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**Probability-of-growth modelling to optimize the use of hurdle
technology to achieve microbiological stability of high moisture
processed cheese**

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Food Technology

at Massey University, Palmerston North,

New Zealand

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2014

Abstract

This thesis reports a study of the application of hurdle technology to high moisture, low acid ambient shelf-stable hot packed processed cheese analogue (PCA). Hurdle technology makes use of a combination of mild stress factors. A combination of these stress factors can be more effective in inhibiting or inactivating the growth of micro-organism than individual stress factors. The current study focused on the application of hurdle technology to inhibit the growth of the food pathogen, *Clostridium botulinum* (*C. botulinum*). This micro-organism poses a hazard for consumers and is capable of growth in low acid food (pH > 4.5). As there are difficulties in working with *C. botulinum* in laboratory trials, *Clostridium sporogenes* (*C. sporogenes*) was used as an analogue of *C. botulinum*. *C. sporogenes* is very similar to *C. botulinum* in growth characteristics but is not dangerous.

The effectiveness of selected preservatives on the growth of the target micro-organism was expressed as the probability of growth and was modelled as function of the concentrations of the selected preservatives in nutrient broth. Nutrient broth was initially used as it can be easily and accurately adjusted and controlled in terms of composition, and allows more rapid growth than is observed in PCA. A combination of salt (sodium chloride), sorbic acid (in the form of potassium sorbate), nisin and lysozyme was selected as stress factor. The inhibitory effect of these preservatives was then observed in the high-moisture nutrient broth at pH 7 (the optimum condition for spore of *C. sporogenes* to germinate) at 37°C for eight weeks. It was found that lysozyme did not have a significant inhibitory effect on *C. sporogenes* in combination with salt, potassium sorbate and nisin. Therefore, the inhibitory effect of salt, sorbic acid and nisin at two different pHs (5.5 and 7) were subsequently evaluated in the nutrient broth at 37°C for eight weeks.

The probability of growth of *C. sporogenes* was modelled as a function of the concentrations of these selected preservatives at each pH. The results demonstrated that a combination of salt, nisin and potassium sorbate at relatively low concentrations can be used to inhibit growth. The inhibitory effects of the preservatives were pH dependent and their inhibitory effect is higher at pH 5.5. The developed models were validated

using a fresh data set. Finally, the applicability of the developed model was checked in high moisture PCA. The results showed that the developed broth model underestimated the probability of growth in the PCA. Therefore, a specific probability of growth model was developed for the PCA using the PCA instead of nutrient broth as the growth medium. This model accurately predicted the probability of growth of *C. sporogenes* in the PCA for given combinations of preservative concentrations.

The model developed for PCA allows the relative levels of preservatives to be easily quantified without the need for time consuming and expensive experimental work. The model would have limitations in the case of strongly varying formulations, since minor changes in processed cheese formulation or its production, could significantly alter its ability to support toxin production. Therefore, the model is applicable only to PCAs that have formulations similar to that used in this study. The general approach described in this thesis could be applied in the development of other high moisture, low acid foods.

Acknowledgments

This research has been possible with the collaboration of amazing people I have met along the way. After four years of PhD adventure, I am glad to have the opportunity to express my gratitude to all of you.

My deepest sense of gratitude is to my main supervisors, Professor Steve Flint and Dr. Owen McCarthy, for all their experience and knowledge they have shared with me throughout the entire period of my doctoral study. I thank them for their conscientious guidance, advice and their intensive review of different documents, including this thesis, during my PhD. It was a rewarding experience to work under their supervision. Their comments were always constructive and positive, guiding me in completing this thesis with a thorough understanding. I would like to thank my co-supervisors Professor Matt Golding and Dr. Jon Palmer for all their support and suggestions during my doctoral study. I am deeply grateful to the Institute of Food Nutrition and Human Health (IFNHH) and Dr. Owen McCarthy for supporting financially this research, some of my training and my PhD scholarship.

I am also thankful to Professor Mark Tamplin for giving me the opportunity to spend some time at the Food Safety Centre at the University of Tasmania. Special thanks to Assoc. Professor Tom Ross for his valuable scientific input and advice during the paper publications. I also acknowledge Emeritus Professor David Ratkowsky and Professor Tom McMeekin for their insightful statistical discussions and wise suggestions during my time at University of Tasmania. Many thanks also to Professor Hugh Morton, Massey University and Duncan Hedderley, Plant and Food Research for helping me with experimental design and statistical analysis.

I am indeed thankful to Ann-Marie Jackson for being an excellent lab manager, providing training, ordering chemicals at the right time and for technical help. I am sincerely grateful to Julia Good for helping me anytime in Micro-suite Lab. I am thankful to all the staff in the IFNHH for their help during this period of my study. Thanks to all my office mates especially Sandra and Yap Sia for their support and company. I express my deep sense of gratitude to Faegheh, Lara, Christine, Heather,

Ximenita, and Fleur for their encouragement and inspiration, always helping me to keep thinking positive even in a discouraging phase of my study. Heartfelt thanks to Anna Wildey for her enormous support and proof assistance.

I am extremely grateful to the pillar of my life, my parents, for their constant motivation and endless guidance. I am deeply thankful to my sister, Elahe, for her invaluable emotional support and encouragement throughout my PhD study. The last but not the least, eternal gratitude goes to my husband, Sina, as the thesis would not have been completed without his constant support and his sacrifice.

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List of publications

Peer-reviewed papers

Khanipour, E., McCarthy, O., Flint, S., Golding, M., Palmer, J. and Tamplin, M. Evaluation of the effects of salt, potassium sorbate, nisin and lysozyme on the probability of growth of *Clostridium sporogenes*, *International Journal of Food Science and Technology*, 2014, 49(6), 1506-1512.

Khanipour, E., McCarthy, O., Flint, S., Golding, M., Palmer, J. Ratkowsky, D. A., Ross, T. and Tamplin, M. (2014). Modelling the combined effects of salt, sorbic acid and nisin on the probability of growth of *Clostridium sporogenes* in nutrient broth. Submitted for publication in *International Journal of Microbiology*.

Khanipour, E., McCarthy, O., Flint, S., Golding, M. & Palmer, J. and Tamplin, M. (2014). The growth probability model of *Clostridium sporogenes* as a function of salt, sorbic acid and nisin in processed cheese (In preparation).

Conference presentations

Khanipour, E., McCarthy, O., Flint, S., Golding, M. & Palmer, J. Production of a cocktail of spores of *Clostridium sporogenes* for growth trials in new generation foods. New Zealand Microbiology Society (NZMS) Conference, Convention Center, Palmerston North, 26-28 November 2011 (Poster presentation).

Khanipour, E., McCarthy, O., Flint, S., Golding, M. & Palmer, J. The combined effect of salt, nisin, sorbate and lysozyme on the survival of *Clostridium sporogenes* in broth. New Zealand Microbiology Society (NZMS) Conference, Otago University, Dunedin, 26-28 November 2012 (Poster presentation).

Khanipour, E., McCarthy, O., Flint, S., Golding, M. & Palmer, J. The combined effects of salt, nisin and potassium sorbate at different pHs on the survival of *Clostridium sporogenes* in broth. New Zealand Institute of Food Science and

Technology (NZIFST) Conference, Hawkes Bay Opera House, Hastings 2-4 July 2013 (Poster presentation).

Khanipour, E., McCarthy, O., Flint, S., Golding, M. & Palmer, J. Modelling the growth/no growth of *Clostridium sporogenes* spores in broth as a function of salt, nisin and potassium sorbate concentrations at two pHs. International Conference on Predictive Modeling in Foods (ICPM) Conference, the Institute Pasteur, Paris, 16-20 September 2013 (Poster presentation).

Khanipour, E., Flint, S., McCarthy, O., Golding, M. & Palmer, J. How does the combination of common preservatives affect the survival of *Clostridium sporogenes*. Second Food Symposium. Research Presentation, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, 15 November 2013 (oral presentation).

Khanipour, E., Flint, S., McCarthy, O., Golding, M. & Palmer, J. Probability-of-growth modelling to optimize the use of hurdle technology to achieve microbiological stability of high moisture food product. University of Tasmania's Postgraduate Research meeting, 25 November 2013, Sandy Bay Campus, Hobart, Tasmania (oral presentation).

Khanipour, E., Flint, S., McCarthy, O., Golding, M. & Palmer, J. Evaluation of the effect of salt, potassium sorbate and nisin on the probability of growth of *Clostridium sporogenes*. University of Tasmania's Food Safety Centre Forum, 2-3 December 2013, Sandy Bay Campus, Hobart, Tasmania (oral presentation).

“All models are wrong but some are useful”

George E. P. Box 1979

Chapter One. Introduction

1.1. Introduction

Many foods are very vulnerable to microbial spoilage because they contain sufficient nutrients for microbial growth. The purpose of food preservation is to create a hostile environment for micro-organisms in order to prevent their growth. However, applying severe preservation techniques to prevent the growth of these micro-organisms may impair the nutritional quality of the food. The demand from consumers and manufacturers for high quality and natural food has led to the use of mild preservation techniques such as hurdle technology. Hurdle technology was developed by Leistner (1995) as a means to extend the shelf stability of food while maintaining quality. This technology uses a combination of preservative factors instead of just one, in order to allow a smaller amount of preservatives to be used.

