Applied Microbiology*, 109(4), 1339–1348.

Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., & Setlow, P. (2006). Role of dipicolinic acid in resistance and stability of spores of *Bacillus subtilis* with or without DNA-protective α/β-type small acid-soluble proteins. *Journal of Bacteriology*, 188(11), 3740–3747.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, 101(3), 514–525.

Setlow, P. (1995). Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annual Review of Microbiology*, 49(1), 29–54.

Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, 6(6), 550–556.

Sonenshein, A. L., Hoch, J. A., & Losick, R. (2002). *Bacillus subtilis* and its closest relatives. From genes to cells. ASM press, USA.

Thomas, A., & Sathian, C. T. (2014). Cleaning-In-Place (CIP) system in dairy plant. *Journal of Environmental Science, Toxicology and Food Technology*, 8(6), 41–44.

Udoh, A. P. (2000). Atomic absorption spectrometric determination of calcium and other metallic elements in some animal protein sources. *Talanta*, 52(4), 749–754.

Vries, Y. P. De. (2004). Minireview the role of Calcium in bacterial spore germination. *Microbes and Environments*, 19(3), 199–202.

Wells-Bennik, M. H. J., Janssen, P. W. M., Klaus, V., Yang, C., Zwietering, M. H., & den Besten, H. M. W. (2019). Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. *International Journal of Food Microbiology*, 291(2), 161–172.

Williams, O. B., & Hennessee, A. D. (2006). Studies on heat resistance.VII. The effect of phosphate on the apparent heat resistance of spores of *Bacillus stearothermophilus*. *Journal of Food Science*, 21(1), 112–116.

DRC 16

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Murali Kumar
Name/title of Primary Supervisor:	Steve Flint
Name of Research Output and full reference:	
Murali Kumar, Steve Flint, Jon Palmer, Paul Pleger. A comparison of the spore heat resistance of dairy isolates of Geobacillus stearothermophilus obtained using a CDC biofilm reactor and a sporulating medium	
In which Chapter is the Manuscript /Published work:	4
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
This paper was the work of the candidate who researched and wrote the paper under the guidance of the four co-authors	
For manuscripts intended for publication please indicate target journal:	
International Dairy Journal	
Candidate's Signature:	Murali Kumar Digitally signed by Murali Kumar Date: 2020.12.16 13:58:20 +13'00'
Date:	16 Dec 2020
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2020.12.16 17:31:31 +13'00'
Date:	16th December 2020

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019

Preface to Chapter 5

In chapter 4, the role of phosphate on the heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 was studied. This study determined the role of phosphate as a chelating agent causing excess release of calcium from the spores thereby lowering their heat resistance. The heat resistance of spores of A1, D1, P3 were significantly lower ($p \leq 0.05$) than ATCC 12980. The results from this study were primarily based on spores produced by a sporulation medium which is not a true representation of a dairy manufacturing environment.

In chapter 5, the heat resistance of spores (D values) of A1, D1, P3 and ATCC12980 obtained from a milk biofilm under continuous flow condition using a CDC reactor was determined and compared with previously obtained D values in chapter 3.

Chapter 5

5 Comparison of the heat resistance of spores obtained from the CDC reactor and a sporulation medium

Abstract

In this study, the heat resistance of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 obtained using the milk biofilm system under continuous flow was compared with previously reported values obtained from spores prepared in a sporulation medium. The average decimal reduction times of A1, D1, P3 and ATCC 12980 were 4.2, 4.9, 4.1 and 7.9 min respectively and were not significantly different ($p \leq 0.05$) to the heat resistance of spores obtained by a sporulating medium. The total calcium content of spores of A1, D1, P3 and ATCC 12980 were 1.9, 2.0, 2.5 and 5.1 ppm respectively. The Dipicolinic Acid (DPA) content of spores of A1, D1, P3 and ATCC 12980 was 0.19, 0.26, 0.33, 1.38 pg per spore respectively. A common method to determine spore heat resistance of dairy isolates involves spores obtained from a sporulation medium. This may not a be a true representation of the heat resistance of spores from their natural environment (e.g. dairy manufacturing plant). The findings presented in this study demonstrate that spore heat resistance is not influenced by the sporulation conditions and that strain variation has the main effect on heat resistance.

5.1. Introduction

G. stearothermophilus (formerly *B. stearothermophilus*) is a major contaminant in the food industry, particularly in milk powder manufacture (Scott et al., 2007). The bacterium is capable of forming biofilms harbouring heat resistant spores on stainless steel surfaces within the evaporator (Burgess et al., 2010; Murphy et al., 2007; Scott et al., 2007).

Previous heat resistance studies have involved different spore test parameters yielding significantly variable outcomes (Kent et al., 2016). These studies used laboratory media to produce spores which are quite different from the true biofilm environment present on processing surfaces in the dairy industry (Sadiq et al., 2016; Stoeckel et al., 2016; Zhao et al., 2013). Wells-Bennik et al. (2019), studied the heat resistance of spores obtained from a milk-biofilm system grown under batch conditions which again is not a true reflection of the conditions in an industrial system. The present study aimed to approximate an industrial system determining the heat resistance of spores more closely of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 isolated from a continuous flow milk-biofilm system using the CDC biofilm reactor. The results were then compared with decimal reduction time (D_{110} value) of spores produced on a sporulation medium (chapter 4). The total calcium and DPA content of spores obtained from the CDC biofilm reactor was also compared with previously obtained values using sporulation medium.

5.2. Materials and Methods

5.2.1. Microorganisms and media

G. stearothermophilus A1, D1 and P3 were previously isolated from the evaporator sections of a New Zealand dairy powder manufacturing facility (Burgess et al., 2014). *G. stearothermophilus* ATCC 12980 was used as a reference strain in this study. An overnight grown culture of A1, D1, P3 and ATCC 12980 was prepared by growing these strains until stationary phase in Trypticase Soy

Broth (TSB; BD Biosciences USA) at 55 °C for 12 h, washed twice and re suspended in phosphate buffer saline (PBS; Oxoid, UK).

5.2.2. Design and assembly of the biofilm reactor

The CDC biofilm reactor (CBR 90) (Biosurface Technologies, USA) was used for these experiments (Fig S.5.1). Stainless steel coupons (dia 10 mm, 3 mm thick, 304 grade with a 2B finish) were used. Stainless-steel coupons were soaked for 12 h in 99.5% acetone (Merck, USA), rinsed with distilled water, suspended in 5% (w/v) Pyroneg solution (Thermofisher scientific, USA) and cleaned using an ultrasonic cleaner (Agar scientific, UK). The coupons were sterilized by autoclaving at 121 °C for 15 min. The reactor was then assembled and autoclaved before use.

5.2.3 Inoculation of the biofilm reactor

Sterile reconstituted skim milk (RSM) was prepared by dissolving 10% (w/v) instant skim milk powder (Fonterra, New Zealand) in water followed by UHT treatment at 142 °C for 8 s. RSM (20 L) was inoculated with 200 mL of the 12 h culture at the start of the experiment. The milk container was kept at 4°C throughout the experiment. The inoculated RSM was delivered at a constant flow rate of 25mL min^{-1} which was selected to ensure that the residence time of milk within the reactor is 14 min, lower than the doubling time of all the bacterial strains so as to ensure growth only in the biofilm and not in the planktonic phase (Table S.5.1).

5.2.4 Operation of the CDC reactor

The incubation temperature and baffle speed were maintained at 55 °C and 100 rpm respectively with a digital hot plate stirrer (Biosurface Technologies, USA). Coupon rods were aseptically removed at 16 h.

5.2.5 Enumeration of spores

Spores were enumerated as described previously (Hayrapetyan et al., 2015). The coupons were washed by dipping in sterile distilled water and the placed in sterile glass vials containing 5 mL of

sterile distilled water and 5 g glass beads (D =100 µm, Sigma Aldrich, USA). The glass vials were then mixed by vortex for 1 min at 100 rpm to dislodge the cells from the coupon and to obtain individual cells. Samples were subjected to a heating step of 90 °C for 30 min to obtain a cell free spore suspension prior to plating on tryptic soy agar plates (TSA; BD diagnostics systems, Germany). Heat treated spore suspensions were stored at 4 °C until further use.

5.2.6 Heat treatment of spores

Spore suspensions were centrifugally washed three times (2500 xg, 4 min, 23 °C) before every heat treatment. The spore suspension (50 µL) was transferred into a soda glass capillary tube (Globe Scientific, USA). The ends of the capillary tube were sealed in the flame and heat treatment was carried out at 110 °C in a thermostat controlled oil bath. After heating for a selected time interval, the tubes were immediately submerged in cold water (< 4 °C) for 30 s. The tubes were then washed in 70% ethanol and the ends of the tubes were aseptically cut and contents were serial diluted using 0.1% buffered peptone water. Serial diluted solutions were then plated on TSA plates and incubated at 55 °C for 24 h. D_{110} values were calculated based on the negative inverse of the slope of the regression line plotted with values of the survival curves. All heating experiments were carried out involving four individual spore suspensions obtained from two separate reactor runs.

5.2.7 Estimation of calcium content of spores

Total calcium content of spores was determined as described previously in chapter 4. Spore suspensions containing 10^8 spores/ mL were autoclaved at 121 °C for 15 min to release the spore calcium and an atomic absorption spectroscope (Xplor AA; GBC Scientific Equipment, Australia) was used to determine the total calcium content of spores. A nitrous oxide acetylene flame was used to minimise chemical interference by phosphate. All data in this analysis represents average of four individual spore suspensions isolated from two separate reactor runs.

5.2.8 Estimation of DPA content of spores

DPA content of spores was determined as discussed previously in chapter 4. The terbium complexation method was used to determine the total DPA content of spores (Jamroskovic et al., 2016). Photo luminescence was measured using a spectrofluorometer (Perkin Elmer LS55, USA) with the following settings – excitation wavelength: 276 nm, emission wavelength: 546 nm, slit width: 15 nm and scan speed: 500 nm/min. A sample containing 30 µM terbium chloride ($TbCl_3$) was used as a blank. All data in this analysis represents average of four individual spore suspensions isolated from two separate reactor runs.

5.2.9. Statistical analysis

Statistically significant differences were determined by student's *t* test ($p \leq 0.05$; Minitab 19)

5.3. Results and Discussion

5.3.1. Heat resistance of spores

D_{110} of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 values (Table 5.1) were calculated based on the survival curves (Fig S.5.2). The D_{110} value of the reference strain ATCC 12980 was the highest whereas the dairy strain P3 had the lowest D_{110} value (Table. 5.1). Variation in the spore heat resistance of strains of *G. stearothermophilus* has been reported previously (Wells-Bennik et al., 2019). The D_{110} values of spores obtained from the CDC reactor and the previously reported values obtained by sporulation medium were not statistically different ($p \leq 0.05$) (Table 5.1).

Table 5.1. Comparison of D_{110} values of spores obtained from sporulation agar and from a CDC reactor

Strain	D_{110} value of spores from sporulation agar (min)	D_{110} value of spores from CDC reactor (min)
A1	5.8 ± 1.9	4.2 ± 1.0
D1	6.7 ± 2.7	4.9 ± 0.8
P3	5.7 ± 1.5	4.1 ± 0.5
ATCC 12980	9.0 ± 2.1	7.9 ± 1.2

5.3.2 Calcium content of spores

The total calcium content of the spores obtained from the CDC reactor was not significantly different from the previously reported values obtained by the sporulation medium ($p \leq 0.05$) (Table 5.2). The reference strain ATCC 12980 had the highest calcium content in comparison with the dairy isolates. This is in accordance with the findings from my previous study (Chapter 4).

Table 5.2. Comparison of the total calcium content of spores obtained from sporulation agar and from a CDC reactor

Strain	Total calcium content of spores obtained from sporulation agar (ppm)	Total calcium content of spores obtained from CDC reactor (ppm)
A1	1.8 ± 0.5	1.9 ± 0.18
D1	1.0 ± 0.34	2.0 ± 0.52
P3	1.9 ± 0.03	2.5 ± 0.74
ATCC 12980	6.4 ± 1.0	5.1 ± 0.44

5.3.3 DPA content of spores

The DPA content per spore obtained from the CDC reactor was not significantly different to the previously reported values obtained by the sporulation medium ($p \leq 0.05$) (Table 5.3). The heat resistance of spores obtained from the CDC reactor is not significantly different ($p \leq 0.05$) to previously obtained values by sporulation medium. This is important information considering that heat resistance studies commonly use spores obtained using a sporulation medium (Jamroskovic et al., 2016; Mazas, Lopez, Martinez, Bernardo, & Martin, 1999; Sadiq et al., 2016). Strain variation among the heat resistance of spores of *G. stearothermophilus* in the present work reflects that reported previously (Jamroskovic et al., 2016). Dairy strains exhibited a lower calcium and DPA content per spore in comparison with the reference strain. There appears to be a correlation between the Ca/DPA content and the heat resistance with the reference strain having the highest Ca/DPA content also having the highest D_{110} value. Wells-Bennik et al. (2019), demonstrated the effect of culturing conditions on the heat resistance of spores of *G. stearothermophilus* and concluded that the highest spore heat resistance was noted

among spores produced on a sporulation medium containing ions of calcium, potassium, magnesium and manganese. This is contrary to the findings from this study, where the effect of culturing conditions did not have a significant influence ($p \leq 0.05$) on the heat resistance of spores. I predict that the observed difference may be due to strain variability and spore culturing conditions between the two studies (see supplementary table S.5.2). Previous studies have discussed the role of genes that encode proteins in sporulation that may cause difference in spore heat resistance (Berendsen et al., 2016; Butler et al., 2017). A comparative genome analysis involving these three dairy isolates may help in identifying the role of genes on the spore heat resistance.

Table 5.3. Comparison of the DPA content per spore obtained from sporulation agar and from a CDC reactor

Strain	DPA content per spore obtained from sporulation agar (pg)	DPA content per spore obtained from CDC reactor (pg)
A1	0.25 ± 0.07	0.19 ± 0.01
D1	0.30 ± 0.1	0.26 ± 0.06
P3	0.27 ± 0.08	0.33 ± 0.05
ATCC 12980	1.6 ± 0.05	1.39 ± 0.35

5.4. Conclusion

This study showed that there is no significant difference ($p \leq 0.05$) between the heat resistance of spores of *G. stearothermophillus* A1, D1, P3 and ATCC 12980 obtained using milk in a CDC reactor and a sporulation medium. The data presented in the study provides insight into the heat inactivation

of *G. stearothermophilus* spores which can be used in the design of thermal processing steps during the manufacture of commercially sterile products.

5.5. Supplementary information

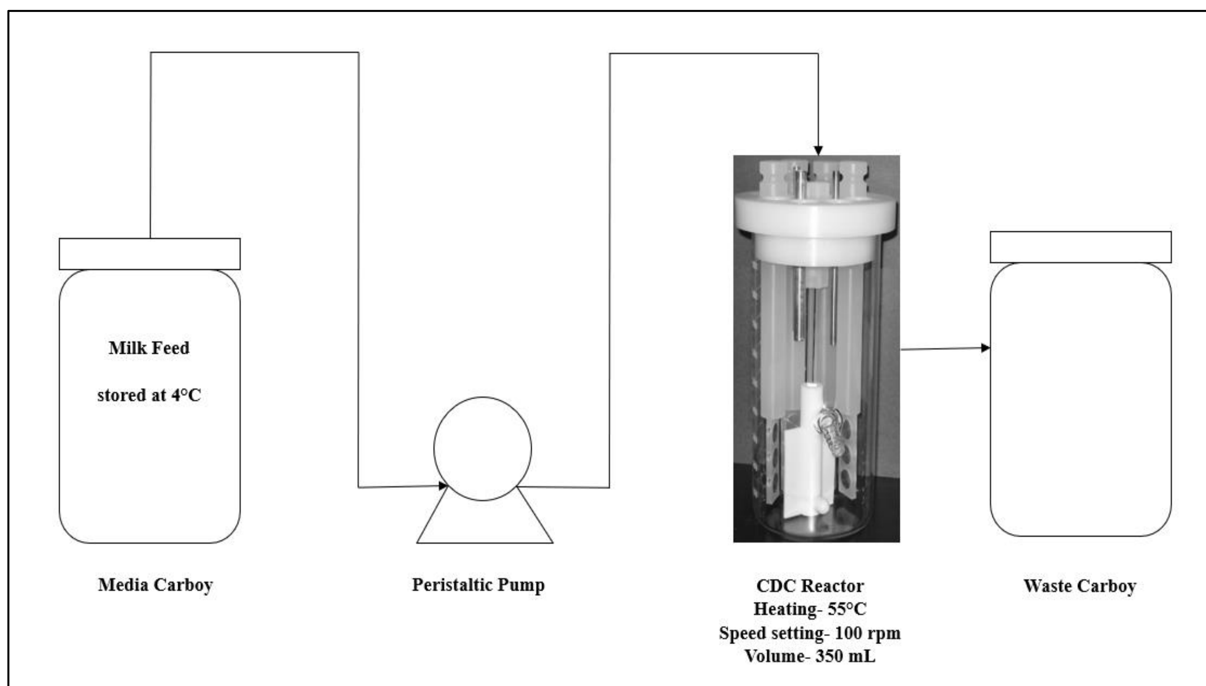


Fig S.5.1. Schematic diagram of the CDC reactor setup

Table S.5.1. Doubling time of *G. stearothermophillus* A1, D1, P3 and ATCC 12980 in 10% (w/v) UHT skim milk at 55 °C