The objective of hurdle technology is to select and combine preservation factors in such a way that microbial stability and safety can be assured. In recent decades, microbiologists and technologists have been working together in food design to determine the type and intensity of hurdles that should be applied to achieve the safety and stability of particular food products. The effect of any one preservative tends to be better when used in combination with one or more other preservatives, than when used by itself. The current study builds on hurdle technology. Demands from health conscious consumers for low salt processed cheese products have led to cheese formulations with higher water activities. In addition, retail distributors of hot packed processed cheese products store the product at ambient temperature. The possible alteration of processed cheese formulations and storage of the products at ambient temperature can compromise their microbial safety and stability, which could be maintained instead by the use of a combination of preservatives. Processed cheese has been a topic of interest to the food scientist as this product traditionally contains a high amount of salt in order to ensure its safety. High salt diets have negative effects on health such as high blood pressure, stroke, heart attack and cardiovascular diseases. Therefore, this study focuses on a combination of commonly used preservatives in order

to reduce the concentration of salt in a high moisture and ambient self-stable processed cheese.

Pasteurized processed cheese has a moisture content of 52-60%, high pH (generally pH 5.4 to 6) and is classified as a low acid food (Tanaka et al., 1986). The botulinum hazard increases if high moisture processed cheese is kept at ambient temperature. Therefore, preservation of processed cheese needs to ensure safety against the growth of *Clostridium botulinum* (*C. botulinum*). Although processed cheeses are not sterile, they have a superb safety record with regard to botulism. This frequently is achieved by adding high amounts of salt (U. S. Patent No. 5670197, 1997) . Salt levels used in processed cheese are generally 5-8% (Ter Steeg, Cuppers, Hellemons, & Rijke, 1995). A few outbreaks, involving botulinum from the consumption of canned processed cheese, have been reported (Glass & Doyle, 2005). All samples related to the outbreaks were found to have high water activity (a_w) (about 0.96 to 0.97) and high pH (about 5.7 to 5.8), which might have been the cause of the production of botulinum toxin in the processed cheese during storage (Briozzo, Amato De Lagarde, Chirife, & Parada, 1983; Glass & Doyle, 2005). Reducing the salt to satisfy consumer needs means, there is an increased risk of such outbreaks. In order to produce such products, one solution to the increased food safety risk, is to use a combination of preservatives. This study was to determine combinations of preservatives to achieve high moisture, ambient shelf-stable processed cheese.

The effectiveness of selected preservatives at preventing the growth of a target micro-organism can be expressed as the probability of a micro-organism being able to grow and produce toxins. In this study, mathematical probabilities of growth models were to be used in order to quantify the effect of the selected preservatives on the probability of growth of *C. botulinum*. The advantage of this model is that it can be used to predict the probability of growth as affected by changes in the levels of the selected preservatives and minimize the use of time consuming product challenge tests. Such models enable the design of safe products by adjusting the concentration of these preservatives in the food formulation. The target micro-organism in this research was *C. botulinum* but there are difficulties in working with this highly potent food poisoning micro-organism in

laboratory trials. *Clostridium sporogenes* (*C. sporogenes*) is very closely related to *C. botulinum* in growth characteristics but is not dangerous. Therefore, *C. sporogenes* was used in this project. *C. sporogenes* is often used to represent *C. botulinum* in studies aimed at controlling the growth of this food borne pathogen (Brown, Tran-Dinh, & Chapman, 2012; Goldoni et al., 1980).

Some studies have investigated and modelled the influence of moisture, pH, disodium phosphate, and salt levels on toxin production in processed cheese (Glass & Doyle, 2005; Tanaka et al., 1986; Whiting, 1995). However, studies on the modelling of the effect of bacteriocins such as nisin in combination with other factors are rare. Currently, no models exist to describe the effect of nisin with other preservatives, such as sorbic acid, on the probability of growth of *C. botulinum*. Nisin and sorbic acid are particularly effective in preventing the outgrowth of spores of *C. botulinum* and are added to cheese to prevent the outgrowth of clostridial spores (Scott & Taylor, 1981a). Therefore, studying the inhibitory effect of these preservatives was of interest.

1.2. Importance of the study

A new generation of high moisture and ambient shelf-stable products have potential financial benefits for a company. In addition, consumers are demanding healthier processed products. This research has commercial importance as dairy companies are interested in producing an ambient stable dairy spread product with a high moisture content that can be distributed and stored without refrigeration. This project aimed to achieve the formulation of a processed cheese product which is high in moisture and low in salt.

1.3. Research hypotheses and aims

The hypothesis for this project was that a high moisture, low acid ambient shelf-stable processed cheese can be made by using combinations of common preservatives. In order to verify this hypothesis the following aims were defined:

- 1) To model the inhibitory effect of the selected preservatives (salt, sorbic acid, nisin and lysozyme) on *C. sporogenes* growth in high moisture and low acid nutrient broth (Chapter 4).
- 2) To develop models of the probability of growth in nutrient broth under the combined effect of selected preservatives.
- 3) To validate one developed model using combinations of the selected preservatives not explicitly investigated experimentally, for growth in nutrient broth.
- 4) To determine the applicability to processed cheese analogue (PCA) of the developed broth probability of growth model for *C. sporogenes*.
- 5) To model the combined effects of the selected preservatives on the probability of growth of *C. sporogenes* in PCA.
- 6) To validate the developed model using combinations of the selected preservatives not explicitly investigated experimentally for growth in the PCA.

1.4. Thesis outline

This thesis includes a review of background information from previous relevant studies (Chapter 2). Chapter 2 covers the principles of hurdle technology and how processed cheese production and ingredients affect the safety of this product. The final sections of this chapter explore predictive microbiology and its application in quantifying the inhibitory effect of hurdles on the growth of micro-organisms.

Chapter 3 describes the general materials and methods used during this research.

Chapter 4 describes the inhibitory effect of four preservatives: salt, sorbic acid, nisin and lysozyme on *C. sporogenes* at the optimum condition for spore of *C. sporogenes* to grow (pH 7) in nutrient broth.

Chapter 5 models the inhibitory effect of salt, sorbic acid and nisin at two different pHs on the growth of *C. sporogenes* in nutrient broth at 37°C. The probability of growth of *C. sporogenes* can be calculated by the developed models as a function of the concentrations of these preservatives for each pH individually. The developed broth models were to be validated using new combinations of preservative which had not been used in the earlier experiments.

Chapter 6 describes the applicability of the developed broth models to *C. sporogenes* growth in PCA.

Chapter 7 models the inhibitory effect of salt, sorbic acid and nisin on the growth of *C. sporogenes* in high moisture and ambient shelf-stable processed cheese analogue at pH 5.5. Finally, the developed model for the processed cheese was validated using new combinations of the preservative concentrations which had not been used in the earlier processed cheese experiments.

The final chapter (Chapter 8) in this thesis provides the summary of the main findings of this research and provides recommendations for future study.

Chapter Two. Literature Review

2.1. Introduction

The principles of hurdle technology and how processed cheese production and ingredients affect the safety of this product are reviewed. The final section of this chapter explores predictive microbiology and its application in quantifying the inhibitory effect of hurdles on the growth of micro-organisms.

2.2. Principles of hurdle technology

Hurdle technology applies a number of mild preservation techniques to achieve a high level of product safety and stability (Alokomi, Skytta, Helandr, & Ahvenaianen, 2000). The hurdle concept has been utilized for stabilizing food with minimal processing. The aim of hurdle technology is to produce foods of high quality that can remain safe and stable even at ambient temperatures. The use of different preservative factors in combination causes minimal damage to product quality and maintains food in a stable and safe condition with acceptable nutritional and organoleptic properties (Leistner, 1978, 2000). The required intensity of hurdles is based on the particular food product and the micro-organisms which are present in that food (Alokomi et al., 2000).

2.2.1. Effect of interaction hurdles

Different combinations of preservative factors can have different microbial effects and have been used as hurdles in the food industry to produce ambient shelf-stable foods. Scientists usually rely on more than one factor to inhibit microbial growth (i.e. multiple hurdle technology) through interaction or synergistic effects (Cleveland, Montville, Nes, & Chikindas, 2001). A synergistic effect means different hurdles in food have an intensifying effect on each other. For example, a_w , pH and preservatives in the food have different targets within the microbial cell (such as disturbance of cell membranes, DNA replication and enzyme systems) and disturb the homeostasis of micro-organisms in several ways. Micro-organisms must overcome different obstacles to start to grow. In this environment, micro-organisms are limited to maintaining homeostasis instead of

multiplying. In contrast, in additive effects, all preservatives have the same target; for instance, all might cause a depression of a_w . This results in no enhanced activity other than what is achieved through lowering the water activity. Therefore, in terms of food quality and safety, a synergistic effect is preferable (Raso & Barbosa-Canovas, 2003).

The preservation of high moisture, low acid, ambient shelf-stable food products that satisfy consumers and manufacturers, is microbiologically challenging. Product having these properties can be developed by using hurdle technology. This approach has been used in different low pH food products such as meat, dairy and canned food products. Shelf-stable meat products can be processed by thermal processing in a sealed container or by adjusting hurdles such as pH, a_w and food preservatives (Kanatt, Chawla, Chander, & Bongirwar, 2002). Processed cheese is also one of the most common ambient shelf-stable products that have a good safety record through using hurdle technology. Microbial safety and stability of processed cheese depends on composition, processing conditions and storage conditions. Demands from health conscious consumers have led to the development of low salt, high moisture processed cheese products. This increases the microbial food safety risk associated with these products. In addition, retail distributors of processed cheese products store the product at ambient temperature. This food safety risk can be mitigated by the use of combinations of preservatives. In the following section, processed cheese production and ingredients that affect safety of this product, are discussed.

2.3. Production of hot packed processed cheese

The idea for processed cheese originated from a desire to extend the shelf-life of natural cheese or to develop a new type of cheese which was milder in taste or more stable than traditional cheeses. Essential steps in the manufacture of processed cheese are melting and heating blends of natural cheese, the addition of emulsifying salts, agitation to produce a homogeneous mixture, followed by packaging and cooling. Heat inactivates starter culture micro-organisms and other bacteria, as well as enzymes present in natural cheese, producing a product with an extended shelf-life. Bacterial spores survive the heat treatment and therefore, are a concern in processed cheese. Pasteurized processed cheeses are classified as low acid foods ($\text{pH} > 4.6$). Low acid foods are considered to be

those with a pH that could allow growth of *C. botulinum*. Processed cheeses are not sterile, but they have a superb safety record with regard to botulism (Tanaka et al., 1986). This is because, for many years, processed cheese manufacturers have relied on high concentrations of sodium chloride to prevent botulism in these products.

Figure 2.1 provides a schematic diagram of the typical steps involved in the manufacturing of processed cheese (Nogueira de Oliveira, Ustunol, & Tamime, 2011).

Processed cheese includes 51-65% of one or more varieties of cheese (such as cheddar, Swiss, mozzarella, old English, Roka blue, etc.), emulsifier and other optional ingredients (Glass & Doyle, 2005). There is potential to produce processed cheese with various ingredients. Optional ingredients include cream, anhydrous milk fat, acidifying agents to adjust the pH, water, salt, artificial colours, enzyme modified cheese (EMC), nisin as a preservative and lecithin as an anti-sticking agent. The composition of processed cheese made with real cheese is difficult to control as with most biological materials, cheese changes with time. Therefore, the use of processed cheese analogues (PCA) facilitates the making of processed cheese of virtually constant composition with greater consistency and more economically than standard processed cheese. This is due to the fact that the processed cheese analogue does not contain real cheese, being made with protein from milk or plant sources and fat from plant sources. The protein sources included sodium and calcium caseinates, rennet casein, milk protein concentrate, and soybean and peanut protein isolates. Vegetable fats such as soya bean, palm kernel, cotton seed, peanut and corn oil are used to replace milk fat. Other common food-grade ingredients used in cheese analogues are water, salt, emulsifiers, acids (sorbic acid, lactic acid) and hydrocolloids (Bachmann, 2001). Formulation involves selecting the correct levels and types of ingredients to give the desired composition and optimal textural and melting properties to analogue cheese products. Each cheese manufacturer has their own specific cheese recipe, usually a closely guarded secret not released in the public domain. In Table 2.1 the range/limits of some key ingredients that have been used in published work are presented (O Riordan, Duggan, O Sullivan, & Noronha, 2011).

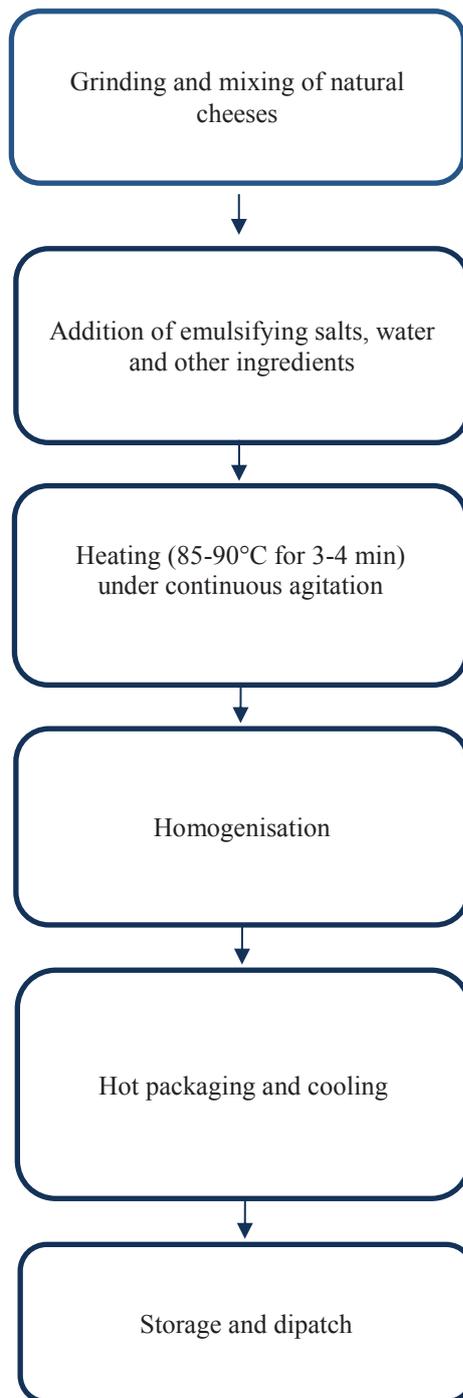


Figure 2.1. Flow diagram showing the stages of manufacturing processed cheese

Table 2.1. A range of ingredients used to formulate processed cheese analogue (Data from (O Riordan et al., 2011))

Ingredient	Additional level (g 100 g ⁻¹)
Water (including condensate)	43.5-60
Casein	11-28
Vegetable oil	0-28
Starch	0-25.8
Emulsifying salts	0.5-4
Acidifying agents	0.1-1.6
Sodium chloride	0.7-2
Preservatives	0.09-1.1
Stabilizer	0.35-5
Colors	0.2-2
Flavor	0.2-5

2.4. Ingredients that affect processed cheese safety

In the following sections, each ingredient individually and in combinations (in section 2.3.1.8) that affect processed cheese safety, will be discussed in detail.

2.4.1. Salt

Salt (sodium chloride) is widely used as a flavouring and preservative agent and the food industry relies on a high concentration of salt to produce ambient shelf-stable processed cheese. Salt has an inhibitory effect on the production of botulin in processed cheese. It has been reported that 8.2-10% w/w salt is necessary in order to inhibit the spores of *C. botulinum* (Lechowich, 1970). The studies of Tanaka (1986) indicated that when the a_w was 0.944 or below, no toxin was detected, and with the $a_w > 0.957$, all the processed cheese products developed toxicity. Between these two values, toxin formation was either negative or positive depending on other factors including salt, phosphate, moisture level and pH. There has been a trend towards reducing or limiting salt in food formulations for health reasons (Russell, Hugo, & Ayliffe, 1992). Russell et al. (1982) reported that salt at lower levels acts synergistically with nitrite, potassium

sorbate and benzoate. This is a promising result. It can be concluded that combinations of processed cheese ingredients can have synergistic effects. Any changes in processed cheese formulations should be done carefully to avoid producing an unsafe product.

2.4.2. pH and weak acids

The growth and metabolism of micro-organisms are affected by pH (Genigeorgis, 1981). Acidic pHs prevent the growth of important food spoilage and food poisoning micro-organisms but do not necessarily kill them (Russell & Gould, 2003). In general, pH directly influences not only the growth and survival of micro-organisms, but also the effects of commonly used preservatives, such as nisin and weak acids (Adams & Moss, 2008). The effect of pH is due either to its direct effect on the micro-organisms or due to the increased effectiveness of some preservatives at lower pH values. The minimum pH for the growth of some proteolytic *C. botulinum* strains is 4.6 to 4.8 and for non-proteolytic species it is 5.0 (Adams & Moss, 2008). A pH range of 5.0- 6.1 is optimal for good processed cheese analogue functionality (Aimutis, 1995).

Lactic acid and sorbic acid are two weak acids that are commonly used in processed cheese. Weak acid preservatives do not kill micro-organisms but inhibit their growth, due to prolonging the lag phase. The antimicrobial effectiveness of a weak acid is associated with the undissociated fraction (Glass & Doyle, 2005). These types of preservatives are more effective at low pH as this increases the concentration of the undissociated acid. The higher antimicrobial effect of the undissociated form of the acid is related to the lipophilic nature of the undissociated form which allows its rapid diffusion through the plasma membrane of the cell. The undissociated form will dissociate into H⁺ and the anion in the bacterial cell. The increased concentration of protons leads to a drop in the internal pH, inhibiting many metabolic functions, whereas the accumulation of the anions may create a high turgor pressure, inhibiting growth by disturbing cell metabolism (Lambert & Stratford, 1999; Stewart et al., 2001; Tienungoon, Ratkowsky, McMeekin, & Ross, 2000).

Lactic acid is a weak acid that prevents botulin formation, and its inhibitory activity is greater at lower pH (Tanaka et al., 1986; Ter Steeg et al., 1995). Sodium and potassium

lactate inhibit botulin production without reducing pH. Sodium lactate (1.5%) significantly delays botulin production in high moisture, low salt processed cheese, compared with a product without lactate (Glass & Johnson, 2004c).

Sorbic acid is another weak acid that inhibits Gram positive and Gram negative bacteria and also inhibits the germination of spores of *Bacillus* spp. and *Clostridium* spp. (Blocher & Busta, 1985). The low water solubility is a disadvantage of sorbic acid. Potassium sorbate is a commonly used salt of sorbic acid and it can be stored as crystals. Potassium sorbate is more convenient to use due to its high solubility in water. Potassium sorbate forms sorbic acid when it is dissolved (Sofos & Busta, 1981). The lower pH and undissociated acid are responsible for the inhibition of outgrowth of *C. botulinum* spores (Sofos & Busta, 1981). Sorbic acid should not be eliminated from a processed cheese formulation unless studies show that doing this is safe (Sofos, 1989).

The use of potassium sorbate in the preservation of more than 40 types of natural cheese and processed cheese products has been one of the most important applications of this preservative (Glass & Doyle, 2005). The antimicrobial activity of potassium sorbate generally increases with a reduction in pH, for the reason given above (Eklund, 1983). Potassium sorbate has been an effective inhibitor of *C. botulinum* in dairy products and its concentration should not exceed 0.2% in processed cheese (Glass & Doyle, 2005; Huhtanen, Talley, Feinberg, & Phillips, 1981). Potassium sorbate has optimum effectiveness at pH values below 6, while it is relatively ineffective at pH values of 7 and above (Sofos, 1989). Storage temperature also influences the inhibitory effect of potassium sorbate (Tompkin, Christiansen, Shaparis, & Bolin, 1974). A combination of potassium sorbate and salt has been recommended for products with a slightly high pH (pH > 4.5) (Tanaka et al., 1986; Yigit & Korukluoglu, 2007). A combination of potassium sorbate and nisin reduces and inhibits the growth of *Listeria monocytogenes* (*L. monocytogenes*) on beef stored under a carbon dioxide environment and in vacuum packed beef (Avery & Buncic, 1997). Citric acid also increases the inhibitory efficacy of potassium sorbate against different common deteriorative micro-organisms (Restaino, Komatsu, & Syracuse, 1981).