Strain	Doubling time (min)
A1	25.85
D1	26.33
P3	28.96
ATCC 12980	31.11

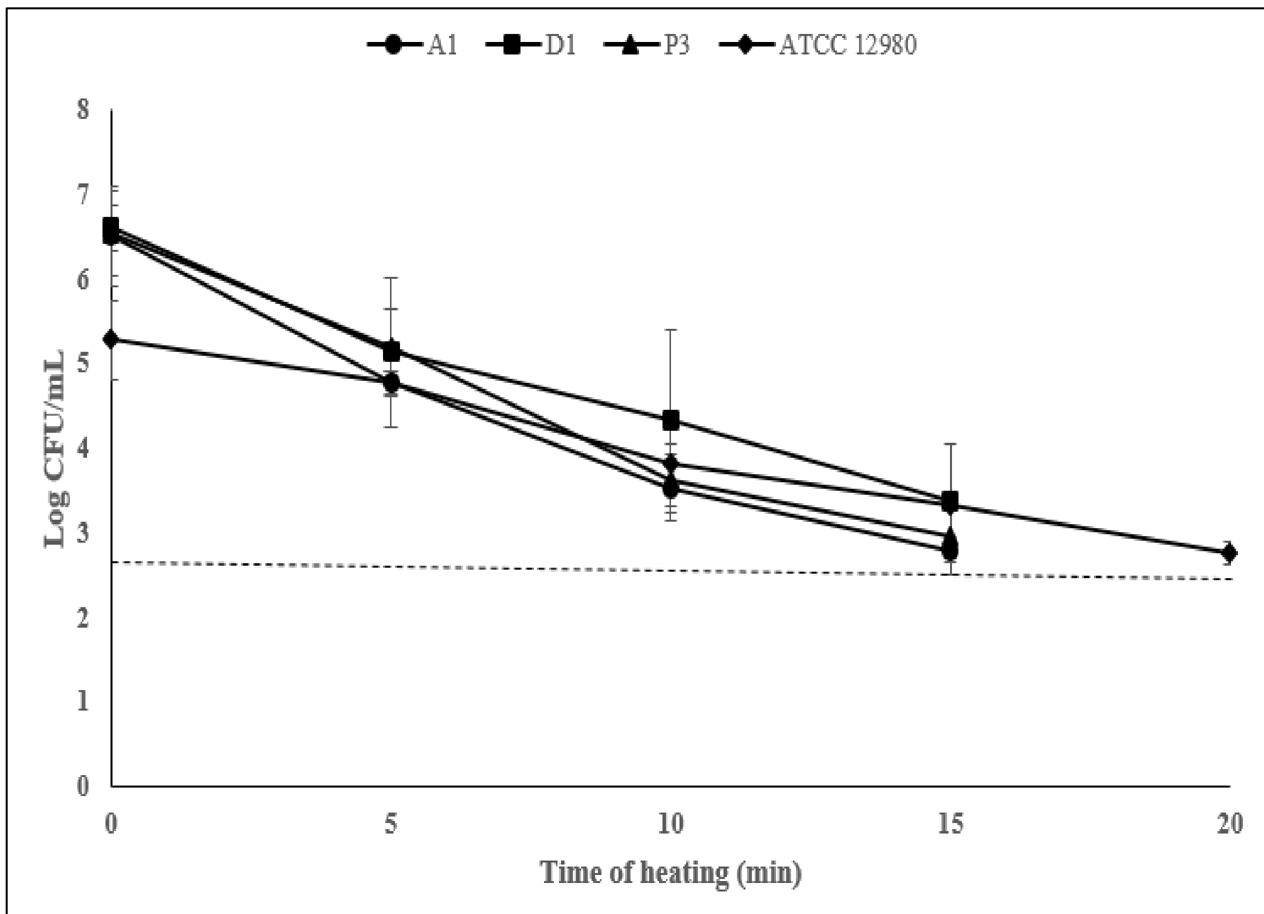


Fig S.5.2. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 °C in distilled water. Error bars represent standard deviation of four individual spore suspensions obtained from two separate reactor runs. Dotted line represents the minimum detection limit of 2.7 Log CFU mL^{-1}

Table S.5.2. Sporulation media composition used in Wells-Bennik et al. (2019) & in this study

Author	Sporulation media composition
Wells-Bennik et al. 2019	Tryptone Soya Agar (Oxoid) supplemented with 0.13 mM $MnSO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 13 mM KCl
This study	Tryptone Soy Agar (BD Scientific, USA) supplemented with (/L) $MgSO_4.7H_2O$, 0.51 g; KCl, 0.97 g; $CaCl_2$. $2H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.003 g; $FeSO_4$. $7H_2O$, 0.55 mg

5.6. References

Berendsen, E. M., Boekhorst, J., Kuipers, O. P., & Wells-Bennik, M. H. J. (2016). A mobile genetic element profoundly increases heat resistance of bacterial spores. *ISME Journal*, 10(11), 2633–2642.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophilic bacilli from a milk powder processing plant. *Journal of Applied Microbiology*, 116(2), 350–359.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215–225.

Butler, R. R., Schill, K. M., Wang, Y., & Pombert, J. F. (2017). Genetic characterization of the exceptionally high heat resistance of the non-toxic surrogate Clostridium sporogenes PA 3679. *Frontiers in Microbiology*, 8(4), 1–11.

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., & Nierop Groot, M. (2015). Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology*, 200(5), 72–79.

Jamroskovic, J., Chromikova, Z., List, C., Bartova, B., Barak, I., & Bernier-Latmani, R. (2016). Variability in DPA and calcium content in the spores of *Clostridium* species. *Frontiers in Microbiology*, 7(11), 1–11.

Kent, D. J., Chauhan, K., Boor, K. J., Wiedmann, M., & Martin, N. H. (2016). Spore test parameters matter: mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. *Journal of Dairy Science*, 99(7), 5180–5191.

Mazas, M., Lopez, M., Martinez, S., Bernardo, A., & Martin, R. (1999). Heat resistance of Bacillus cereus spores : effects of milk constituents and stabilizing additives. *Journal of Food Protection*, 62(4), 410-413.

Murphy, P. M., Lynch, D., & Kelly, P. M. (2007). Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. *International Journal of Dairy Technology*, 52(2), 45–50.

Sadiq, F. A., Li, Y., Liu, T. J., Flint, S., Zhang, G., Yuan, L., Pei, Z., & He, G. Q. (2016). The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *International Journal of Food Microbiology*, 238(12), 193–201.

Sala, F. J., Ibarz, P., Palop, A., Raso, J., & Santiago, C. (1995). Sporulation temperature and heat resistance of *Bacillus subtilis* at different pH values. *Journal of Food Protection*, 58(3), 239–243.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, 60(2), 109–117.

Stoeckel, M., Lücking, G., Ehling-Schulz, M., Atamer, Z., & Hinrichs, J. (2016). Bacterial spores isolated from ingredients, intermediate and final products obtained from dairies: thermal resistance in milk. *Dairy Science and Technology*, 96(4), 569–577.

Wells-Bennik, M. H. J., Janssen, P. W. M., Klaus, V., Yang, C., Zwietering, M. H., & den Besten, H. M. W. (2019). Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. *International Journal of Food Microbiology*, 291(2), 161–172.

Zhao, Y., Caspers, M. P. M., Metselaar, K. I., De Boer, P., Roeselers, G., Moezelaar, R., Groot, M. N., Montijn, R. C., Abee, T., & Kort, R. (2013). Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. *Applied and Environmental Microbiology*, 79(18), 5652–5660.

Chapter 6

6 Overall Discussion and Summary

This study discusses the effect of temperature and the total dissolved solids on the biofilm and spore formation as well as characterizing the spores based on their chemical composition and heat resistance.

6.1. Effect of temperature and total dissolved solids concentration

In chapter 3, the roles of incubation temperature and the total dissolved solids on the biofilm and spore formation of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 were determined. The preferred temperature for biofilm and spore formation among A1, D1, P3 and ATCC12980 is 65 °C. Increasing the total dissolved solids concentration to 20% (w/v) delayed the onset of biofilm and spore formation at 55°C. The effect of temperature and total solids was cumulative. The addition of milk protein (whey protein and Na- caseinate) in excess, delayed the onset of detectable biofilm and spore formation of A1 at 55°C until 12 and 8h respectively. The effect of pre-conditioning of the stainless-steel surface with the milk proteins had a transient effect in the attachment of bacteria, which may explain the apparent delay that was observed in the CDC reactor experiments. In this study, UHT milk was used as the feed material for the CDC reactor experiments as opposed to the industrial scenario wherein raw/ pasteurised milk is used. Previous studies have discussed the effect of UHT treatment on the degradation of milk proteins which may influence the attachment of bacteria onto processing surfaces thus impacting the biofilm and spore formation kinetics (Douglas et al., 1981; Singh et al., 2019). Results from this study are based on skim milk that was subjected to UHT treatment, a severe heat treatment in comparison with pasteurization that is commonly employed in the dairy industry. Hence, we predict that the statistical growth model that has been

constructed based on the results from this study are robust and can be readily employed in the design of thermal processing in the dairy industry.

6.2. Heat resistance of spores and the effect of phosphate

Given the difference in spore formation among A1, D1, P3 and ATCC 12980 observed in chapter 3, heat resistance studies involving these spores were of focus in chapter 4. The three dairy strains produced similar D110 values but significantly different ($p \leq 0.05$) to the D110 value of spores of ATCC 12980. Calcium and DPA content of spores of A1, D1, P3 were significantly lower ($p \leq 0.05$) than ATCC 12980. From the result it appears that the Ca/DPA ratio might influence the heat resistance of *G. stearothermophilus* spores. The pH of the heating medium influenced the heat resistance of spores. This study demonstrates the effect of phosphate as a chelating agent when present in the heating medium and the reduction in the heat sensitivity of spores in a phosphate solution. The CIP used in the dairy industry includes the use of sodium hydroxide (NaOH) at 1-5 % followed by a 0.5-1% acid wash using nitric acid with other acids or surfactants (Bremer et al., 2006; Flint et al., 1997). Tri sodium phosphate has been used in the past in conjunction with the detergents to sequester metal ions and decreasing soil redisposition (Thomas & Sathian, 2014). The role of phosphate on fouling and biofilms have been discussed in previous studies and this work identifies the role of phosphate on the heat resistance of spores.

6.3. Comparison of heat resistance of spores obtained by two different methods

In chapter 4, the heat resistance of spores was obtained by using spores prepared in a sporulation medium. This is not a true representation of a dairy manufacturing environment where spores are produced within biofilms formed on stainless steel surfaces. To address this issue, the D_{110} value of spores obtained in a milk biofilm system under continuous flow using the CDC reactor was determined. The D_{110} values of spores obtained by the milk biofilm system are not significantly

different ($p \leq 0.05$) to the values obtained using spores prepared in sporulation medium. This was supported by the fact that the calcium and DPA content of spores

obtained in the milk biofilm are not significantly different ($p \leq 0.05$) to values obtained by spores prepared in a sporulation medium. The calcium and DPA content of spores of A1, D1, P3 were significantly lower ($p \leq 0.05$) than ATCC 12980, reflecting the relative heat sensitivity of these strains and with results in Chapter 4. The role of DPA on the heat resistance of spores has been discussed in chapter 1 and results from this chapter indicates that spores of ATCC 12980 with the highest amount of DPA also show the highest heat resistance thus in conformance with previous studies.

6.4. Summary

In summary, this study supported all the previously stated hypotheses of this study including the role of temperature. total solids and milk proteins on the biofilm and spore formation and the effect of phosphate on the heat resistance of spores. In addition, this study demonstrates that strains of *G. stearothermophilus* originating from the same milk powder manufacturing facility may vary in their ability to form biofilms and spores. A continuous flow through system using the CDC biofilm reactor in this study to closely resemble the dairy manufacturing environment. However, one of the limitations of this study is the lack of a turbulent flow within the reactor unlike in the dairy manufacturing environment. This study presents evidence on the role of phosphate as a chelating agent in lowering the heat resistance of spores. The heat resistance was not significantly different ($p \leq 0.05$) between spores obtained by either a milk biofilm system or a sporulation medium, but further investigation is needed to completely understand this observation considering the varying levels of minerals between the two media. The heat resistance of spores of dairy isolates were significantly lower ($p \leq 0.05$) than the reference strain which may reflect the adaptation to the dairy

environment. Further work involving a study on the differences in genes and genes expression relating to sporulation

among the dairy isolates and the reference strain would help further understanding the difference between dairy strains and the reference strain.

6.5. Practical contribution of this study to the dairy industry

1. The variation in the biofilm and spore formation of strains of *G. stearothermophilus* isolated from the same milk powder manufacturing facility may have implications in the cleaning regime. The findings from this study highlights the need to include multiple strains while designing new cleaning regimes, targeting the most resistant spores.

2. The role of phosphate in lowering the heat resistance of spores was discussed in this study. Further investigation is required to study the feasibility of using a phosphate based cleaning agent to more effectively reduce spores in biofilm within dairy manufacturing plant, enabling improved product quality and extending the run length of dairy manufacturing plant before the spore numbers in the product exceed specification limits.

3. There was no significant difference ($p \leq 0.05$) in the heat resistance of spores obtained from a milk biofilm and from a sporulation medium. This shows that test results obtained from spores obtained in a laboratory sporulation medium are relevant to the dairy industry.

6.6. Future recommendations

1. Investigate the effect of currently adopted CIP practices reducing heat resistant spores in biofilm on stainless steel surfaces

2. Investigate the feasibility of a phosphate-based cleaning agent in improving the reduction of spores in biofilm on stainless steel surfaces

3. Identify genes involved in sporulation pathways and gene expression differences between dairy isolates and other strains of *G. stearothermophilus* under the influence of abiotic factors such as temperature and total solids

4. Confirming the results from the present trials that suggest adaptation to a dairy environment influences the heat sensitivity of spores G. stearothermophilus by testing more strains from dairy and other sources.

5. Determine the genes that are up regulated during biofilm and spore formation of *G. stearothermophilus* using RNA sequencing

6. Investigate the factors i.e. hydrophobicity, zeta potential and electrostatic charge that may influence the attachment of bacteria to stainless steel surface preconditioned with whey protein and caseins

7. Investigate the biofilm and spore formation of *G. stearothermophilus* in the presence of whole milk and compare with the results from this study that were limited to skim milk.

6.7. References:

Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology*, 106(3), 254–262.

Douglas, F. W., Greenberg, R., Farrell, H. M., & Edmondson, L. F. (1981). Effects of ultra-high-temperature pasteurization on milk proteins. *Journal of Agricultural and Food Chemistry*, 29(1), 11–15.

Flint, S. H., Bremer, P. J., & Brooks, J. D. (1997). Biofilms in dairy manufacturing plant - description, current concerns and methods of control. *Biofouling*, 11(1), 81–97.

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (2019). Ultra high temperature (UHT) stability of

casein-whey protein mixtures at high protein content: heat induced protein interactions. *Food Research International*, 116(12), 103–113.

Thomas, A., & Sathian, C. T. (2014). Cleaning-in-place (CIP) system in dairy plant- review. *Journal of Environmental Science, Toxicology and Food Technology*, 8(6), 41–44.

Chapter 7

---

7 Appendix

7.1. Ingredients

Instant skim milk powder, milk protein concentrate, whey protein concentrates 80% and sodium caseinate of The NZMP brand was previously purchased from Davis Trading, Palmerston North, New Zealand. The whey protein nitrogen index (WPNI) value of the instant skim milk powder was determined as 3.0, thus confirming it as medium heat skim milk powder.

7.2. UHT treatment

Based on the experiment design in chapter 3 , the necessary milk ingredients were reconstituted in hot water (60°C) and were hydrated for 60min in a jacketed Cowles mixer. Following hydration, the reconstituted product was subjected to UHT treatment (142°C for 8s). A 10 s holding tube was used and the flow rate was maintained at 1 L/min. The temperature of the preheater and the heater section was set at 85 and 144°C respectively. Following UHT treatment, the sterile product was filled in sterile 20 L carboy cans and were stored at 4°C.

7.3. Attachment trial

Coupons were immersed in 20 mL of the respective medium in sterile centrifuge tubes at 55°C 100 rpm for 2h prior to inoculation. The coupons were then carefully transferred into 20 mL of the respective fresh media in sterile centrifuge tubes and were inoculated with 10% of overnight grown culture of A1, followed by incubation at 55°C at 100 rpm. The coupons were removed after 2h and then rinsed 3x in phosphate buffer saline and the total number of cells attached to the SS coupons were quantified using bead vortexing method and plating on TSA and colonies were counted after incubation at 55°C for 18h.

7.4. Heat resistance of spores of A1, D1, P3 and ATCC 12980 in MOPS and citrate phosphate buffer at pH 7.0 and 110°C

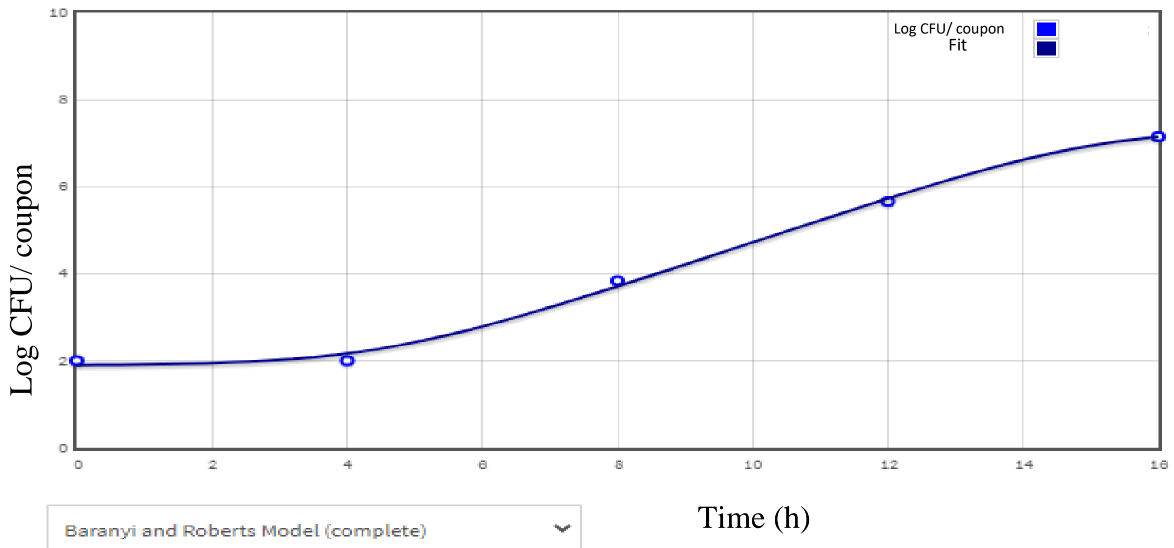
Strain	D_{110} value (min) in MOPS buffer at pH 7.0	D_{110} value (min) in citrate-phosphate buffer at pH 7.0
A1	4.7 ± 0.89	2.21 ± 0.20
D1	5.1 ± 1.1	2.37 ± 0.29
P3	5.55 ± 1.2	2.41 ± 0.48
ATCC 12980	7.62 ± 2.30	2.24 ± 0.09

*** All values are mean ± standard deviation of three independent spore suspensions**

7.5. Kinetic models for biofilm and spore formation of A1, D1, P3 and ATCC 12980. Combase software was used for the creation of these models.

*Assumptions of 2.0 and 1.0 log CFU/coupon were made for observations for total viable cells and spores per coupon below the detection limit respectively.

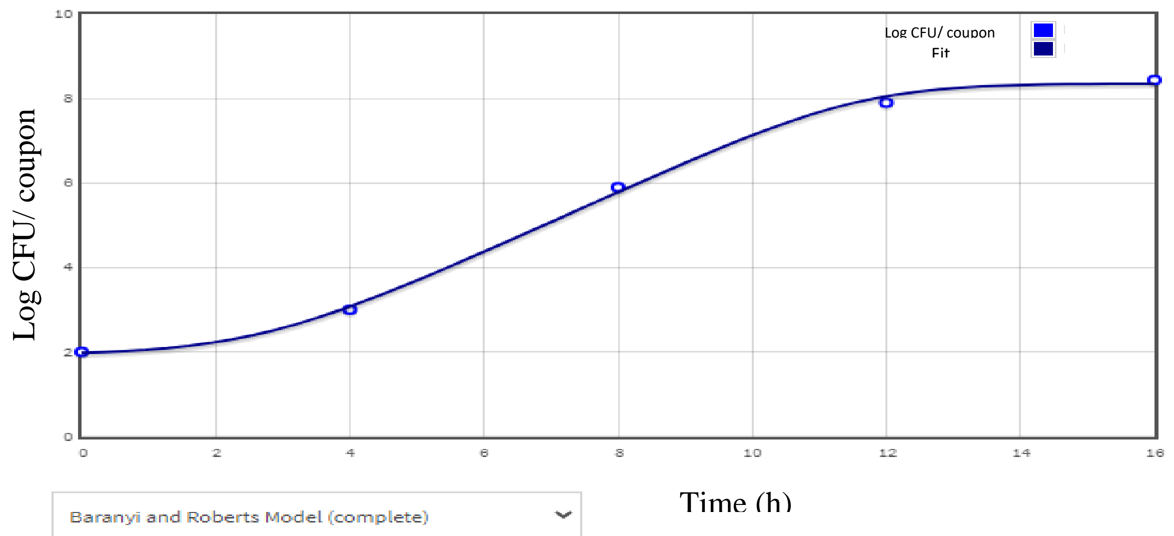
A1 55°C 10% Solids



R-square:	0.989
SE of Fit:	0.243

Initial value	1.9 ± 0.222
Lag/shoulder	4.543 ± 1.0682
Maximum Rate	0.517 ± 0.0768
Final Value	7.332 ± 0.449

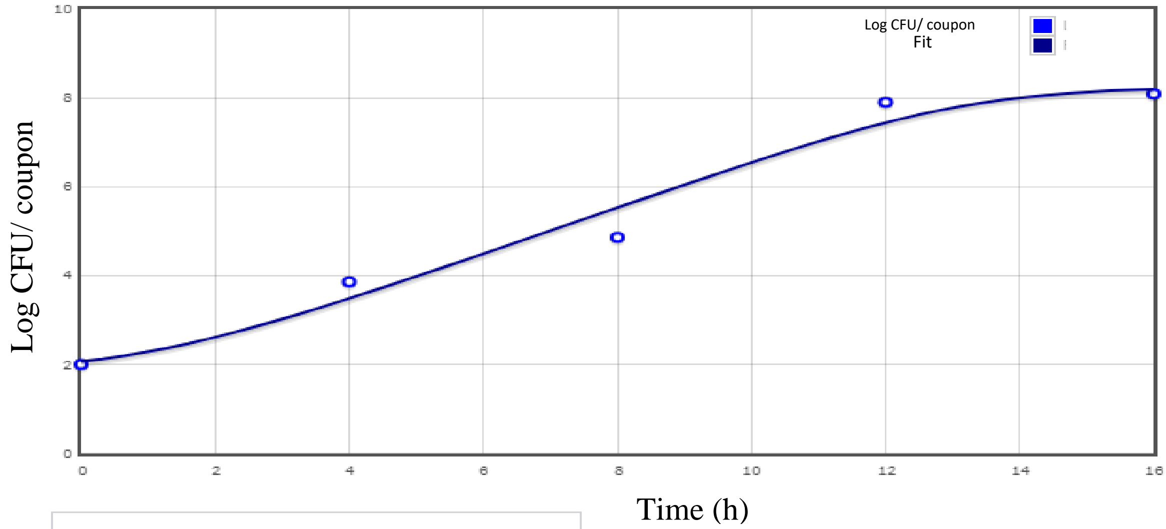
A1 60°C 10% Solids



R-square:	0.994
SE of Fit:	0.224

Initial value	1.976 ± 0.223
Lag/shoulder	2.699 ± 0.637
Maximum Rate	0.719 ± 0.0764
Final Value	8.36 ± 0.212

A1 65°C 10% Solids

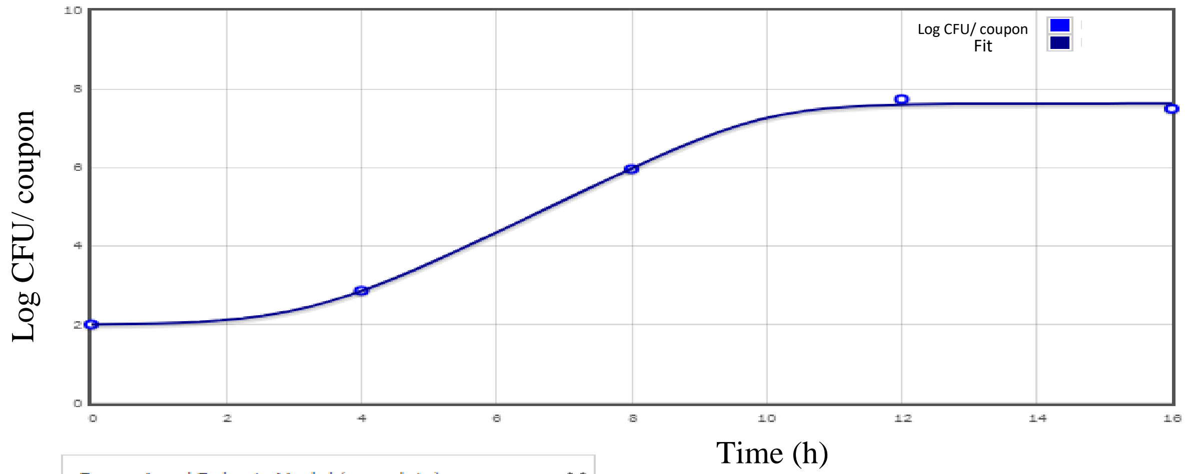


Baranyi and Roberts Model (complete)

R-square:	0.883
SE of Fit:	0.901

Initial value	2.0614 ± 0.899
Lag/shoulder	1.4 ± 3.35°
Maximum Rate	0.526 ± 0.
Final Value	8.249 ± 1.

D1 55°C 10% Solids

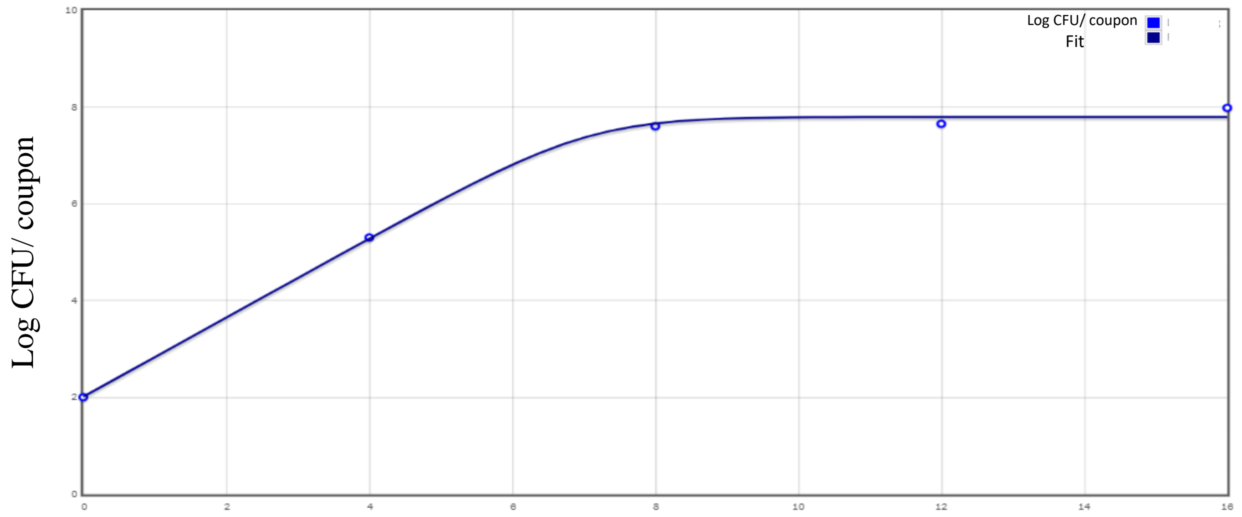


Baranyi and Roberts Model (complete)

R-square:	0.995
SE of Fit:	0.189

Initial value	2.00438 ± 0.189
Lag/shoulder	3.217 ± 0.493
Maximum Rate	0.836 ± 0.0832
Final Value	7.628 ± 0.138

D1 60°C 10% Solids

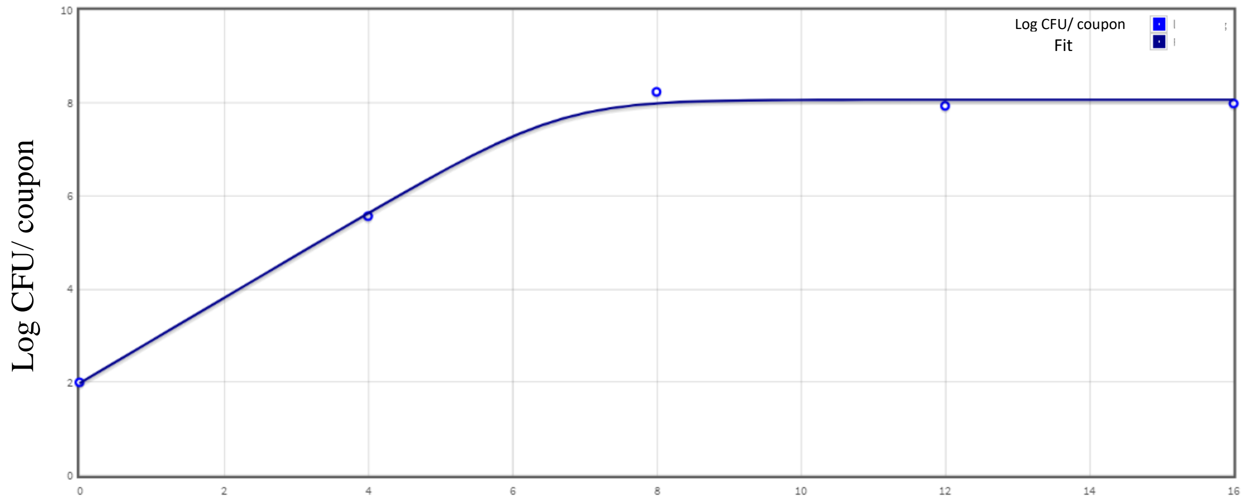


Baranyi and Roberts Model (no lag)

Time (h)

R-square:	0.995
SE of Fit:	0.172
Initial value	2.00927 ± 0.17
Maximum Rate	0.818 ± 0.0578
Final Value	7.794 ± 0.11

D1 65°C 10% Solids

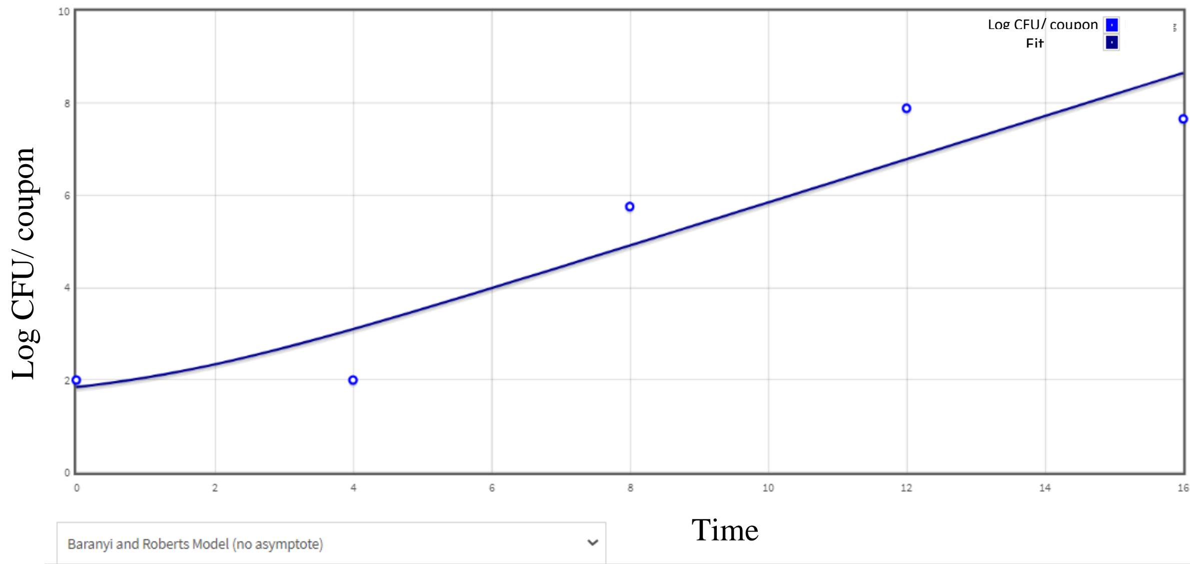


Baranyi and Roberts Model (no lag)

Time (h)

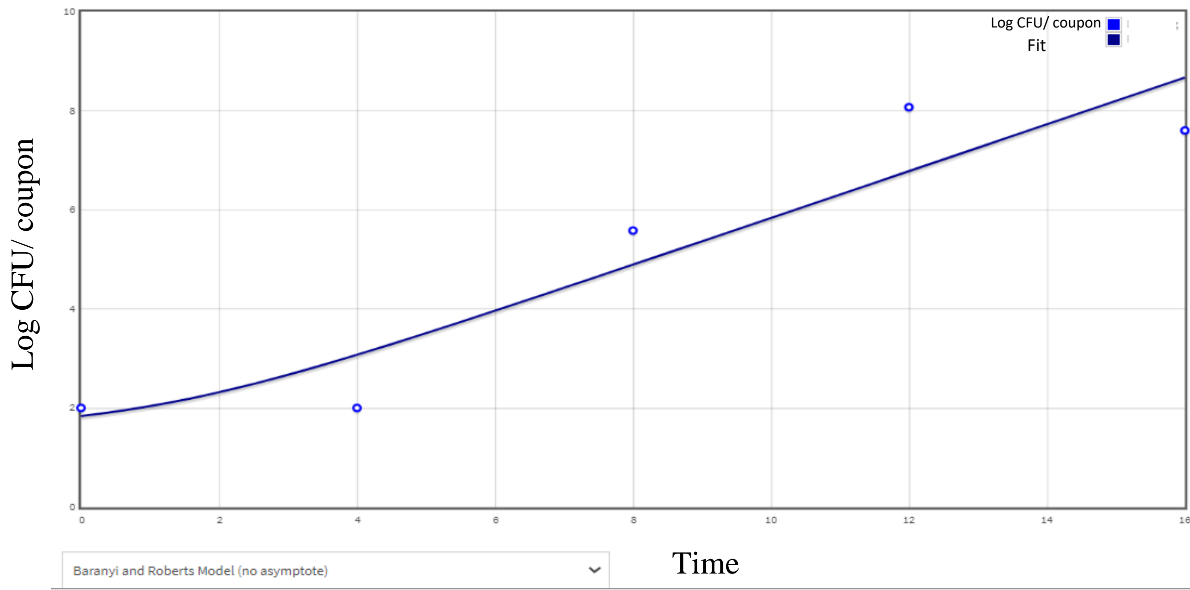
R-square:	0.994
SE of Fit:	0.209
Initial value	1.98 ± 0.209
Maximum Rate	0.915 ± 0.0737
Final Value	8.0691 ± 0.128

P3 55°C 10% Solids



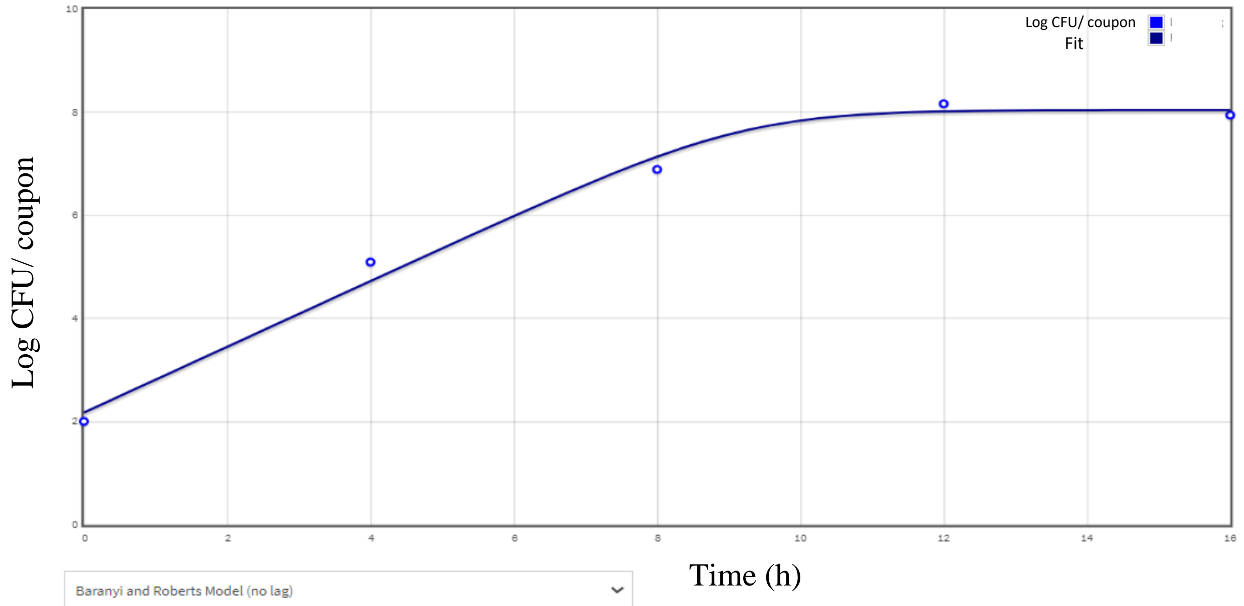
R-square:	0.755
SE of Fit:	1.444
Initial value	1.847 ± 1.439
Lag/shoulder	1.453 ± 5.155
Maximum Rate	0.468 ± 0.177

P3 60°C 10% Solids



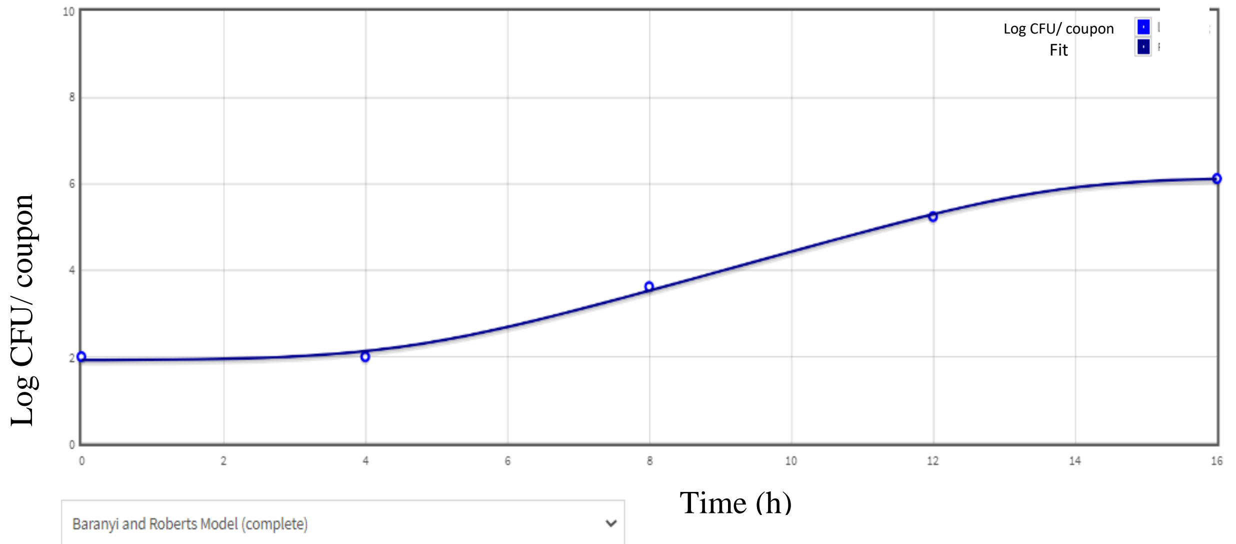
R-square:	0.742
SE of Fit:	1.493
Initial value	1.837 ± 1.487
Lag/shoulder	1.545 ± 5.316
Maximum Rate	0.473 ± 0.185