2.4.3. Nisin

Nisin is a low molecular weight polypeptide produced by *Lactococcus lactis*. Nisin is antibacterial against Gram positive bacteria, including spore formers, but it shows little or no activity against Gram negative bacteria (except when chelating agents are used), yeast and moulds (World Patent No. WO9723136 (A1), 1997) . Nisin binds to the phospholipid head groups of the bacterial cell membrane in Gram positive bacteria. It also prevents spore germination at the pre-emergent swelling stage, making the spores sensitive to heat processing (Delves-Broughton, 2005; Roberts & Zottola, 1993; Schillinger, Geisen, & Holzapfel, 1996). The other two modes of action of nisin are inhibiting cell wall synthesis and disturbing the activity of autolytic enzymes (Morris, Walsh, & Hansen, 1984). Higher concentrations of nisin are required to inhibit spores than vegetative cells (Russell & Gould, 2003). Nisin's action against spores is predominantly sporostatic rather than sporicidal.

Nisin is the only bacteriocin approved by the (food and drug administration) FDA for food use. It has been more widely used in processed cheese products than in any other food type. The amount of nisin should not exceed 250 ppm in cheese products according to (U. S. Department of Agriculture) USDA regulations (Zottola, Yezzi, Ajao, & Roberts, 1994). Roberts and Zottola (1993) determined the shelf-life of high moisture (60%) and low moisture (53%) processed cheese made from cheddar cheese, manufactured with a nisin producing cheese starter culture during ambient storage (22°C and 37°C). The results showed that nisin at less than 250 ppm extended the shelf life of processed cheese at both moisture contents. However, shelf life extension was much greater at 53% moisture than at 60%. Taylor et al. (1986) pointed out that nisin is an effective antibotulinal agent in high moisture processed cheese. It is effective in delaying or preventing *C. botulinum* in pasteurized processed cheese over a 48 week period at 30°C. High levels of nisin are required in those formulations of processed cheese that have higher moisture contents and lower levels of sodium chloride and phosphate (Somers & Taylor, 1987). Somers and Taylor (1987) showed that nisin produced shelf-stable processed cheese with 1.4% disodium phosphate and in the absence of salt. Processed cheese containing 58% moisture, 1.45% disodium phosphate

and 1.2% salt with 100 ppm nisin prevented outgrowth of and toxin production by *C. botulinum* (U.S. Patent No. 45841999, 1986) .

Temperature and pH can influence the efficacy of nisin. Nisin is stable when protected from direct sunlight and stored at temperatures not exceeding 22°C (Huhtanen & Feinberg, 1980; Jack, Tagg, & Ray, 1995; Parente, Giglio, Ricciardi, & Clementi, 1998; Restaino et al., 1981; Sofos & Busta, 1981; Yigit & Korukluoglu, 2007). Nisin is most effective at < pH 7 (Glass & Doyle, 2005; Scott & Taylor, 1981b). This is linked to the solubility and stability of nisin in acidic environments. Autoclaving at 121°C for 15 minutes reduces the activity of nisin whereas nisin is stable at pasteurization temperatures (Delves-Broughton, 2005; Rilla, Martinez, Delgado, & Rodriguez, 2003; Scott & Taylor, 1981a).

Nisin activity is also reduced by some components of complex media such as food systems (Henning, Metz, & Hammes, 1986). High fat contents (more than 20%) reduce nisin effectiveness because fat has a protective effect on micro-organisms (Jung, Bodyfelt, & Daeschel, 1992). The presence of cations such as Mg²⁺ and Ca²⁺ in foods may reduce the efficacy of nisin against Gram-positive bacteria. These cations interact with the negatively charged phospholipid groups in the cytoplasmic membrane of the cell and prevent inhibitory interactions between nisin and these groups (Abee, Rombouts, Hugenholtz, Guihard, & Letellier, 1994). The solubility of cation salts such as Mg²⁺ and Ca²⁺ in a food system increase by decreasing the pH and this can have an impact on nisin activity.

A combination of salt and nisin shows a significant inhibitory effect ($p < 0.05$) at pH 5.5 and 7 (Mazzotta, Crandall, & Montville, 1997; Parente et al., 1998). The presence of salt can enhance the preservative effect of nisin against *L. monocytogenes* (Harris, Fleming, & Klaenhammer, 1991; Thomas & Wimpenny, 1996). On the other hand, salt can reduce the inhibitory effect of nisin in preventing the outgrowth of *Bacillus lincheniformis* spores (Bell & Delacy, 1985). Bouttefroy et al. (2000) reported that salt reduced the inhibitory effect of nisin against *L. monocytogenes*. Conflicting results from previous studies on the effectiveness of salt and nisin in combination could be strain

dependent. Some micro-organisms including: *L. monocytogenes*, *Bacillus subtilis* (*B. subtilis*), *Bacillus licheniformis*, *Bacillus cereus* (*B. cereus*), *Salmonella aureus*, and *C. botulinum* show resistance to nisin (Mazzotta et al., 1997).

Nisin resistance of Gram-positive bacteria can occur either because they synthesize nisinase, which can inactivate nisin, or because the cells adapt to nisin. These adaptations develop from alterations in the cell envelope, at the level of the cytoplasmic membrane and peptidoglycan in the bacterial cell wall (Jarvis, 1967). Nisin sensitivity varies greatly among *Clostridium* strains and at different pHs (Montville & E., 1994; Rayman, Aris, & Hurst, 1981; Scott & Taylor, 1981a). The resistance of *Clostridium* spores to nisin can be reduced by lowering the pH (Rayman et al., 1981). It can be concluded that nisin individually cannot ensure the microbial safety of food. Nisin may help to reduce harsh preservation treatments and extend the shelf life of products when used in combination with other inhibitory factors.

2.4.4. Emulsifiers

Emulsifying salts may prevent the germination and outgrowth of *C. botulinum* by binding calcium (Karahadian, Lindsay, Dillman, & Deibel, 1985). The type of emulsifier may play an important role (Eckner, Dustman, & Rodriguez, 1994; Glass & Doyle, 2005; Karahadian et al., 1985; Tompkin, 1983). Phosphate emulsifiers inhibit growth and toxin production by *C. botulinum* by sequestering iron, magnesium and calcium ions. Phosphate in combination with potassium sorbate in culture media was more effective than either of these individually in delaying growth of *C. botulinum* (Ivey & Robach, 1976). Tanaka et al. (1986) showed that antibotulinal effects of salt and disodium phosphate (DSP) were similar and additive. Botulin production was delayed in media with 2% polyphosphate emulsifiers compared with controls, and media with DSP or disodium orthophosphate. There is evidence that the pH levels of products with polyphosphates were 0.15 to 0.35 pH units lower than those with DSP (Eckner et al., 1994; Glass, Kaufman, & Johnson, 1998). However, it is unlikely this small extent of pH reduction has a significant inhibitory effect. Polyphosphates may also interact physically with bacterial cells by forming channels in the cell wall and

increasing cell wall permeability to inhibitory compounds, which promotes leakage and cell lysis (Tompkin, 1983).

Sodium citrate emulsifiers have a less inhibitory effect on *C. botulinum* growth compared with sodium phosphate emulsifiers. The spoilage results of using different emulsifier salts in processed cheese showed that the cheese blends with citrate or orthophosphate had the least stability compared to those using polyphosphate (Karahadian et al., 1985; Tompkin, 1983). It can be concluded that emulsifying phosphates indirectly have antimicrobial properties in foods.

2.4.5. Fat

Fat decreases the efficacy of several components such as monolaurin, polyphosphate, nisin, sorbic acid, potassium sorbate and enzyme modified component (EMC) (McLay, Kennedy, O'Rourke, Elliot, & Simmonds, 2002). Botulin production was also delayed in high moisture, reduced fat (5%), and fat free (<1%) products compared with full fat (>20%) products with similar moisture content (59%), salt content, emulsifier content and pH (Glass & Doyle, 2005; Glass & Johnson, 2004b; Tanaka et al., 1986). Glass and Johnson (2004) investigated factors that contribute to the botulinal safety of reduced-fat and fat-free processed cheese. For example, no botulin was detected for up to 56 weeks in processed cheese products containing less than 5% fat, while botulin was detected in a similar but full-fat product after only four weeks storage. There have been several explanations for the apparent protective effects of fat. Fat may provide micro-environments for the bacteria that protect bacteria from antimicrobials in the water phase of a product. The lipophilic portion of some antimicrobials interacts with fat in full fat (> 20%) processed cheese products rather than with the phospholipids in bacterial cell membranes, thereby reducing their antimicrobial effects. For this reason, the antibotulinal effect is enhanced in reduced fat processed cheese compared with full-fat processed cheese (Glass & Doyle, 2005; Glass & Johnson, 2004a). Another possible reason that the reduced fat product delays botulin production is that some ingredients which have been used to replace fat, such as whey protein, might provide an antibotulinal system through binding water and reducing the a_w of the product (Glass & Doyle, 2005).

On the other hand, the study by Ter Steeg et al. in 1995 showed that fat does not have an antibotulinal effect and does not contribute to the botulinum stability of processed cheese. Increased fat levels more than 41% can reduce moisture without concomitant increase in stability. This can be due to the high percentage of fat, as in the study the level of fat varied from 0.1- 41% w/w.

2.4.6. Flavour enhancers and other food additives

No single component of processed cheese is responsible for the safety of this product. Rather, safety depends on a combination of ingredients. EMC is used as a flavour enhancer in cheese products. It can significantly delay botulinum toxin production in cheese products (Glass & Doyle, 2005). Glucono delta lactone (GDL) is used as a flavouring ingredient in reduced salt processed American cheese foods and spreads and has an inhibitory effect on *C. botulinum*. A study showed that no toxin could be detected in samples of cheese containing GDL, but reduced salt samples without GDL were toxic after 84 days at 30°C (Karahadian et al., 1985). GDL reduces the pH of cheese and helps to inhibit *C. botulinum*.

Lysozyme is an enzyme present in milk, eggs, tears and other secretions. It is used in cheese, frankfurters, cooked meat and poultry products and inhibits the growth of *C. botulinum* and other Gram positive bacteria. Lysozyme degrades the cell wall by attacking peptidoglycans and hydrolysing the glycosidic bond that connects N-acetylmuramic acid with the fourth carbon atom of N-acetylglucosamine (Delves-Broughton, 2005; Graham, Mason, & Peck, 1996).

Lysozyme and nisin in combination have a synergistic inhibitory effect against Gram positive bacteria. The combination attacks two different targets in the bacteria. Nisin disturbs the cell membrane while lysozyme cause hydrolysis of peptidoglycan in the cell wall (Chung & Hancock, 2000; Leistner & Gorries, 1995). The bactericidal effects on *L. monocytogenes* using 10 International Units (IU) of nisin combined with 25 µg/mL of lysozyme were similar to those obtained with either 200 IU of nisin or 50 µg/mL of lysozyme used alone (European Patent No. 89123445, 1982; Chung &

Hancock, 2000; Gill & Holley, 2000; Nattress, Yost, & Baker, 2001; Roller & Board, 2003). A combination of nisin and lysozyme used to inhibit *Brochothrix thermosphacta* (*B. thermosphacta*) was a more effective antimicrobial than either nisin or lysozyme individually (Nattress et al., 2001). Lysozyme in combination with sorbic acid has a synergistic effect (Akashi & Oono, 1972).

2.4.7. Effect of Maillard reaction products

The Maillard (non-enzymatic browning) reaction, a chemical reaction between amino groups and reducing sugars, produces several reaction products depending on the reaction conditions (heating time and temperature), physicochemical properties of the system (pH, a_w), chemical composition and nature of reagents. The antimicrobial activity of Maillard reaction products has been studied in model systems (Einarsson, Snygg, & Eriksson, 1983). Maillard reaction products include brown melanoidins, formed at the last stage of the reaction as well as compounds with antioxidant and antimicrobial properties (Ames, 1992; Einarsson et al., 1983).

Melanoidin acts against *Bacillus stearothermophilus*, *E.coli*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *B. subtilis* (Lanciotti, Anese, Sinigaglia, Severini, & Massini, 1999; Rufian-Henares & Morales, 2007; Rufian-Henares & Morales, 2008; Rufian-Henares & Morales, 2008). The antimicrobial activity of melanoidins is due to their ability to chelate some metal ions such as iron and magnesium (Rufian-Henares & Morales, 2007). These metals ions are necessary for growth and survival of microorganisms. Aminoreductone, another Maillard reaction product found in milk and dairy products, inhibits the growth of *Helicobacter pylori*. No study has described the inhibitory effect of Maillard reaction products on the growth of and toxin production by *C. botulinum*.

2.4.8. Combined effects of cheese ingredients

Processed cheeses can be stored at ambient temperatures for up to three months with a combination of potassium sorbate, salt, a_w lower than 0.90, and pH between 5.4 and 5.5 (Aguilera & Arias, 1992). Growth of and toxin production by *C. botulinum* can be

influenced by moisture content, salt, pH, type and concentration of emulsifiers (phosphate and/ or citrate) and preservatives such as nisin and potassium sorbate (Briozzo et al., 1983; Eckner et al., 1994; Karahadian et al., 1985; Roberts & Zottola, 1993; Somers & Taylor, 1987; Tanaka, 1982; Tanaka, Goepfert, Traisman, & Hoffbeck, 1979; Tanaka et al., 1986). Ambient shelf-stable processed cheeses with high moisture (51-58%) and high pH (pH 5.3-6) are preserved by adding salt, emulsifying phosphate and sodium lactate in sufficient amounts to achieve stable ambient storage for at least 180 days (Adrianson et al., 1997). It can be concluded that different combinations of processed cheese ingredients all ensure the safety of this product if properly set.

Tanaka et al. (1986) attempted to clarify the effect of sodium chloride, sodium phosphate, pH and moisture, individually and in combination, on toxin production by *C. botulinum*. Their results showed that a high moisture product can be produced safely if the pH of the product is 6 and the salt plus phosphate concentration is 4.64%. For the first time, Tanaka et al. (1986) applied a logistic regression approach to model the effects of sodium chloride, sodium phosphate, pH and moisture, individually and in combination, on toxin production by proteolytic *C. botulinum* in pasteurized processed cheese. The model of Tanaka et al. underestimates the time for toxin production in the high moisture varieties and predicts stability for some unstable low-moisture compositions (Tanaka et al., 1986).

Ter Steeg et al. (1995) investigated additional factors (temperature, pH, emulsifying salts (citrate and phosphate), a_w , lactate in moisture, and the percentage of total fat) that affect *C. botulinum*. Their results showed that polyphosphate appeared to have a greater inhibitory effect than citrate on *C. botulinum*. Salt, emulsifying salts and lactic acid affect the stability of processed cheese (Ter Steeg & Cuppers, 1995; Ter Steeg et al., 1995). Ter Steeg et al. (1995) attempted to incorporate additional factors related to ingredients of processed cheese in order to improve the predictive accuracy of Tanaka's model for botulinal stability. Their study evaluated the inhibitory effect of temperature, pH, emulsifying salts (citrate and phosphate), a_w , lactate in moisture, and the percentage of total fat on *C. botulinum*. Their developed model estimates the time for a 100-fold increase in cell numbers and it is capable of predicting the observed stability of

compositions derived from the literature (Ter Steeg & Cuppers, 1995; Ter Steeg et al., 1995). Their model can assess processed cheese safety.

It can be concluded that the growth of micro-organisms can be suppressed by the applications of combinations of intrinsic and extrinsic factors. Some of the ingredients reviewed above act as antimicrobial hurdles. Each antimicrobial on its own has limited influence but together they can inhibit the growth of micro-organisms or even inactivate them. In order to determine which combinations of antimicrobials are sufficient to prevent growth of a target micro-organism, mathematical models have been developed that describe the probability of growth as a function of antimicrobial factors. In this way, microbiologically safe, high moisture and ambient shelf-stable processed cheese products can be obtained.

2.5. Microbiological considerations

The production method for processed cheese and PCA includes temperatures of 90°C for 10 minutes, and a hot fill step, which destroys all yeasts, moulds and vegetative cells. Proteolytic spores of *C. botulinum* survive pasteurization temperatures of 90°C and, as most processed cheeses have an a_w above 0.95, have an anaerobic environment within the product, there is a potential botulism hazard if these products are kept at ambient temperature (Glass & Johnson, 2004c; Jay, 2000; Lycken & Borch, 2006; Tanaka et al., 1986). It is important to ensure foods are safe with respect to *C. botulinum* because this pathogen produces an extremely powerful neurotoxin, botulin. The *C. botulinum* must multiply and produce toxin in the food in order to cause food poisoning. Spores of the non-proteolytic strains are much less heat resistant than those of the proteolytic strains (Russell & Gould, 2003). Consequently, spores of the proteolytic *C. botulinum* are the major concern for ambient shelf-stable and low acid food products.

Three sequential processes are involved in the transformation of a dormant bacterium spore into a vegetative cell: activation, germination and outgrowth. Optimum conditions for the activation of proteolytic spores of *C. botulinum* are pH 7 and 37°C. An activated spore germinates into a vegetative cell, grows and eventually sporulates, at which time

toxin is produced. A vegetative cell develops from a germinated spore in a process known as outgrowth (Kim & Foegeding, 1993). Proteolytic spores of *C. botulinum* are widely used in inoculated pack studies designed for low acid foods (Brown et al., 2012).

The target micro-organism in this research is *C. botulinum*, and specifically their proteolytic spores, but there are difficulties in working with this highly potent food poisoning micro-organism in laboratory trials. *C. sporogenes* is genetically and physiologically similar to the proteolytic spores of *C. botulinum* in growth characteristics but is not dangerous. *C. sporogenes* can be used to represent *C. botulinum* in studies aimed at controlling the growth of this food borne pathogen (Brown et al., 2012; Goldoni et al., 1980). Therefore, a cocktail of five spores of *C. sporogenes* were used in this project.

A cocktail of spores of different strains is preferable to a single strain, to cover possible variability between strains (Koutsoumanis, Kendall, & Sofos, 2004b). Models that have been developed with a mixture of strains can be considered relatively safer than those developed with a single strain. In this project, a cocktail of five spore strains of *C. sporogenes* was used.

Inoculum size can influence the location of the growth/no growth boundary, which shifts to less stressful conditions at lower inoculation levels (Dang et al., 2010; Koutsoumanis & Sofos, 2005; Masana & Baranyi, 2000; Parente et al., 1998; Pascual, Robinson, Ocio, Aboaba, & Mackey, 2001; Razavilar & Genigeorgis, 1998; Robinson et al., 2001; Robinson, Ocio, Kaloti, & Mackey, 1998; Skandamis et al., 2007). It is necessary to inoculate the food with at least 10^6 CFU mL⁻¹ of spores or vegetative cells in order to confirm the effect of a preservation system in food, and demonstrate that growth and toxin formation do not occur during the shelf-life of the product or in abusive conditions that might occur in practice (Lund, 1993). This relatively high inoculum level is chosen to ensure that preservative levels that are found to be necessary to prevent growth are conservatively high (Koutsoumanis & Sofos, 2005; Masana & Baranyi, 2000; Parente et al., 1998; Robinson et al., 2001). However, this large inoculum size is greater than the expected contamination in food products, so the probability of growth would be lower under realistic conditions. Thus, developed

models will be fail safe (Vermeulen, Daelman, Van Steenkiste, & Devlieghere, 2012). In this project, the high inoculum size of *C. sporogenes* was used to investigate a worst case scenario. It can be assumed that if *C. sporogenes* does not grow at a high inoculation level, foods would remain safe in real situations where initial contamination is likely to be very low.