P3 65°C 10% Solids



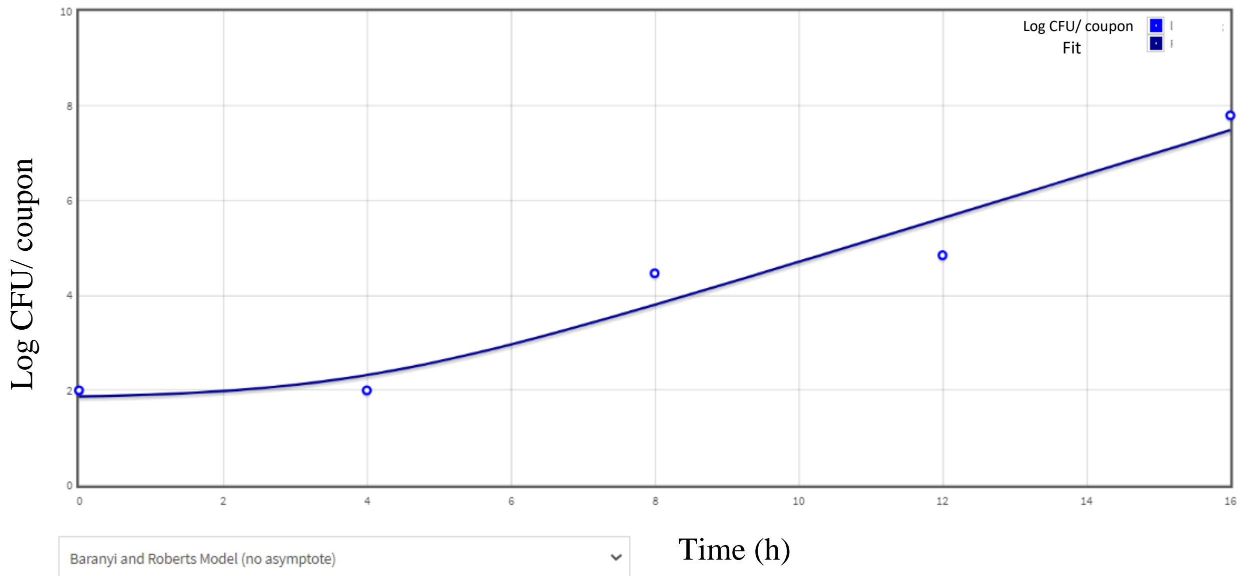
R-square:	0.98
SE of Fit:	0.36
Initial value	2.171 ± 0.335
Maximum Rate	0.638 ± 0.0743
Final Value	8.0395 ± 0.264

ATCC 12980 55°C 10% Solids

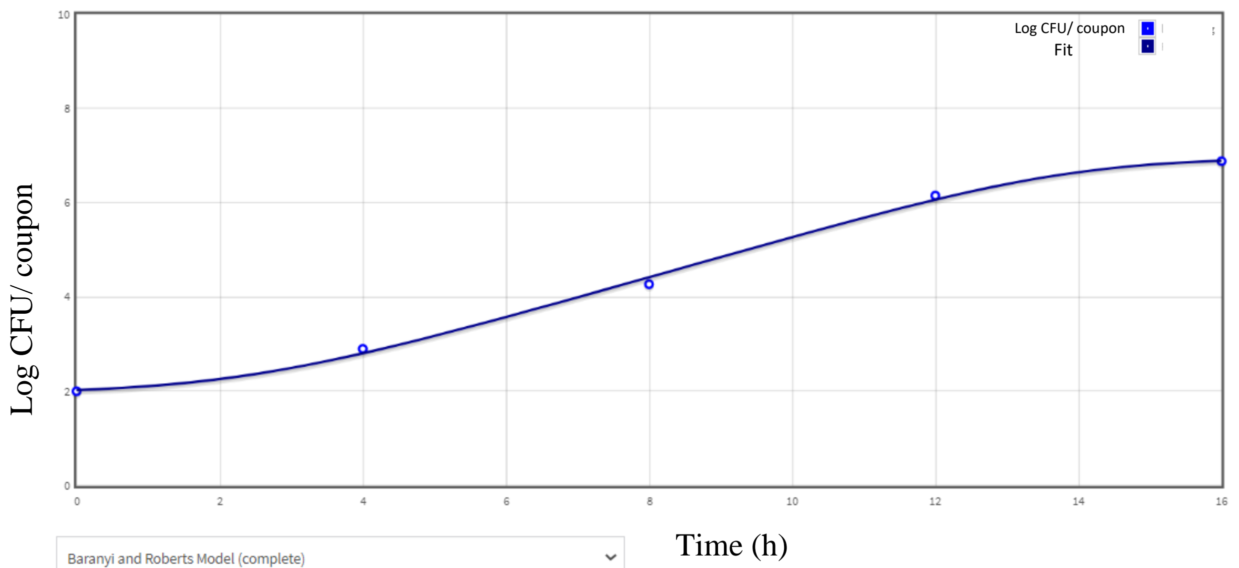


R-square:	0.99
SE of Fit:	0.185
Initial value	1.92 ± 0.167
Lag/shoulder	4.54 ± 0.913
Maximum Rate	0.46 ± 0.0607
Final Value	6.149 ± 0.214

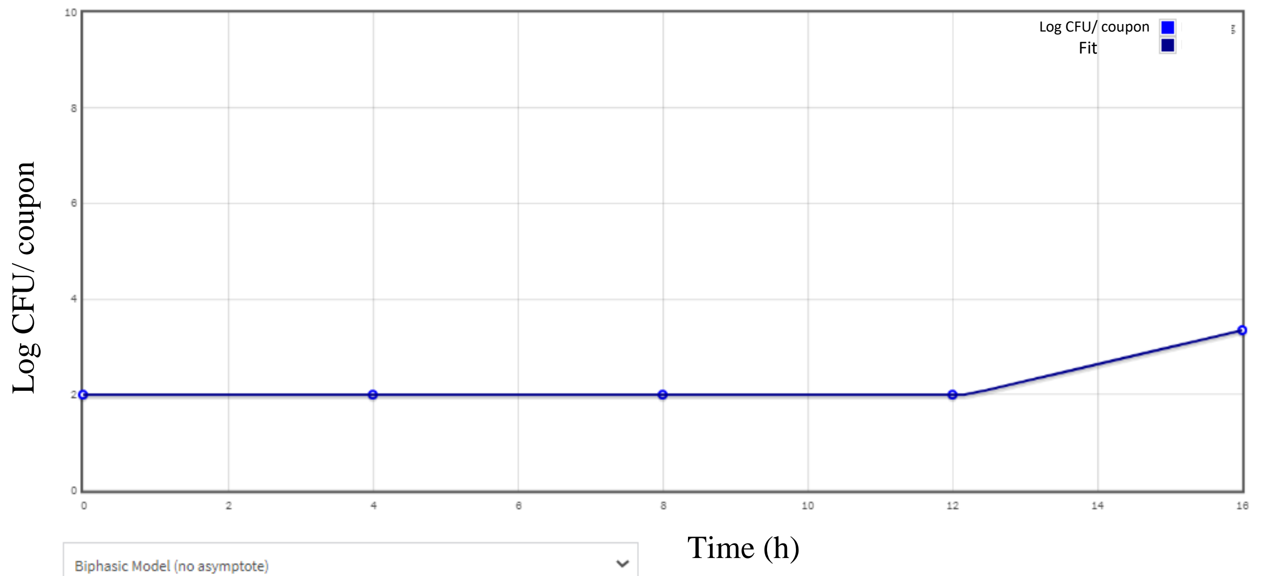
ATCC 12980 60°C 10% Solids



ATCC 12980 65°C 10% Solids

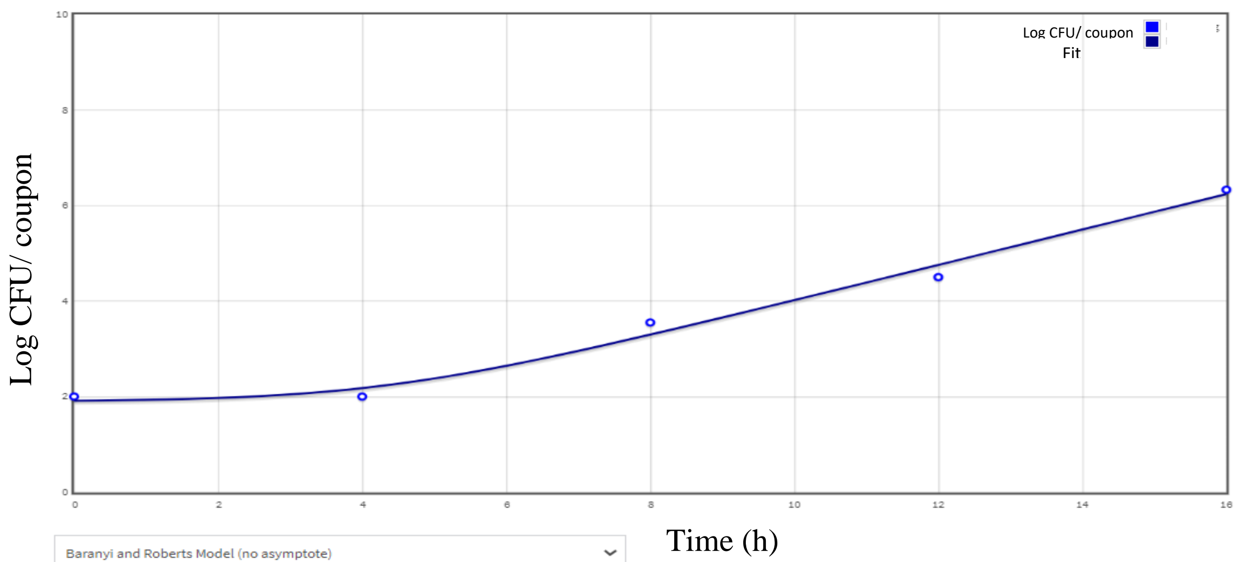


A1 55°C 20% Solids



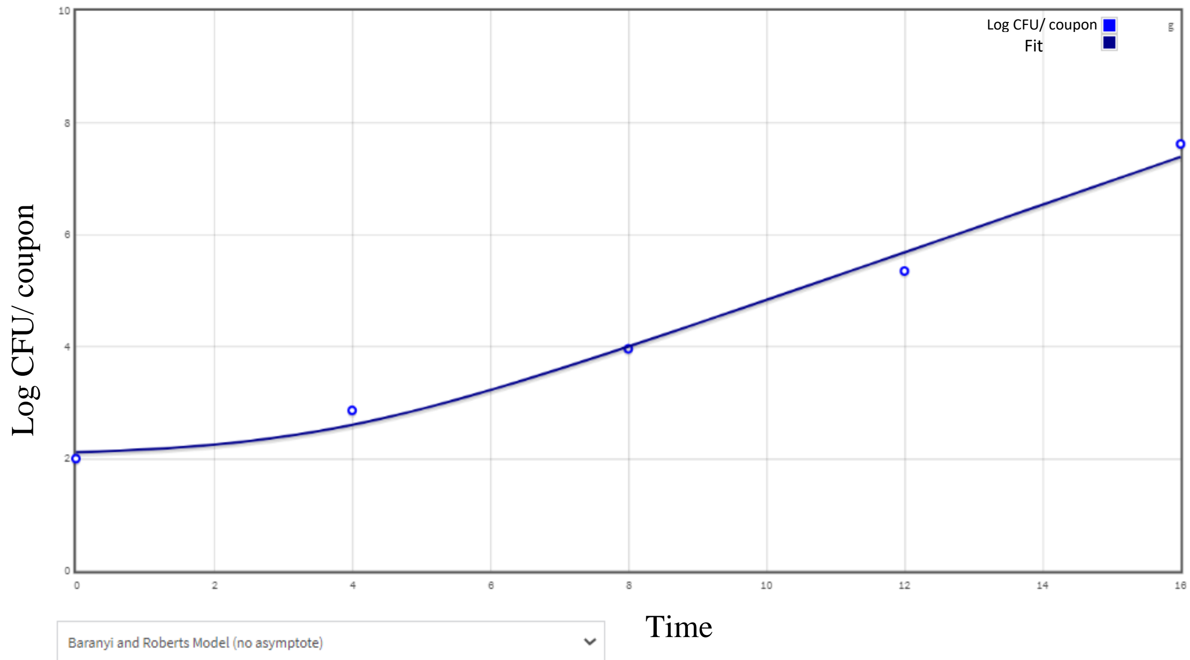
R-square:	1
SE of Fit:	3.14e-16
Initial value	2 ± 1.57e-16
Lag/shoulder	12.21 ± 8.37e-8
Maximum Rate	0.356 ± 7.86e-9

A1 60°C 20% Solids



R-square:	0.974
SE of Fit:	0.297
Initial value	1.912 ± 0.271
Lag/shoulder	4.338 ± 1.484
Maximum Rate	0.371 ± 0.0485

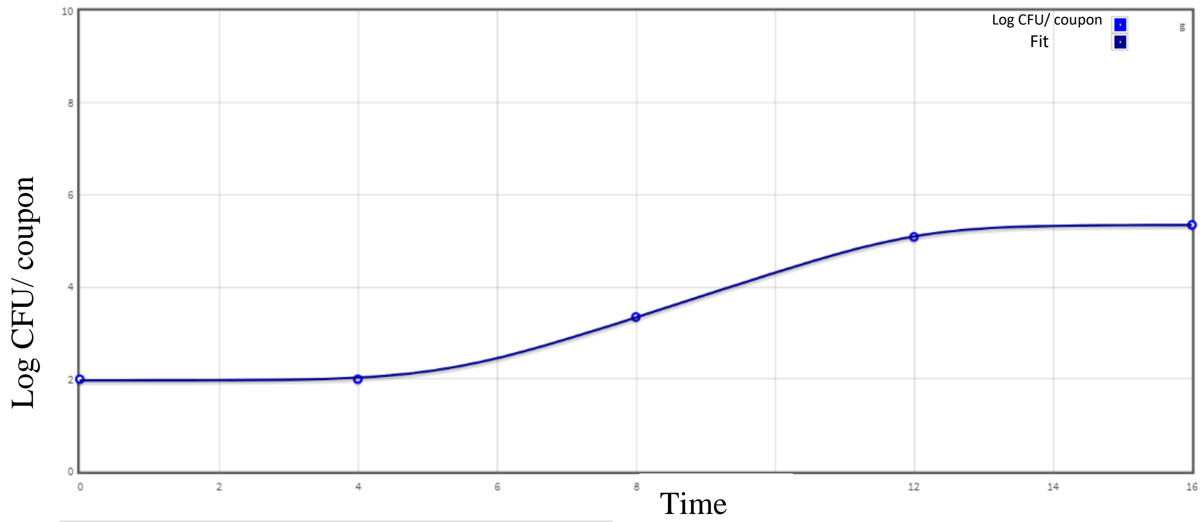
A1 65°C 20% Solids



R-square:	0.975
SE of Fit:	0.35

Initial value	2.114 ± 0.333
Lag/shoulder	3.649 ± 1.513
Maximum Rate	0.427 ± 0.053

D1 55°C 20% Solids

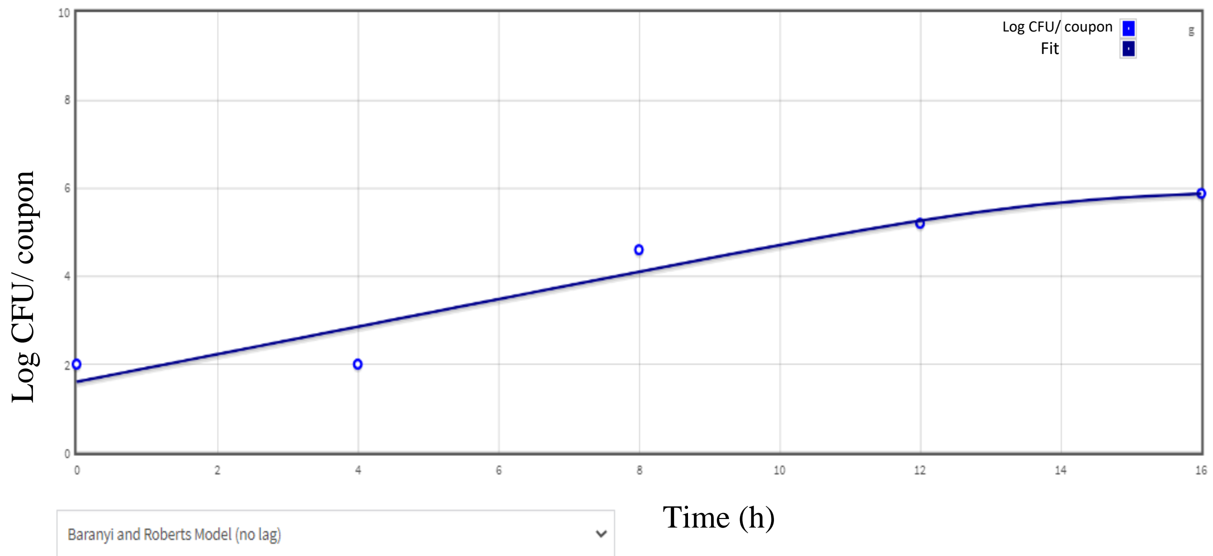


Baranyi and Roberts Model (complete)

R-square:	0.999
SE of Fit:	0.0468

Initial value	1.971 ± 0.0369
Lag/shoulder	5.269 ± 0.23
Maximum Rate	0.498 ± 0.026
Final Value	5.347 ± 0.0471