2.5.1. Microbial challenge testing

Challenge trials testing are a “last resort” strategy for assessing the safety of specific food products and processes. Well-designed challenge studies can provide critical information on the microbiological safety and stability of a food formulation. However, in these types of trials, mimicking actual processing is challenging and introducing pathogenic bacteria to processing plant, is unacceptable for public health reasons. The history of analogous products and the use of predictive microbiology models can be used as guidelines to conduct of challenge trials relevant to product manufacture (Ross, 2011).

2.6. Predictive microbiology

Predictive microbiology brings together the disciplines of food microbiology and mathematics to provide useful predictions about microbial behaviour in food systems. It has been widely used to determine the effect of combinations of factors on bacterial growth in the design of multi-component food preservation systems. Predictive microbiology is used to help manage food safety and quality. One of the main advantages of predictive microbiology is cost effectiveness compared with traditional microbiological tests which are used to determine shelf life and safety of food products. Predictive models are able to predict microbial behaviour under conditions previously not examined (that is, where no experimental data exists) (McMeekin, Ross, & Olley, 2002). There are three steps to developing a predictive model. The first step is experimental design and data collection, while the next step is fitting the mathematical model that best describes the experimental data. The final step is the validation of the model via the use of data collected under experimental conditions interpolated and/or

extrapolated relative to the original conditions. However, extrapolation is risky (Buchanan, 1993; Dodds, 1993).

Whiting and Bunchanan (1993) classified predictive models into three types: primary, secondary and tertiary models. The primary model describes the change in the bacterial population size number, over time under fixed environmental and cultural conditions. The response can be measured directly by total viable count, toxin formation, substrate level or metabolic products, or indirectly by optical density or impedance. Secondary models describe the dependence of parameters of the primary model on intrinsic and extrinsic factors (e.g. pH, temperature, water activity) (Ross & Dalgaard, 2004). Tertiary models are computer programs based on primary and secondary models. A computer program provides an interface between the mathematical models and the user, allowing model inputs to be easily entered, and results displayed in simple graphical outputs (Tamplin, Baranyi, & Paoli, 2004). These programs consist of large quantities of data on microbial growth responses to various combinations of intrinsic and extrinsic factors. Several predictive tools have been developed such as the Pathogen Modelling Program (PMP), ComBase and Food Micro Model (FMM). These programs can be used for quick and easy prediction of the rate at which a micro-organism will grow in a food system as well as the shelf life of food products.

From a food safety point of view the ability to predict whether or not micro-organisms could grow or could produce toxins is more important than predicting growth rate because any growth implies a possibility of food-borne disease, food intoxication or food spoilage. It is of economic interest to the food industry to be able to predict (without the need for time consuming and expensive challenge testing) the potential for growth of specific micro-organisms in a food or to establish product formulations excluding growth (McKellar & Lu, 2001). This has led researchers to seek methods to predict probability of growth. Probability of growth models are an important component of predictive microbiology in the case of food safety and microbiological shelf life issues.

2.6.1. Probability models

Probability models are a practical approach to assessing the effect of combinations of preservative factors in food products. Models which predict the likelihood of growth of a micro-organism as a function of intrinsic and extrinsic factors were first described in 1971 (Genigeorgis, Martin, Franti, & Riemann, 1971). For the evaluation of probability of growth, mathematical models have been developed as a function of independent variables. Several reviews of probability modelling were presented in the early 1990s (Baker, 1993; Baker & Genigeorgis, 1993; Dodds, 1993; Lund, 1993; Ross & McMeekin, 1994; Whiting, 1995; Whiting & Oriente, 1997; Zhao, Chen, & Schaffner, 2001).

The use of a probability model is a convenient way to predict the likelihood of a microbial event occurring. The purpose of this type of model is to predict the location of the interface between growth/no growth accurately or determine the probability of growth in food. Masana and Baranyi (2000) developed a graphical growth/no growth interface of *B. thermosphacta* as a function of pH and water activity. The growth/no growth data near the limit of growth for *B. thermosphacta* were used to demonstrate a growth/no growth interface for the micro-organism. Several techniques can be used to establish probability models in order to predict the probability of growth and establish the growth/no growth interface.

2.6.1.1. Techniques used for establishing probability models

In probability modelling the data observed from experiments is usually the response (e.g. growth, detectable toxin production) or lack of response, i.e. binary data is collected. For instance, if toxin production was the response, it would be coded 1, if toxin was observed or coded 0, if not; or coded between 0 and 1, if replicated observations have been made. Probability models quantify the effects of various hurdles (as independent variables) on probability of growth as the dependent variable.

Logistic regression

Logistic regression is commonly used to investigate the relationship between a binary response variable (only two possible outcomes) and a set of independent variables (Gysemans et al., 2007). Regression is the best technique to use to quantify the relationship between variables when the value of one variable is affected by changes in the values of other variables. Large numbers of replicates are required in logistic regression in order to provide a good determination of the probability as binary data are less informative than other forms of data (Salter, Ratkowsky, Ross, & McMeekin, 2000).

Zhao et al. (2001) compared the performance of linear and logistic regression. They pointed out that logistic regression provided a more accurate description of data than linear regression which should not be used for binary data. Three problems arise when linear regression is used for binary data: 1) the variance of the error term is not constant, 2) the error term is not normally distributed and 3) there is no restriction requiring the prediction to fall between 0 and 1 (Zhao et al., 2001).

Logistic regression is categorized into non-linear and linear (ordinary) logistic regression (Gysemans et al., 2007).

Non-linear logistic regression must be used if the right-hand side of the intended model is a non-linear function of the parameters to be estimated. Equation (2.1) is an example of a non-linear logistic regression model.

$$\text{logit}(p) = \ln\left(\frac{p}{1-p}\right) = \alpha + \frac{\beta \times X}{X + \gamma} \quad (2.1)$$

where $\text{logit}(p)$ is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), X is variable and α , β and γ are three parameters to be estimated.

In predictive microbiology, a growth/no growth probability model can be derived from a square root-type kinetic model using non-linear logistic regression. This type of non-

linear logistic regression was introduced by Ratkowsky and Ross (1995) and has been used by other researchers. A kinetic model was transformed into a probability model by taking the natural logarithm of both sides of the original kinetic equation and then replacing one side with the logit of the probability of growth (Lanciotti, Sinigaglia, Gardini, Vannini, & Guerzoni, 2001; Presser, Ratkowsky, & Ross, 1998; Salter et al., 2000; Tienungoon et al., 2000; Vermeulen et al., 2009). The non-linear logistic regression model retains some elements of simplicity despite this apparent complexity (Ratkowsky, 2002). From this approach one may use data from kinetic experiments to model the detectable growth/no growth boundary within a given time (Ratkowsky & Ross, 1995). Alternatively, one may use data derived from experiments which specifically determines whether growth is possible or not (Presser et al., 1998). Kinetic and probability models are closely related as the probability of detectable growth within a specific time depends on kinetic parameters such as germination, lag and generation time (Ratkowsky & Ross, 1995; Ross & Dalgaard, 2004). Non-linear logistic regression has the advantage of containing biologically meaningful parameters. These parameters, however, appear in a non-linear way, hampering parameter estimation (Ratkowsky, 2002).

Linear logistic regression is used when the model form is linear, e.g. the polynomial expression like that shown in equation (2.2). The advantage of linear logistic regression models is that they are easy to fit (Ross & Dalgaard, 2004).

$$\text{Logit}(p) = \ln\left(\frac{p}{1-p}\right) = \alpha + \beta_1 f_1(X) + \beta_2 f_2(X) + \beta_3 f_3(X) + \dots \quad (2.2)$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), $f_1(X)$, $f_2(X)$, etc. are functions of the explanatory variable X. These functions must not contain any parameters. Thus, the right-hand side of the model is a linear expression in terms of the parameters α , β_1 , β_2 , β_3 , etc.

To test whether a model is linear or non-linear, the derivative of the left-hand side of the model with respect to each of the parameters separately should be calculated. In a non-linear model, at least one of these derivatives will be a function of at least one of the

parameters. This contrasts with a linear logistic regression model, in which none of the derivatives is a function of a parameter.

Probability models using linear logistic regression have been extensively used to predict the probability of growth of important food-borne pathogens, although spoilage of yeast and fungi are currently receiving increasing attention in shelf-stable products. The probability of growth of different fungi have been studied: *Monascus ruber* in green table olives (Panagou, Skandamis, & Nychas, 2005), *Byssochlamys fulva* and *Byssochlamys nivea* on malt extract agar (Panagou, Chelonas, Chatzipavlidis, & Nychas, 2010), *Aspergillus carbonarius* in pistachio nuts (Marín, Hodžić, Ramos, & Sanchis, 2008), *Aspergillus flavus* (*A. flavus*) in chili powder (Marín, Colom, Sanchis, & Ramos, 2009), *Aspergillus carbonarius* on synthetic grape juice medium (Tassou, Natskoulis, Magan, & Panagou, 2009), *Colletotrichum gloeosporioides* in microwave treatments (Sosa-Morales, Garcia, & López-Malo, 2009), *A. flavus*, *Aspergillus ochraceus*, *Aspergillus paraciticus* on potato dextrose agar medium (Palou & López-Malo, 2006) and *Aspergillus niger*, *Penicillium spinosum* in cold filled ready to drink beverages (Battey, Duffy, & Schaffner, 2001).

Linear logistic regression has also been used to define the probability of growth of yeasts such as *Zygosaccharomyces ballii* under individual and combination of different preservatives (Battey, Duffy, & Schaffner, 2002; Cole & Keenan, 1986; Dang et al., 2010; Dang et al., 2011; Jenkins, Poulos, Cole, Vandeven, & Legan, 2000; López-Malo & Palou, 2000; Mertens et al., 2012; Vermeulen et al., 2008), *Saccharomyces cerevisiae* (*S. cerevisiae*) (Arroyo-López, Bautista-Gallego, Romero-Gil, Rodríguez-Gómez, & Garrido-Fernández, 2012; Arroyo López, Durán Quintana, & Garrido Fernández, 2007; Belletti et al., 2007; Lopez-Malo, Guerrero, & Alzamora, 2000) and *Issatchenkia Occidentalism* olive spoilage yeast (Arroyo López, Quintana, & Fernández, 2007).

This type of technique has been used to define the growth probability of important food-borne pathogens such as *L. monocytogenes* (Belessi, Gounadaki, Schwartzman, Jordan, & Skandamis, 2011; Bolton & Frank, 1999; Boziaris, Chorianopoulos, Haroutounian, & Nychas, 2011; Boziaris & Nychas, 2006; Boziaris, Skandamis, Anastasiadi, & Nychas, 2007; Gysemans et al., 2007; Ingham, Borneman, Ané, & Ingham, 2010; Koutsoumanis

& Sofos, 2005; Parente et al., 1998; Valero, Carrasco, Pérez-Rodríguez, García-Gimeno, & Zurera, 2006; Yoon et al., 2009), *E. coli* (McKellar & Lu, 2001; Skandamis et al., 2007; Uljas, Schaffner, Duffy, Zhao, & Ingram, 2001), *Salmonella* (Koutsoumanis et al., 2004b; Yoon et al., 2012), *Staphylococcus aureus* (Borneman, Ingham, & Ane, 2009; Stewart et al., 2002). Only a few studies used probability of growth models for spore formers. A growth probability model for *B. cereus* was developed as a function of pH and temperature (Ding, Wang, Park, Hwang, & Oh, 2013) and also as a function of water activity, pH, storage time and temperature (Daelman et al., 2013).

Dodds (1993) developed a linear logistic regression model to predict the probability of growth of *C. botulinum* in terms of pH, a_w and storage time in cooked, vacuum packed potatoes stored at 25°C. The result of this research illustrated all factors (time storage, pH and a_w) had highly significant effects on the probability of toxigenesis. The interaction effects between pH- a_w , and a_w -time were also highly significant, as was a quadratic term for a_w . They also modelled the effect of modified atmosphere packaging (MAP), irradiation and storage temperature on *C. botulinum* toxin production. The statistical analysis showed that temperature and O₂ concentration were highly significant. In addition, irradiation and final CO₂ concentration and several interactions were also significant (Dodds, 1993). The proposed model predicts the probability of growth under a given set of conditions and can be used to calculate preservative concentrations needed to achieve given probabilities of growth.

Linear logistic regression models are characterised by their flexibility. These types of regression models are more sensitive to anomalies in the data (Gysemans et al., 2007). The anomalous data points need to be eliminated before selecting linear logistic regression to fit data. The dangers of over-fitting and of getting microbiologically illogical results are the disadvantages of the linear logistic regression. A way of solving these problems is the use of constrained polynomial models, meaning a combination of the flexibility of a polynomial and the incorporation of a priori microbiological knowledge as constraints during parameter estimation (Geeraerd et al., 2004).

Artificial neural networks

The use of artificial neural networks (ANN) is a potential alternative to logistic regression analysis. These are computationally patterned on the structure of the human brain (Guerriere & Detsky, 1991; Hinton, 1992). The advantages of artificial neural networks are that the method is easy to learn and it detects many different relationships or interactions among variables. The drawbacks are a greater computational burden, the tendency to over fit data and a lack of ability to show which relationships are causal (Jack, 1996). Artificial neural networks are used for the same aim as logistic regression modelling: they make predictions based on values of certain selected variables (Specht, 1990). They are appropriate for research that is focussed on outcome prediction.

Hajmeer et al. (2002) applied artificial neural networks to the classification of bacterial growth/no growth data and to modelling the probability of growth. The application of artificial neural networks was used to predict *C. botulinum* growth in *Zataria multiflora* as a function of essential oil content, pH, salt and temperature. This is a plant belonging to the *Lamiaceae* family that grows only in Iran. Gunvig et al. (2013) also used this method to develop a mathematical model for predicting growth/no growth of psychotropic *C. botulinum* in pasteurised meat products packed in modified atmosphere for combinations of storage temperature, pH, NaCl, added sodium nitrite and sodium lactate. They also developed a logistic regression model in order to compare with ANN. They used a root mean square method (RMS) to compare these two techniques. However, based on the RMS value, the ANN was preferred. Logistic regression is still a more informative model compared to ANN for the identification of causal relationships and specific links between predictor variables and outcomes (Gunvig, Hansen, & Borggaard, 2013; Hajmeer & Basheer, 2003; Jack, 1996).

2.6.1.2. Factors influencing probability of growth modelling

Probability modelling is used in order to define the probability that detectable toxins or growth occurs within a specified time and under specified product composition or storage conditions (Salter et al., 2000). However, there is a time factor to consider. Hence, the probability of growth should be more accurately referred to as the

probability of growth (Koutsoumanis, 2008; Koutsoumanis et al., 2004b; Le Marc, Pin, & Baranyi, 2005; Lund, Graham, & Franklin, 1987; Masana & Baranyi, 2000; McKellar & Lu, 2001; Presser et al., 1998; Tienungoon et al., 2000).

Inoculum size affects the probability of growth and this should be taken into consideration during the modelling process (Bidlas, Du, & Lambert, 2008; Koutsoumanis & Sofos, 2005; Lindroth & Genigeorgis, 1986; Masana & Baranyi, 2000; Parente et al., 1998; Pascual et al., 2001; Robinson et al., 2001). Whiting and Oriente (1997) developed time to turbidity and probability of growth models for non-proteolytic type B *C. botulinum* in broth media with varying temperature, pH, salt and inoculum size. The probability of growth of and toxin formation by *C. botulinum* was strongly affected by inoculum size (Whiting & Oriente, 1997). The result of this study was similar to the result of Zhao et al. (2002) who reported a probability of growth model for *C. botulinum* under different conditions of inoculum size, pH and sodium chloride concentration. All tested factors and only one interaction term (salt-inoculum size) were retained in the final model. Their study indicates that when inoculum size increased, the probability of growth of *C. botulinum* increased (Zhao, Montville, & Schaffner, 2002). A high inoculum size is preferable if the aim is to determine absolute limits to growth and to ensure a low probability of growth. In addition, the use of high inoculum size can partially compensate for relatively short incubation periods in experimental trials (Masana & Baranyi, 2000; McKellar & Lu, 2001; Presser et al., 1998; Salter et al., 2000; Tienungoon et al., 2000). Models that incorporate inoculum size are better than models that do not (Vermeulen et al., 2009). The use of a mixture of strains, covering the possible variability encountered between strains should also be considered in these types of models. A mixture of strains (a cocktail) avoids the bias that may occur through individual strain variability, and therefore models prepared using cocktails are considered safer than those prepared with individual strains (Koutsoumanis et al., 2004b).

2.7. Summary

The growth of micro-organisms can be suppressed by hurdle technology. A combination of preservatives is more effective than using preservatives individually (Leistner, 2002).

In developing preservation technologies, scientists usually rely on more than one factor to inhibit microbial growth by taking advantage of interactions or synergistic effects. In terms of food quality and safety a synergistic effect is preferable (Raso & Barbosa-Canovas, 2003).

The preservation of high moisture, low acid ambient shelf-stable food products that satisfy consumers and manufacturer is challenging. Processed cheese product is a product of this type. The safety of such food products can be achieved through using hurdle technology. *C. botulinum* is the micro-organism of concern in low acid and ambient shelf-stable processed cheese products.

Microbial safety and stability of processed cheese depend on its composition and storage conditions. The review on processed cheese ingredients that affect the safety of this product showed that different combinations of processed cheese ingredients can insure safety if properly set. Tanaka et al. (1986) and Ter Steeg et al. (1995) evaluated the inhibitory effect of processed cheese ingredients, pH and storage temperature individually and in combination on the growth of *C. botulinum* in processed cheese. Their studies did not attempt to achieve high moisture and ambient shelf-stable processed cheese. The inhibitory effects of processed cheese ingredients on micro-organisms, particularly proteolytic *C. botulinum*, revealed that preservatives such as salt, sorbic acid, lysozyme and nisin individually had inhibitory effects on *C. botulinum*. No previous study has investigated the inhibitory effect of a combination of salt, sorbic acid, nisin and lysozyme on *C. botulinum*. Some interaction effects between these preservatives were observed against other micro-organisms (Mazzotta et al., 1997; Parente et al., 1998; Robach, 1980; Roberts, Gibson, & Robinson, 1982; Sofos & Busta, 1981; Thomas, 1999). Therefore, the objective of this study was to achieve high moisture and ambient shelf-stable processed cheese by applying a combination of these selected preservatives using probability of growth models. Such models allow qualitative setting of preservative concentrations to give any desired probability of growth. Conversely, they allow the probability of growth to be predicted for a given set of preservative concentrations.

Chapter Three. Materials and methods

3.1. Introduction

General materials and methods used during this project are discussed in this chapter. Specific methods relating to the work described in Chapters 4 to 7 are covered in these chapters.