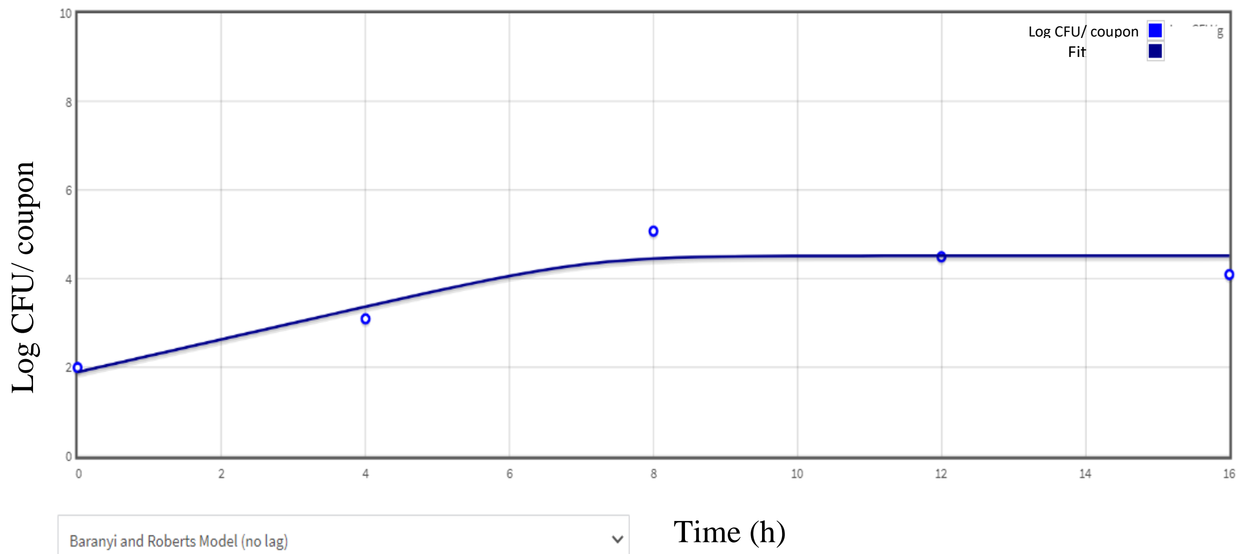
D1 60°C 20% Solids



R-square:	0.829
SE of Fit:	0.755

Initial value	1.598 ± 0.651
Maximum Rate	0.313 ± 0.099
Final Value	5.961 ± 1.044

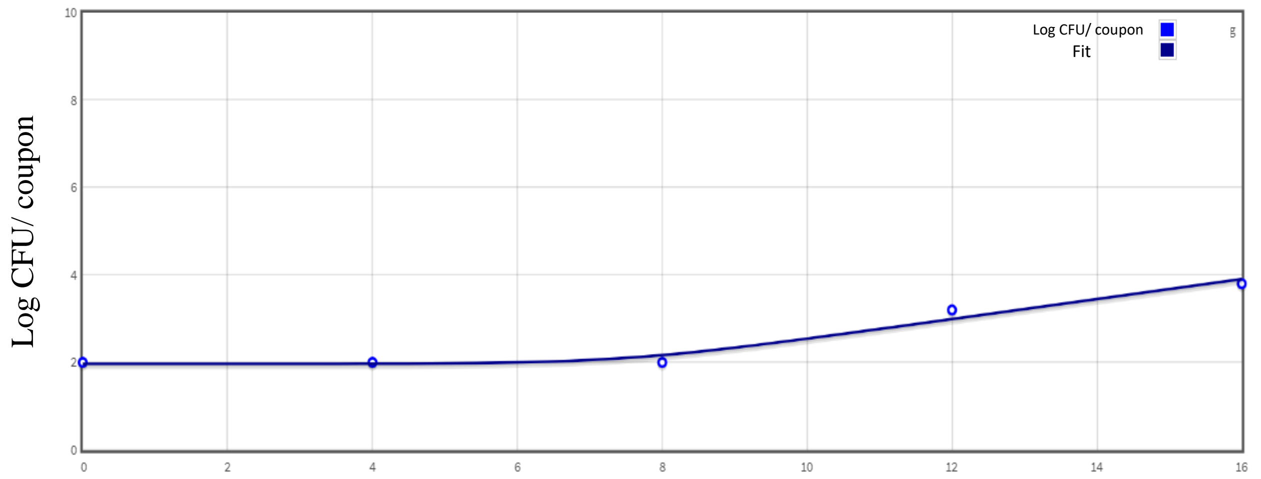
D1 65°C 20% Solids



R-square:	0.781
SE of Fit:	0.571

Initial value	1.896 ± 0.566
Maximum Rate	0.37 ± 0.191
Final Value	4.524 ± 0.367

P3 55°C 20% Solids

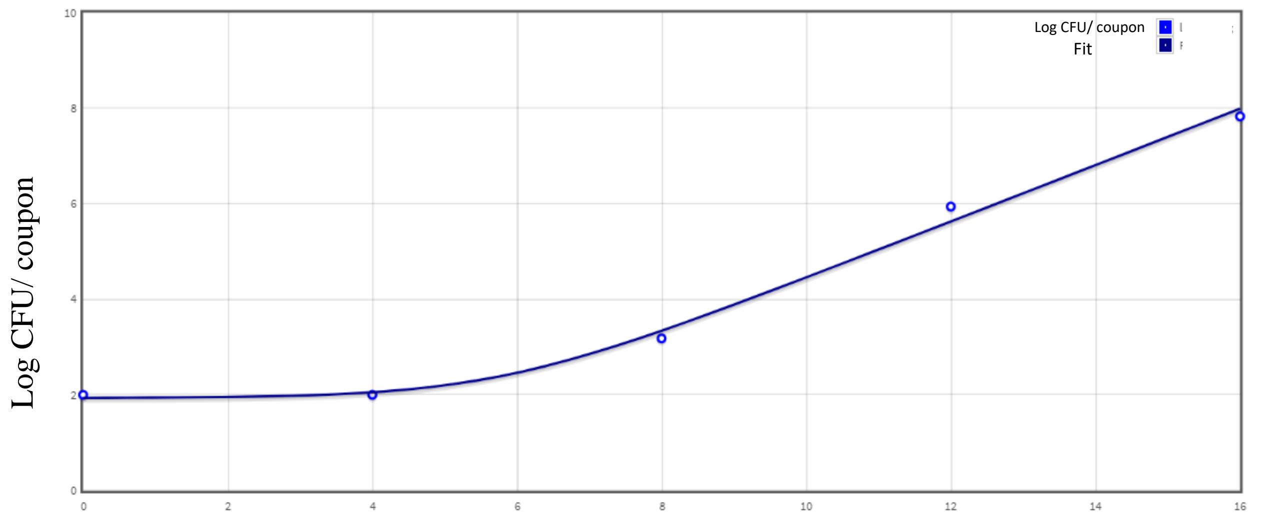


Time (h)

Baranyi and Roberts Model (no asymptote)

R-square:	0.941
SE of Fit:	0.205
Initial value	1.968 ± 0.146
Lag/shoulder	7.535 ± 1.561
Maximum Rate	0.229 ± 0.0471

P3 60°C 20% Solids

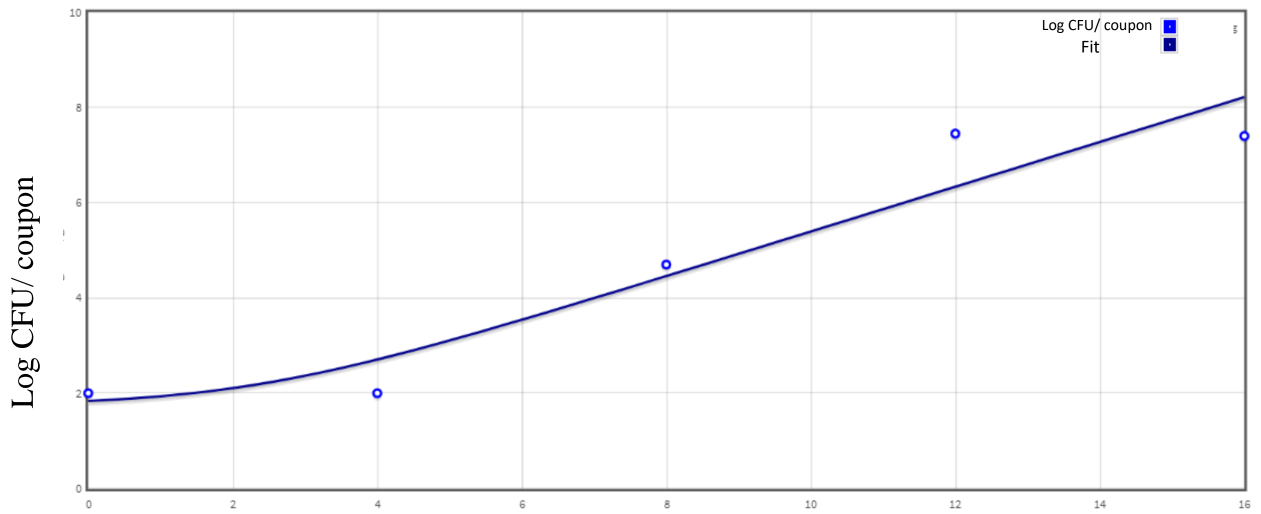


Time (h)

Baranyi and Roberts Model (no asymptote)

R-square:	0.988
SE of Fit:	0.279
Initial value	1.938 ± 0.22
Lag/shoulder	5.756 ± 0.82
Maximum Rate	0.591 ± 0.0522

P3 65°C 20% Solids

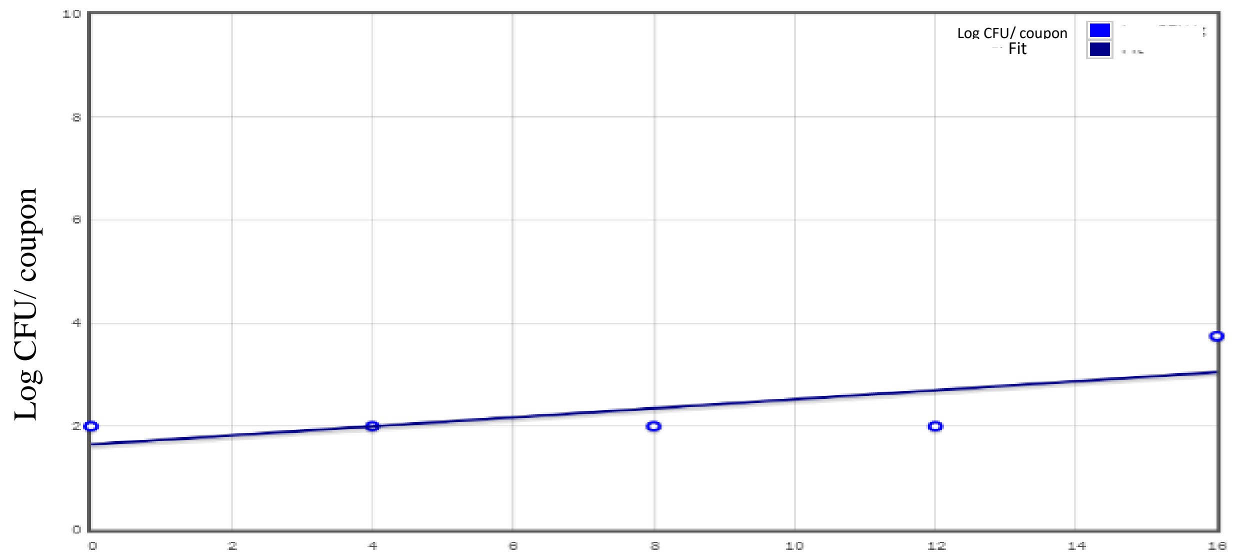


Time (h)

Baranyi and Roberts Model (no asymptote)

R-square:	0.83
SE of Fit:	1.117
Initial value	1.836 ± 1.105
Lag/shoulder	2.44 ± 4.0196
Maximum Rate	0.47 ± 0.144

ATCC 12980 55°C 20% Solids

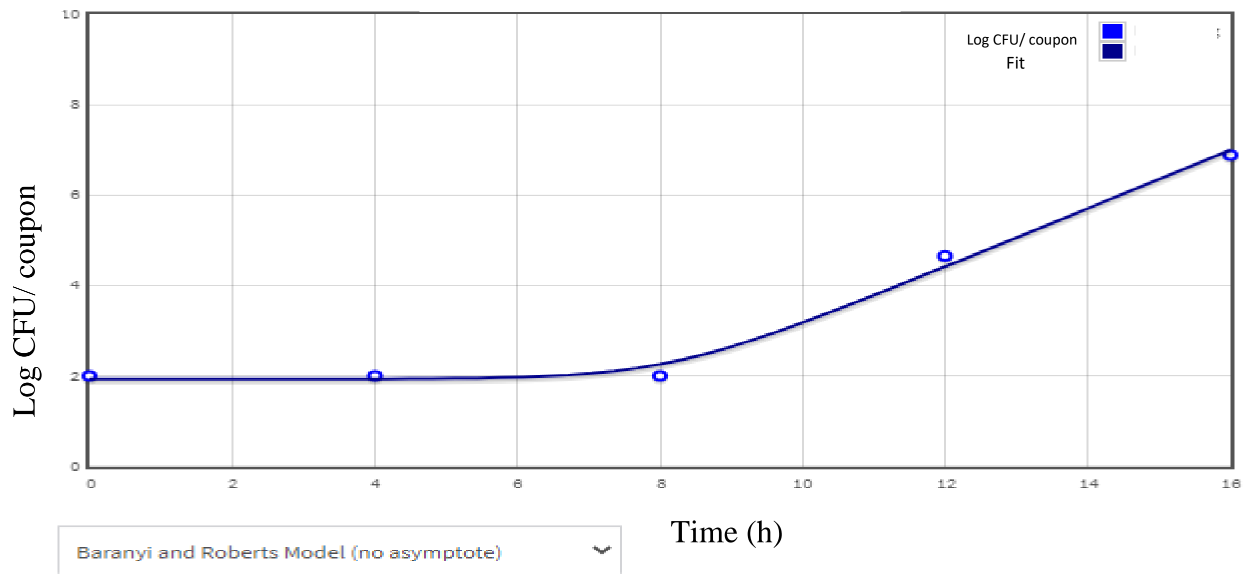


Time (h)

Linear Model

R-square:	0.333
SE of Fit:	0.639
Initial value	1.65 ± 0.495
Maximum Rate	0.0875 ± 0.0505

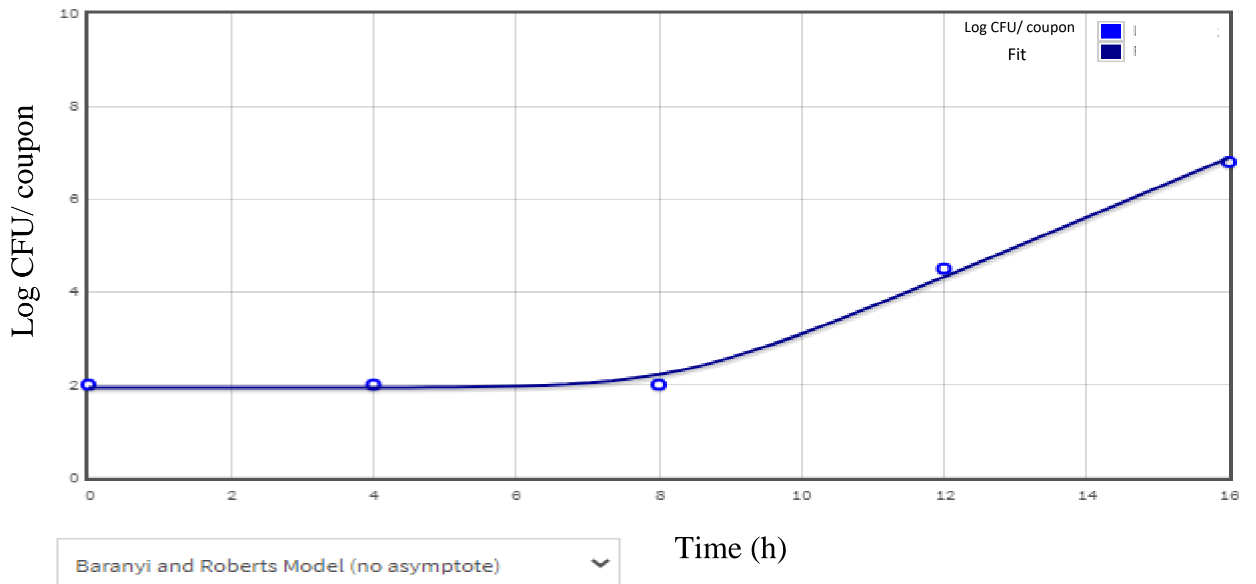
ATCC 12980 60°C 20% Solids



R-square: 0.985
SE of Fit: 0.267

Initial value 1.929 ± 0.184
Lag/shoulder 8.157 ± 0.791
Maximum Rate 0.646 ± 0.0722

ATCC 12980 65°C 20% Solids

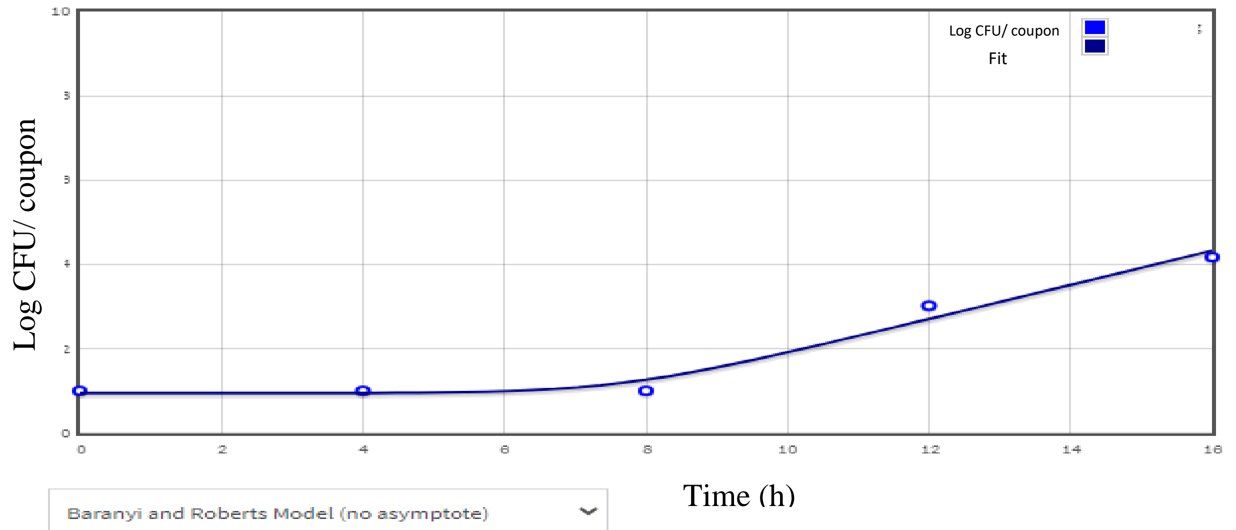


R-square: 0.99
SE of Fit: 0.218

Initial value 1.935 ± 0.148
Lag/shoulder 8.301 ± 0.66
Maximum Rate 0.643 ± 0.0613

Spore formation:

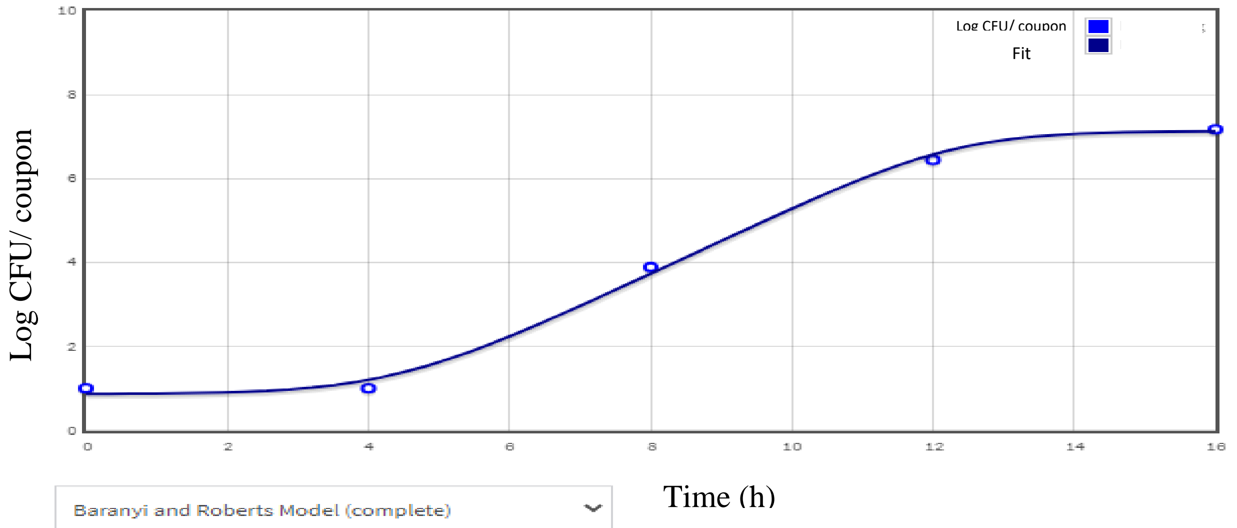
A1 55°C 10% Solids



R-square: 0.955
SE of Fit: 0.315

Initial value 0.945 ± 0.222
Lag/shoulder 7.664 ± 1.373
Maximum Rate 0.406 ± 0.0743

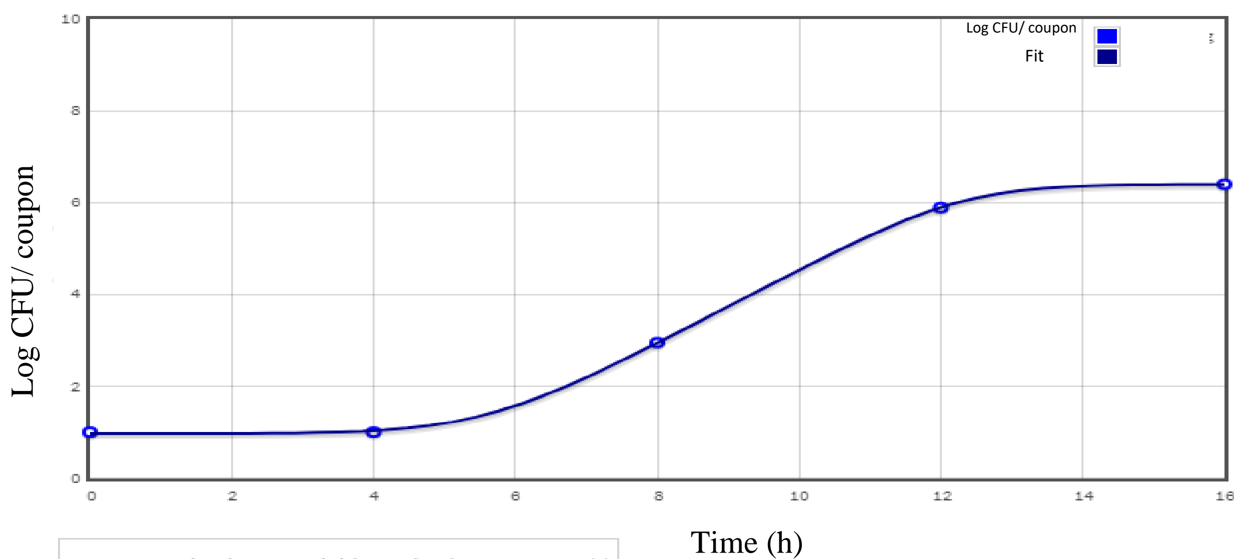
A1 60°C 10% Solids



R-square: 0.988
SE of Fit: 0.318

Initial value 0.87 ± 0.29
Lag/shoulder 4.403 ± 0.956
Maximum Rate 0.794 ± 0.128
Final Value 7.131 ± 0.322

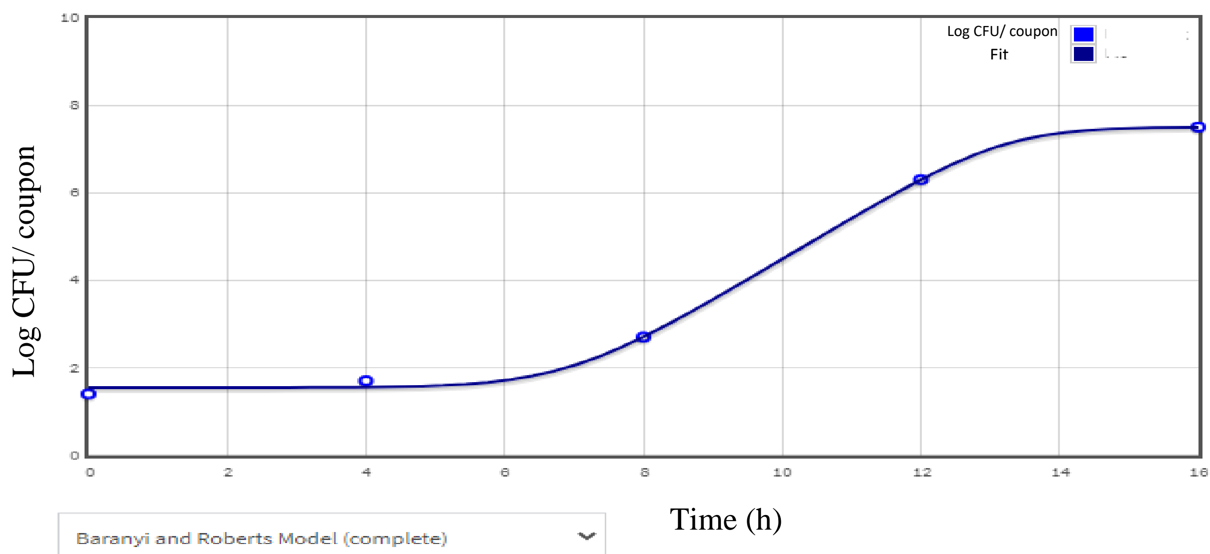
A1 65°C 10% Solids



Baranyi and Roberts Model (complete)

R-square:	1
SE of Fit:	0.0471
Initial value	0.969 ± 0.0356
Lag/shoulder	5.596 ± 0.128
Maximum Rate	0.812 ± 0.0236
Final Value	6.4 ± 0.0476

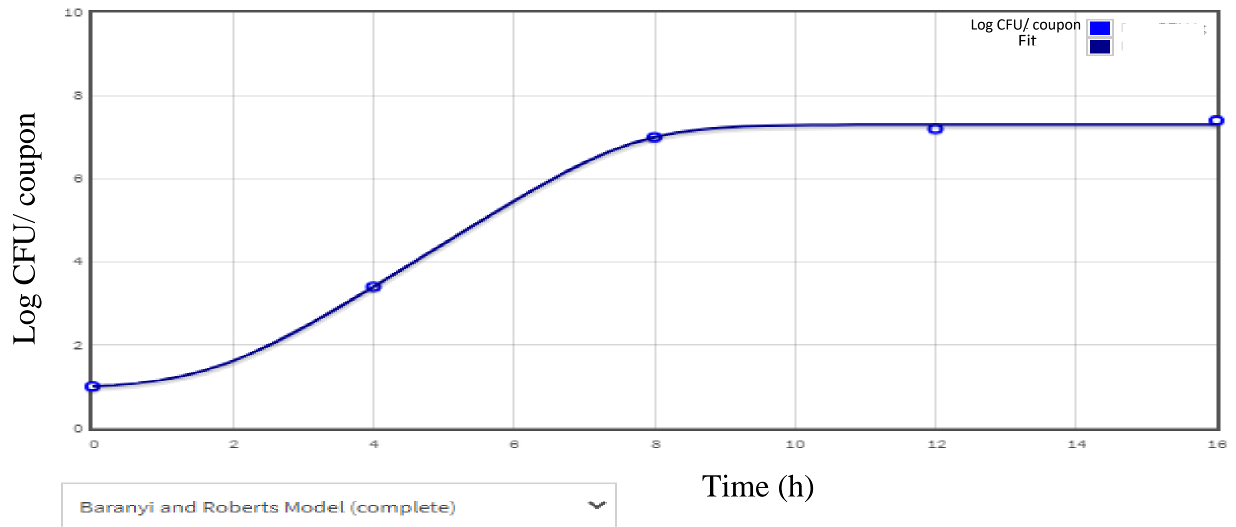
D1 55°C 10% Solids



Baranyi and Roberts Model (complete)

R-square:	0.995
SE of Fit:	0.204
Initial value	1.543 ± 0.146
Lag/shoulder	6.92 ± 0.397
Maximum Rate	0.952 ± 0.0867
Final Value	7.507 ± 0.209

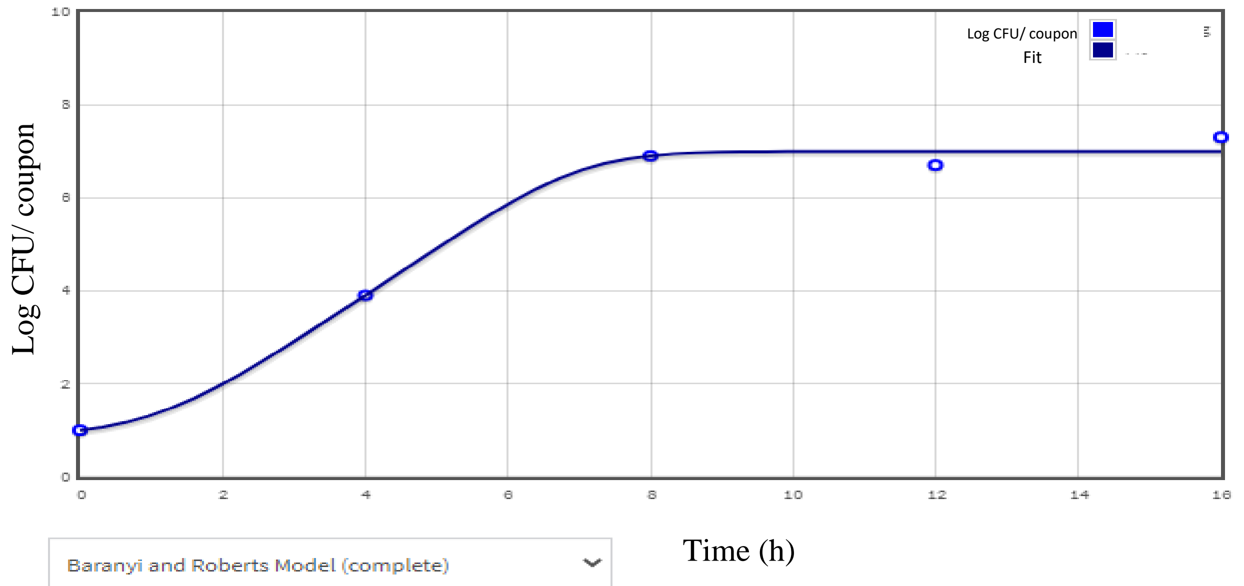
D1 60°C 10% Solids



R-square:	0.998
SE of Fit:	0.141

Initial value	1 ± 0.141
Lag/shoulder	1.751 ± 0.332
Maximum Rate	1.0578 ± 0.108
Final Value	7.3 ± 0.0999

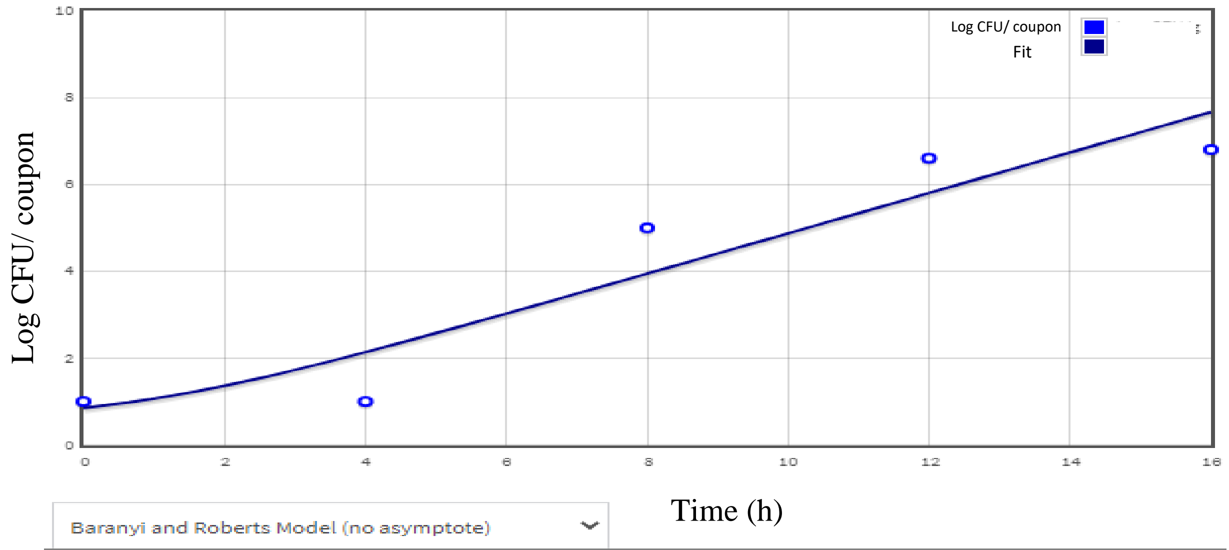
D1 65°C 10% Solids



R-square:	0.975
SE of Fit:	0.424

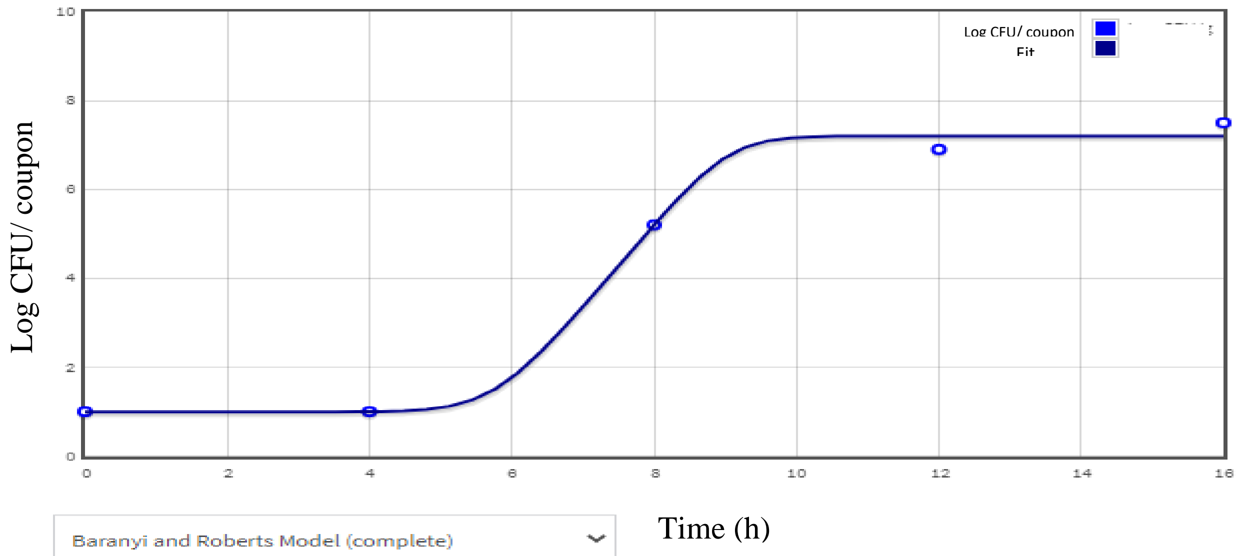
Initial value	1 ± 0.424
Lag/shoulder	1.192 ± 2.331
Maximum Rate	1.0308 ± 0.809
Final Value	7.000407 ± 0.3

P3 55°C 10% Solids



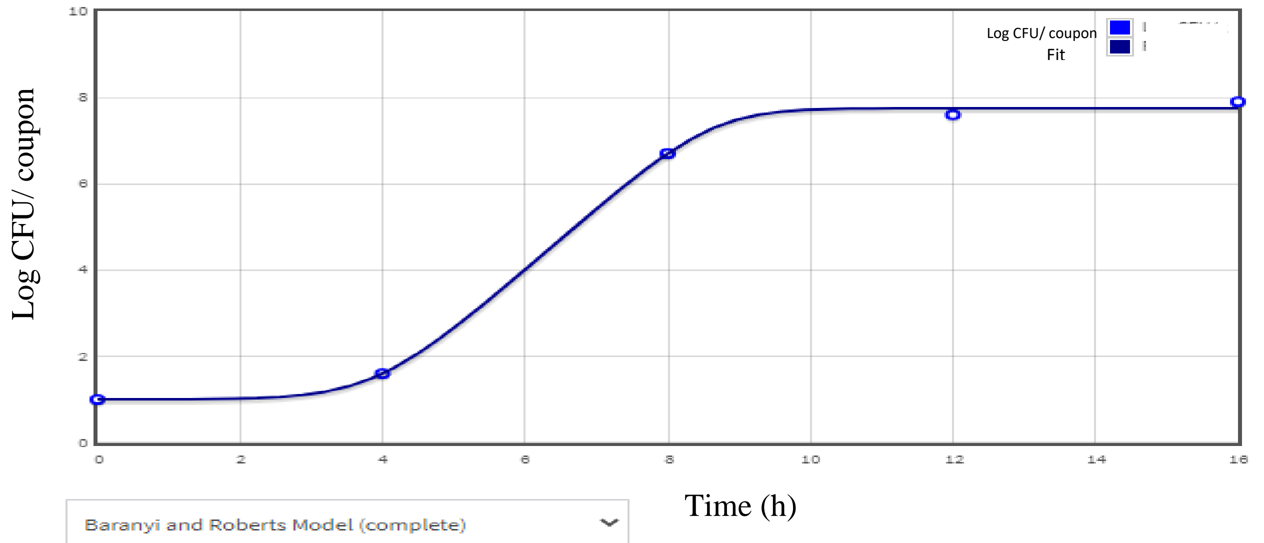
R-square:	0.773
SE of Fit:	1.38
Initial value	0.854 ± 1.376
Lag/shoulder	1.368 ± 4.939
Maximum Rate	0.465 ± 0.168

P3 60°C 10% Solids



R-square:	0.982
SE of Fit:	0.424
Initial value	0.996 ± 0.424
Lag/shoulder	5.752 ± 17.512
Maximum Rate	1.88 ± 14.519
Final Value	7.2 ± 0.3

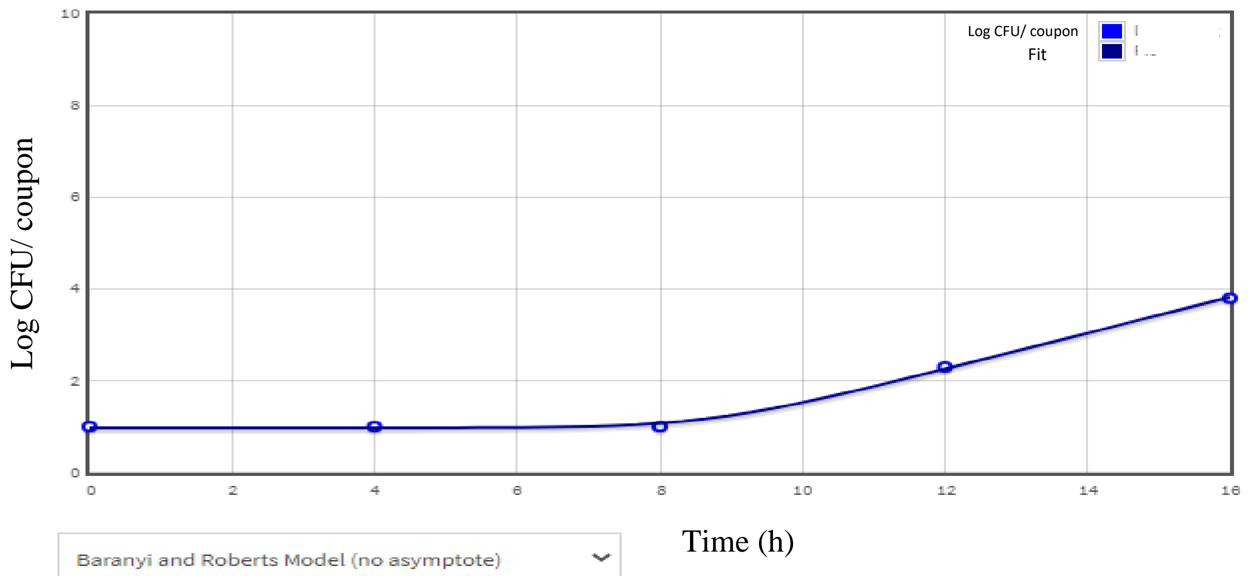
P3 65°C 10% Solids



R-square:	0.996
SE of Fit:	0.212

Initial value	1 ± 0.212
Lag/shoulder	3.907 ± 0.393
Maximum Rate	1.432 ± 0.126
Final Value	7.75 ± 0.15

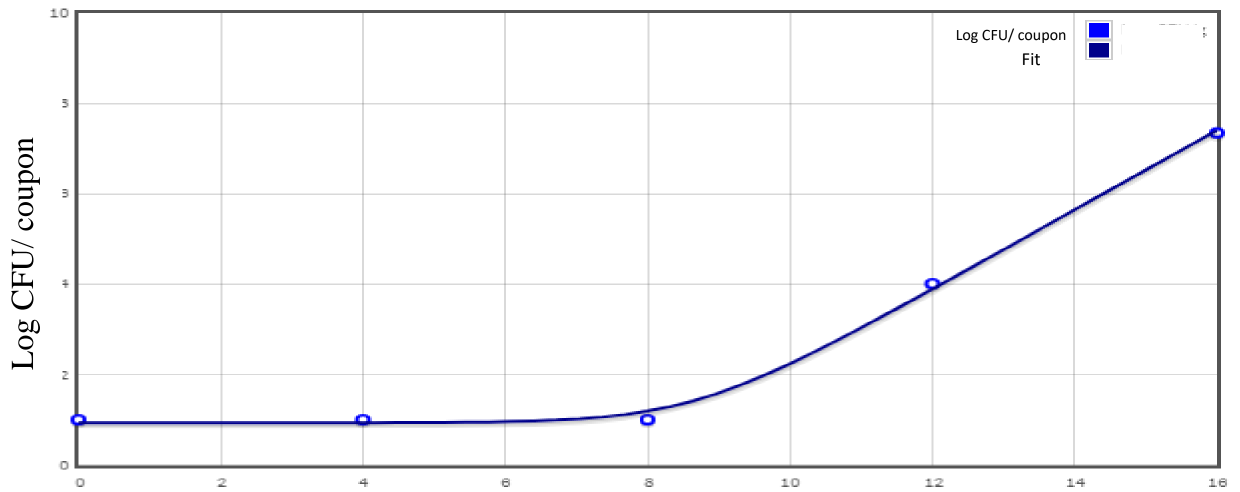
ATCC 12980 55°C 10% Solids



R-square:	0.997
SE of Fit:	0.069

Initial value	0.973 ± 0.045
Lag/shoulder	8.741 ± 0.357
Maximum Rate	0.392 ± 0.0218

ATCC 12980 60°C 10% Solids



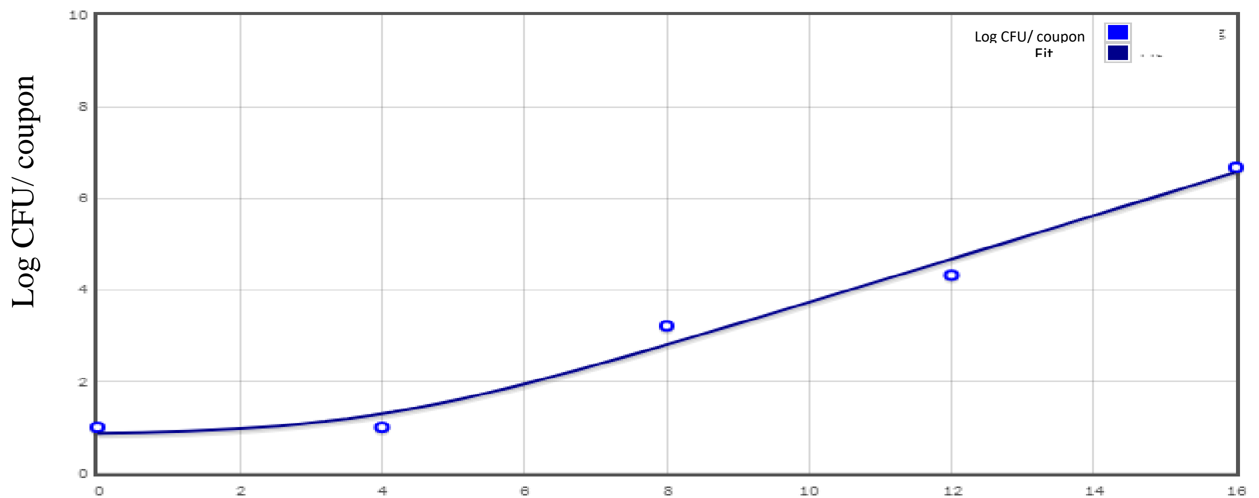
Time (h)

Baranyi and Roberts Model (no asymptote)

R-square:	0.996
SE of Fit:	0.177

Initial value	0.933 ± 0.117
Lag/shoulder	8.653 ± 0.406
Maximum Rate	0.881 ± 0.0548

ATCC 12980 65°C 10% Solids



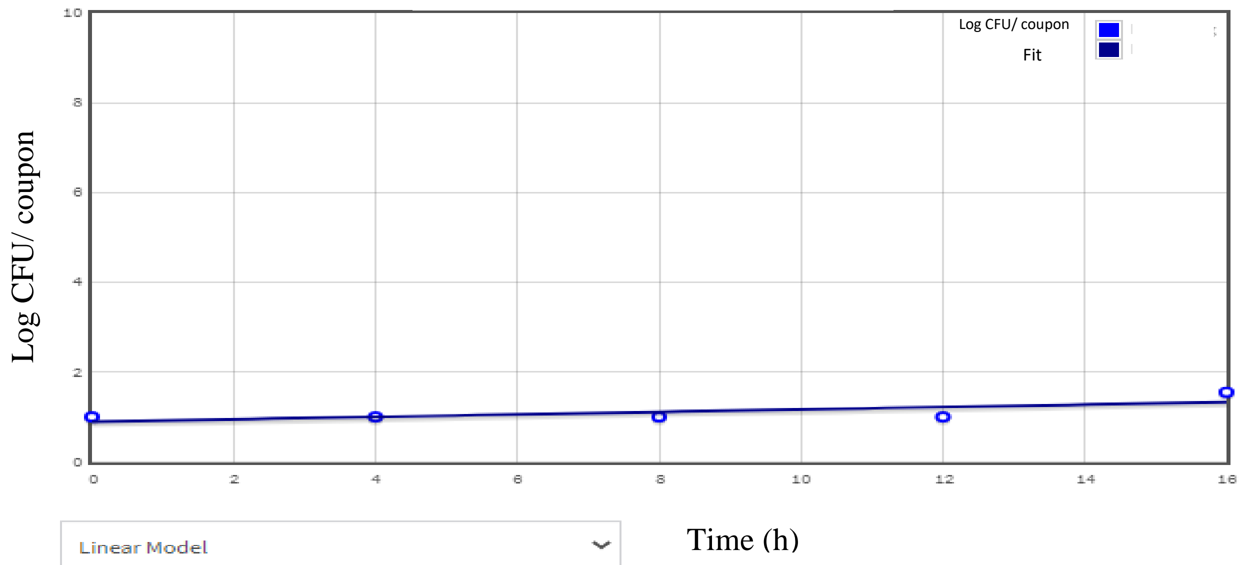
Time (h)

Baranyi and Roberts Model (no asymptote)

R-square:	0.965
SE of Fit:	0.451

Initial value	0.868 ± 0.422
Lag/shoulder	3.977 ± 1.754
Maximum Rate	0.474 ± 0.0704

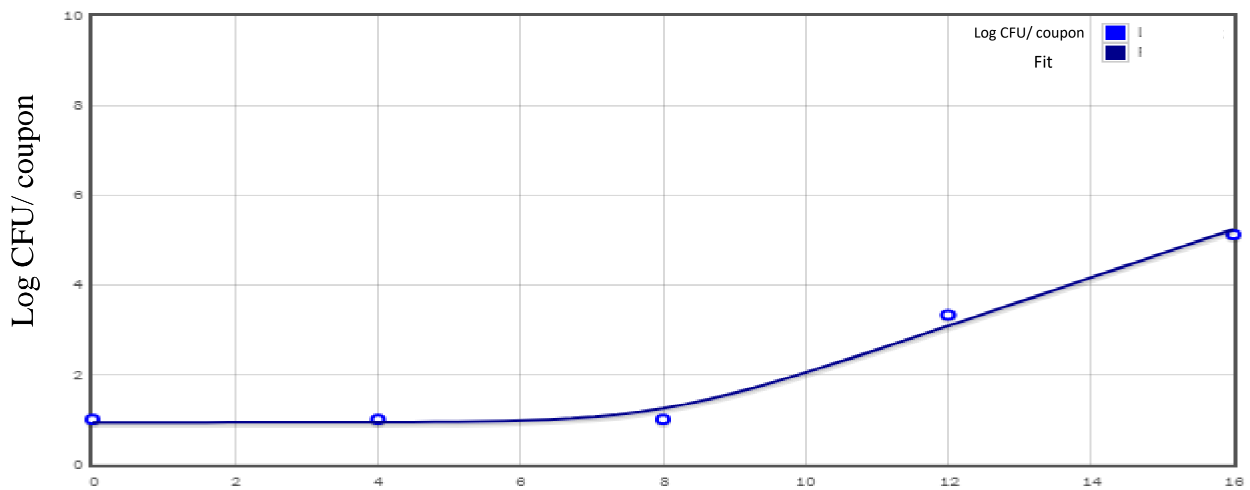
A1 55°C 20% Solids



R-square: 0.333
SE of Fit: 0.201

Initial value 0.89 ± 0.156
Maximum Rate 0.0275 ± 0.0159

A1 60°C 20% Solids



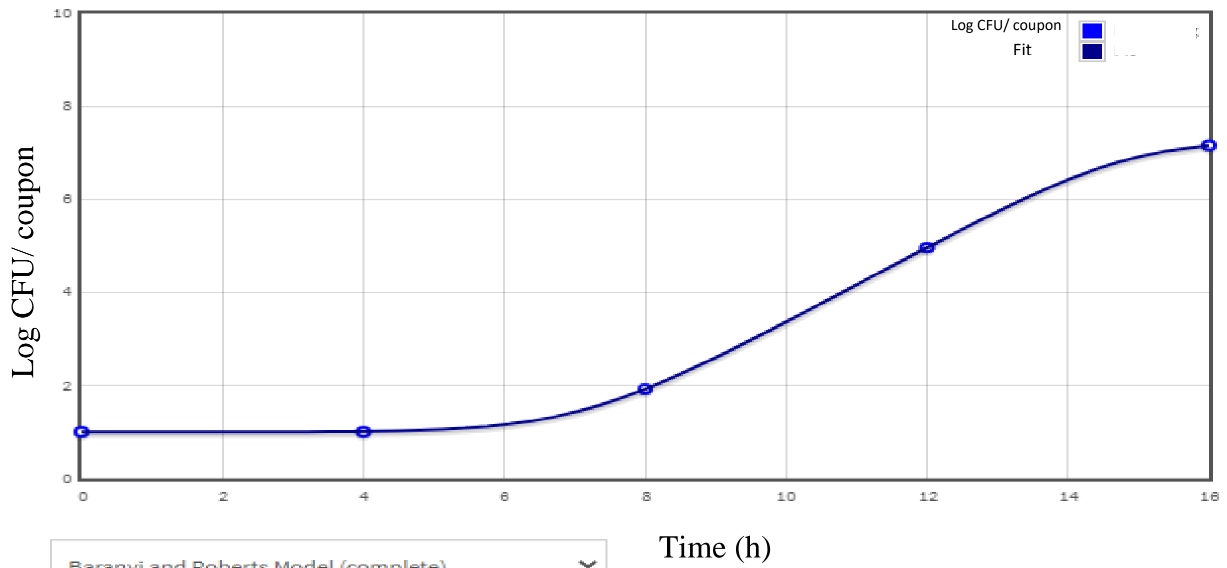
Baranyi and Roberts Model (no asymptote)

Time (h)

R-square: 0.98
SE of Fit: 0.267

Initial value 0.936 ± 0.185
Lag/shoulder 8.0239 ± 0.927
Maximum Rate 0.54 ± 0.0694

A1 65°C 20% Solids

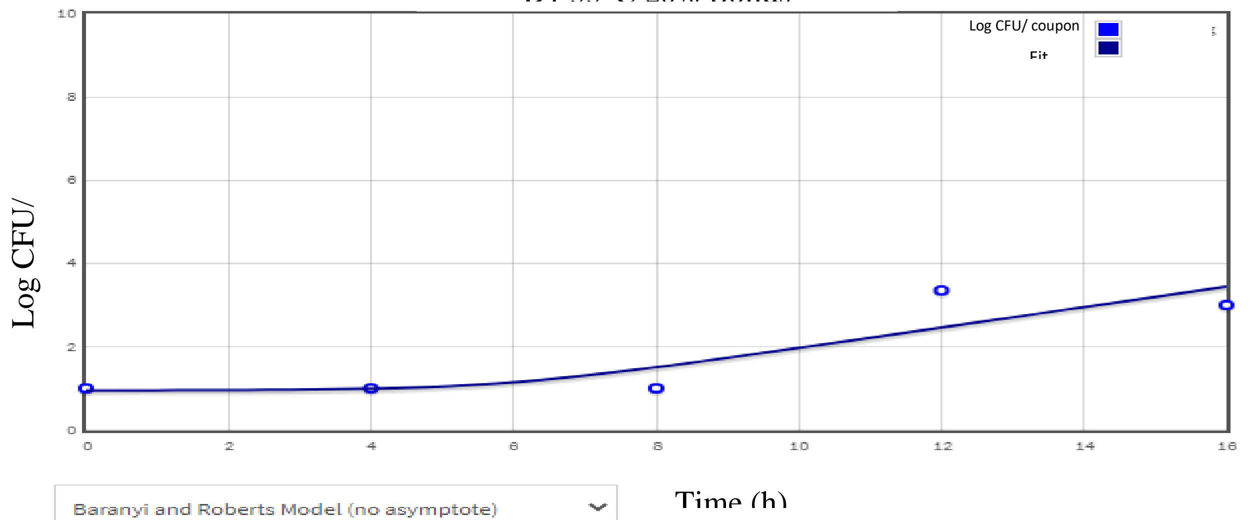


Baranyi and Roberts Model (complete)

R-square:	1
SE of Fit:	0.0117

Initial value	0.992 ± 0.00845
Lag/shoulder	7.112 ± 0.0287
Maximum Rate	0.815 ± 0.00506
Final Value	7.286 ± 0.0168

D1 55°C 20% Solids

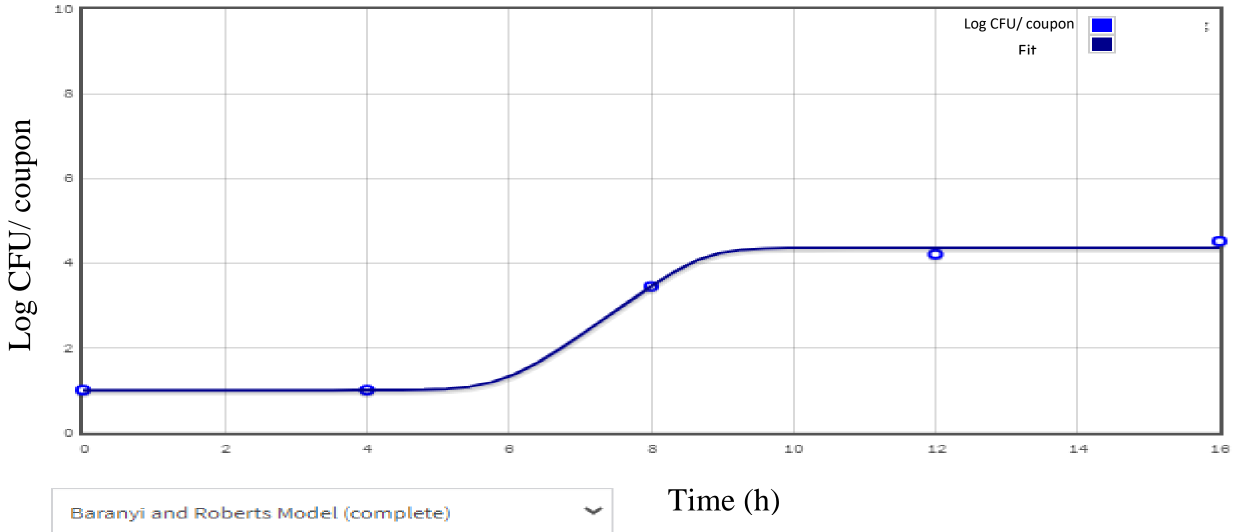


Baranyi and Roberts Model (no asymptote)

R-square:	0.565
SE of Fit:	0.79

Initial value	0.95 ± 0.613
Lag/shoulder	5.875 ± 5.486
Maximum Rate	0.246 ± 0.149

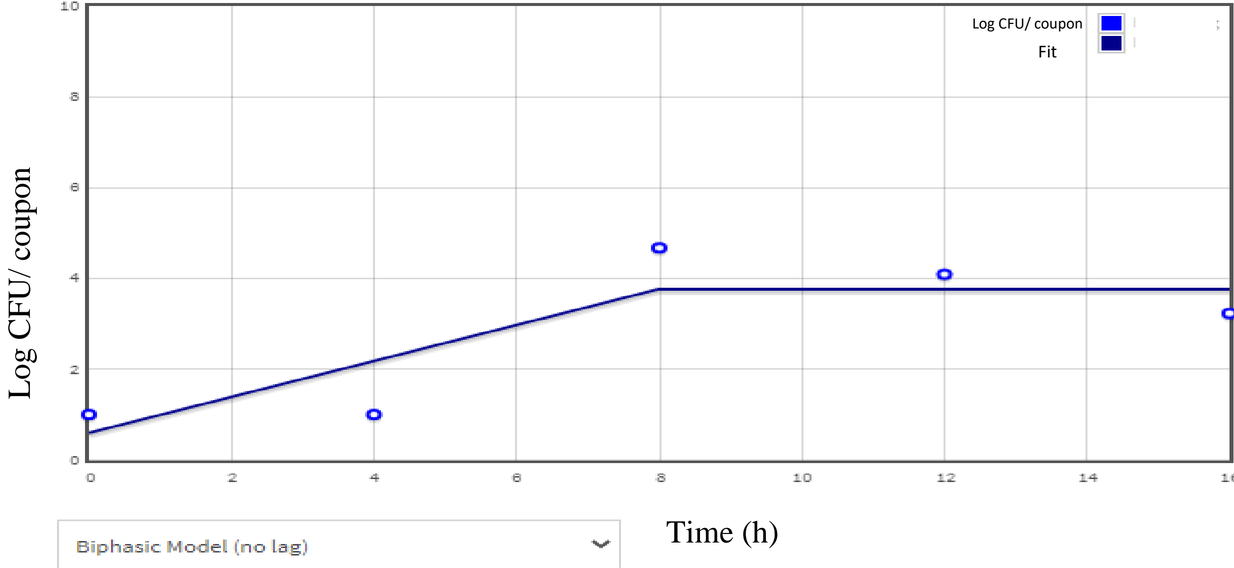
D1 60°C 20% Solids



R-square:	0.984
SE of Fit:	0.219

Initial value	0.999 ± 0.219
Lag/shoulder	5.93 ± 49.234
Maximum Rate	1.194 ± 28.359
Final Value	4.365 ± 0.155

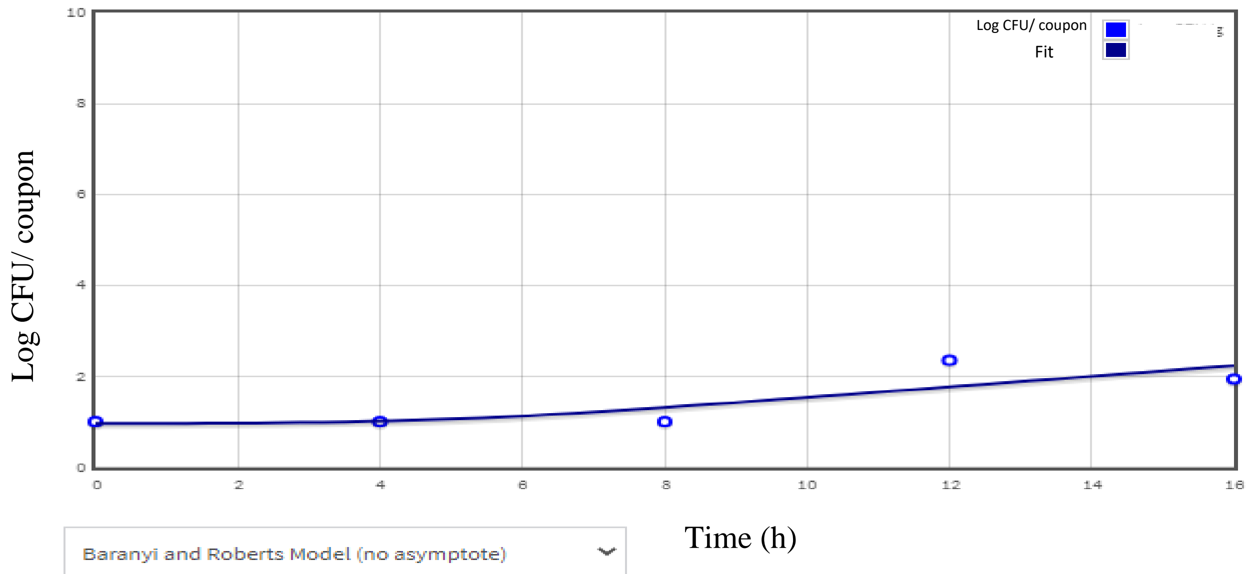
D1 65°C 20% Solids



R-square:	0.532
SE of Fit:	1.177

Initial value	0.597 ± 1.0741
Maximum Rate	0.396 ± 0.208
Tmax	8 ± 3.436

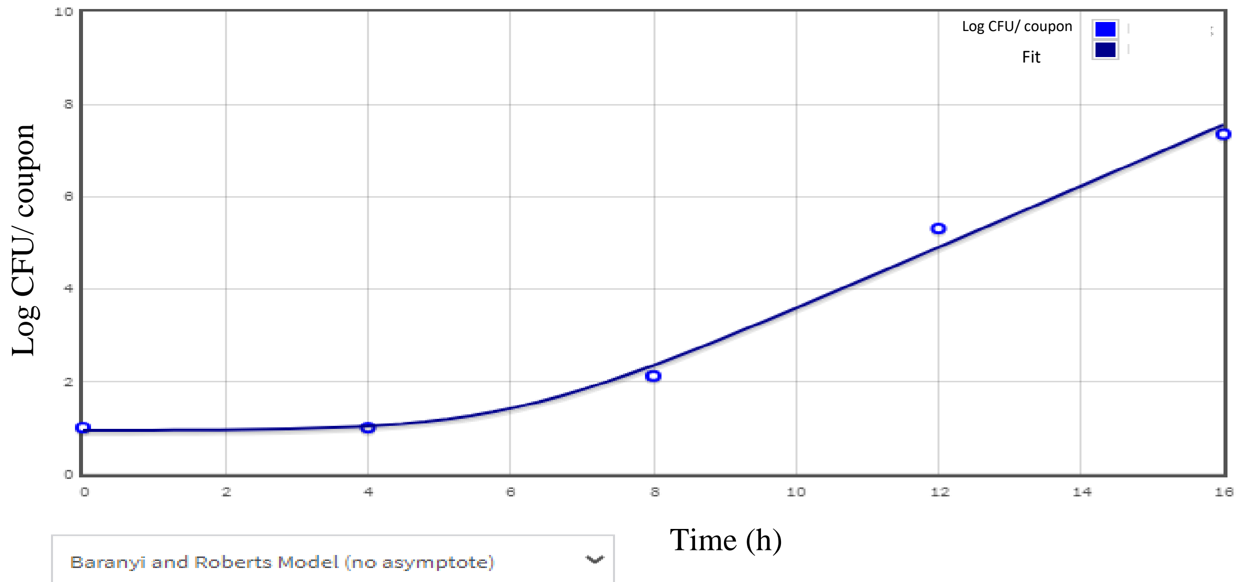
P3 55°C 20% Solids



R-square:	0.365
SE of Fit:	0.513

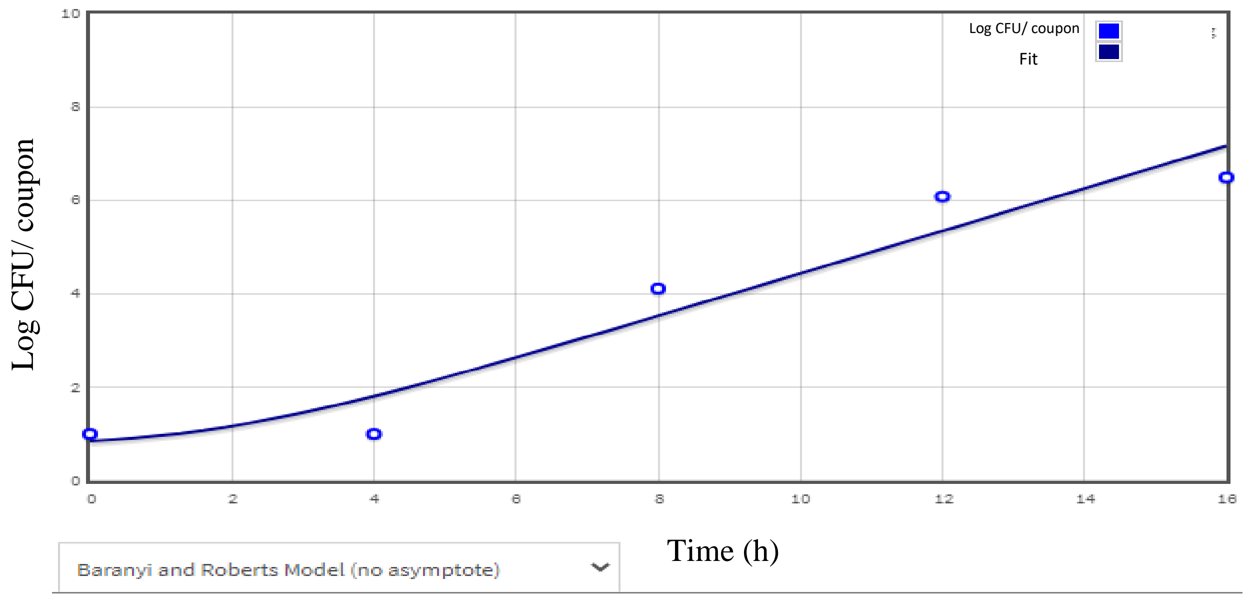
Initial value	0.963 ± 0.438
Lag/shoulder	5.102 ± 8.121
Maximum Rate	0.116 ± 0.0914

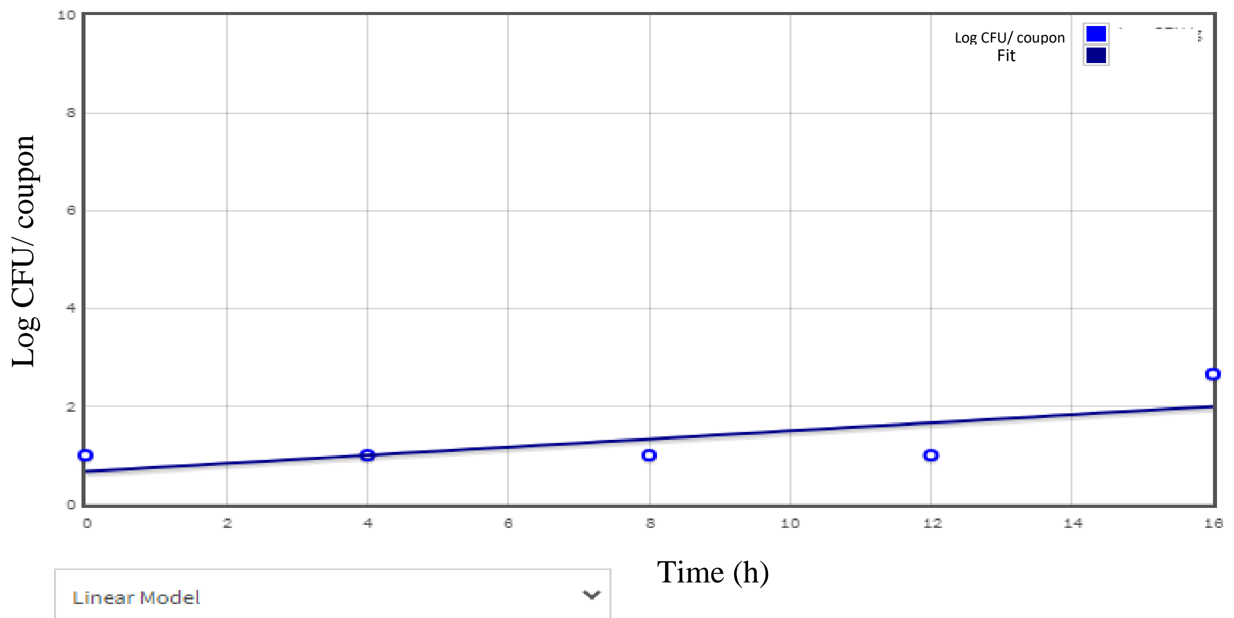
P3 60°C 20% Solids



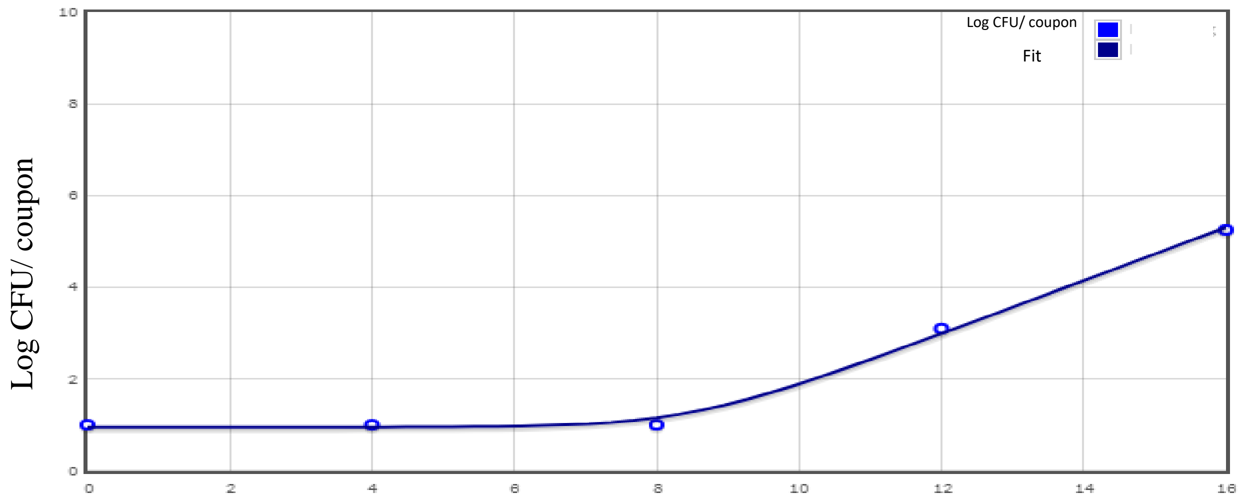
R-square:	0.984
SE of Fit:	0.364

Initial value	0.94 ± 0.28
Lag/shoulder	6.0341 ± 0.933
Maximum Rate	0.664 ± 0.0694


P3 65°C 20% Solids
Log CFU/ coupon
Fit
Log CFU/ coupon
Time (h)
Baranyi and Roberts Model (no asymptote)
R-square: 0.857
SE of Fit: 1.00388
Initial value 0.842 ± 0.996
Lag/shoulder 2.121 ± 3.714
Maximum Rate 0.455 ± 0.127


ATCC 12980 55°C 20% Solids
Log CFU/ coupon
Fit
Log CFU/ coupon
Time (h)
Linear Model
R-square: 0.333
SE of Fit: 0.606
Initial value 0.668 ± 0.47
Maximum Rate 0.083 ± 0.0479

ATCC 12980 60°C 20% Solids

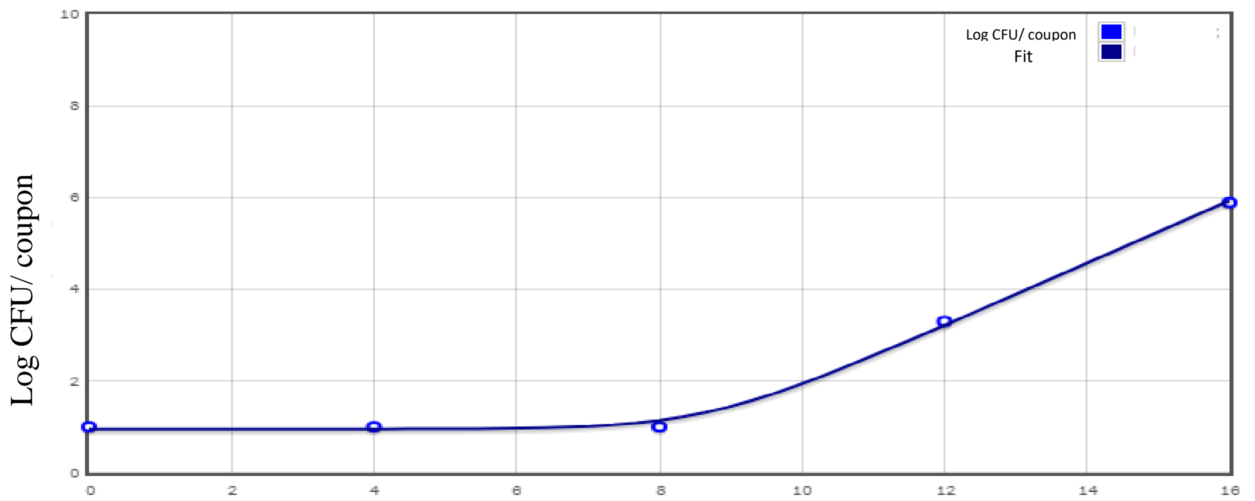


Baranyi and Roberts Model (no asymptote)

Time (h)

R-square:	0.994
SE of Fit:	0.148
Initial value	0.949 ± 0.0992
Lag/shoulder	8.494 ± 0.51
Maximum Rate	0.58 ± 0.044

ATCC 12980 65°C 20% Solids



Baranyi and Roberts Model (no asymptote)

Time (h)

R-square:	0.996
SE of Fit:	0.13
Initial value	0.95 ± 0.0853
Lag/shoulder	8.688 ± 0.386
Maximum Rate	0.681 ± 0.0405

7.6. Consolidated curve fit parameters

Maximum growth rate and lag phase of biofilm formation of A1, D1, P3 and ATCC 12980 in UHT skim milk at 55, 60 and 65°C

Strain	Total solids concentration (%w/v)	Temperature (°C)		
		55	60	65
A1	10	0.51 ± 0.07 (4.5 ± 1.0)	0.71 ± 0.07 (2.6 ± 0.63)	0.52 ± 0.21 (1.4 ± 3.3)
	20	0.35 (12.21)	0.37 ± 0.04 (4.3 ± 1.4)	0.42 ± 0.05 (3.6 ± 1.5)
D1	10	0.83 ± 0.08 (3.2 ± 0.49)	0.81 ± 0.05 (no lag)	0.91 ± 0.07 (no lag)
	20	0.49 ± 0.02 (5.2 ± 0.23)	0.31 ± 0.09 (no lag)	0.37 ± 0.19 (no lag)
P3	10	0.46 ± 0.18 (1.4 ± 5.1)	0.47 ± 0.18 (1.5 ± 5.1)	0.63 ± 0.07 (no lag
	20	0.22 ± 0.04 (7.5 ± 1.5)	0.59 ± 0.05 (5.7 ± 0.82)	0.47 ± 0.14 (2.4 ± 4)
ATCC 12980	10	0.46 ± 0.06 (4.5 ± 0.91)	0.46 ± 0.12 (3.8 ± 3.1)	0.43 ± 0.04 (2.4 ± 0.89)
	20	0.08 ± 0.05 (no lag)	0.64 ± 0.07 (8.1 ± 0.79)	0.64 ± 0.06 (8.3 ± 0.66)

* All values are mean ± standard deviation

* Lag phase values are represented within ( )

* some values have a high standard deviation which is to be noted carefully

Spore formation:

Maximum growth rate and lag phase of spore formation of A1, D1, P3 and ATCC 12980 in UHT skim milk at 55, 60 and 65°C

Strain	Total solids concentration (%w/v)	Temperature (°C)		
		55	60	65
A1	10	0.40 ± 0.07 (7.6 ± 1.3)	0.79± 0.12 (4.4 ± 0.95)	0.81 ± 0.02 (5.5 ± 0.12)
	20	0.02 ± 0.01 (no lag)	0.54 ± 0.06 (8 ± 0.92)	0.81 ± 0.05 (7.1 ± 0.02)
D1	10	0.95 ± 0.08 (6.9 ± 0.39)	1 ± 0.1 (1.7 ± 0.33)	1 ± 0.80 (1.1 ± 2.3)
	20	0.24 ± 0.14 (5.8 ± 5.4)	1.1 (5.93)	0.39 ± 0.2 (no lag)
P3	10	0.46 ± 0.16 (1.3 ± 4.9)	1.8 ± 14 (5.7 ± 17.5)	1.4 ± 0.12 (3.9 ± 0.39)
	20	0.11 ± 0.09 (5.1)	0.66 ± 0.06 (6 ± 0.93)	0.45 ± 0.12 (2.1)
ATCC 12980	10	0.39 ± 0.02 (8.7 ± 0.35)	0.88 ± 0.05 (8.6 ± 0.4)	0.47 ± 0.04 (3.9 ± 1.75)
	20	0.08 ± 0.04 (no lag)	0.58 ± 0.04 (8.4 ± 0.51)	0.68 ± 0.04 (8.6 ± 0.38)

* All values are mean ± standard deviation

* Lag phase values are represented within ( )

* some values have a high standard deviation which is to be noted carefully