3.2. Inoculum preparation

The properties of *Clostridium* spores may differ significantly between batches of the same strain (Lund et al., 1987). Therefore, it was essential to have the ability to repeatedly grow large numbers of spores of *C. sporogenes*. One single batch of each strain was used for the entire project in order to ensure spore consistency.

Five strains of *C. sporogenes* were purchased from the New Zealand reference culture collection (254, NZ isolate 1966; 1097 NCTC 532 (ATCC 19404, DSM 1664, NCIB 532); 1221, Wadsworth VA Hospital, Los Angeles, USA, Wadsworth strain No. 2253; 2997, NCIB 8053 (ATCC 7955, DSM 767, NCTC 8594); 3502, ATCC 3584 (DSM 795, NCIMB 10696)). All five stocks were revived from freeze dried pellets in a small volume (5-6 mL) of reinforced clostridial medium (RCM) at the optimal growth temperature of 37°C overnight. A schematic of the process used to produce spores is shown in Figure 3.1.

This procedure was used to produce a high population of spores which could readily be purified to give pure spore suspensions of *C. sporogenes* spores. A high population of spores (80-90% of vegetative cells sporulated) was achieved after three weeks of incubation for all five strains. All five cultures were harvested as described in section 3.3.2.

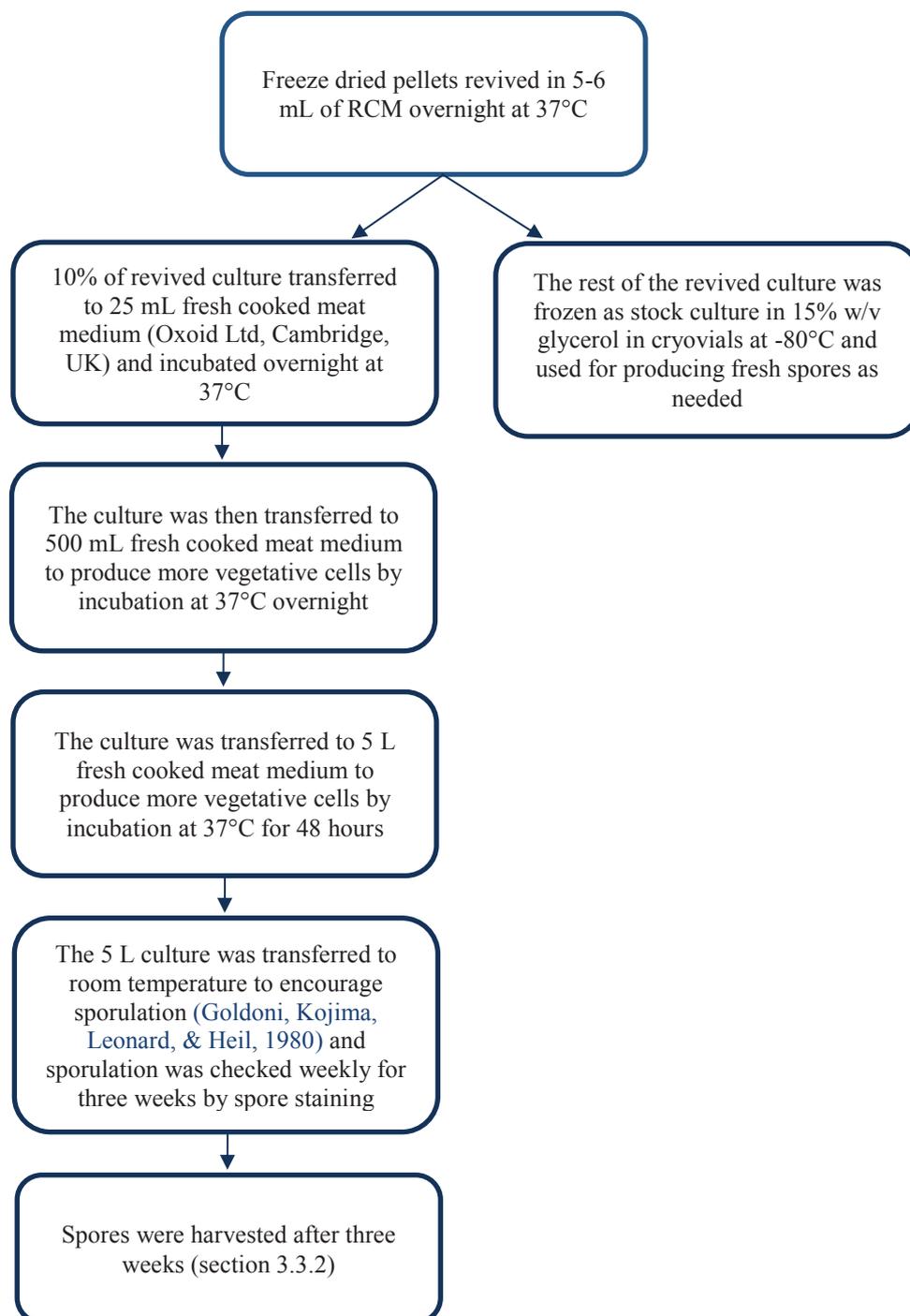


Figure 3.1. Schematic of the process used to produce spores.

3.2.1. Spore staining

The population of produced spores was examined weekly by spore staining to ensure that 80-90% of vegetative cells had sporulated. A smear of culture was prepared by streaking a loopful onto a glass microscope slide. This was allowed to dry in air, and then heated by passing through a flame. The heat-fixed smears were covered by small pieces of blotting paper and placed on a rack over a boiling water bath.

The blotting paper pieces were covered with 5% malachite green (Merck KgaA, Darmstadt, Germany) and the slides were allowed to warm thoroughly over the water bath for 10 minutes with the dye being replaced as it evaporated. Blotting papers were removed carefully from the slides after 10 minutes and the slides cooled to room temperature for 2 minutes. The slides were then washed with cold tap water, counter stained by adding 0.5% safranin (Merck) and left to stand for 2 minutes. They were then rinsed thoroughly in cold water, dried with paper towels and examined microscopically using an Olympus microscope (model BX53F, Olympus UK Ltd., London, United Kingdom) in order to observe the percentage sporulation. The gradual increase in spore numbers during incubation is illustrated in Figure 3.2.

3.2.2. Spore harvesting

The following procedure was used to recover spores from the cooked medium in which they were produced. Sporulation cultures were aseptically filtered, after three weeks of incubation at room temperature, through four layers of sterile cheesecloth placed in a sterile funnel over a sterile flask, as shown in Figure 3.3.

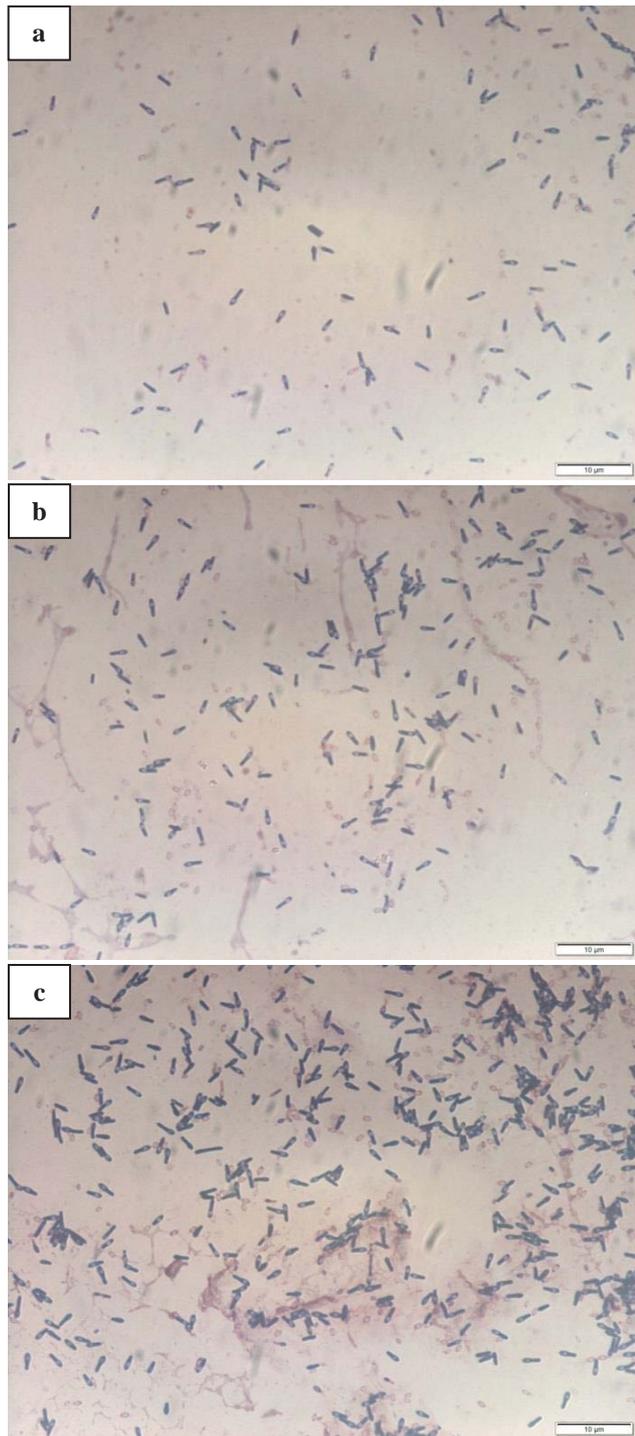


Figure 3.2. Images of stained spores at the first (a), second (b) and third (c) week of incubation at room temperature.

Sterilized aluminium foil was used as a cover during filtration to avoid airborne contamination. The filtrates were centrifuged at $14\ 800\times g$ for 10 minutes at 4°C . The sedimented spores were washed once by re-suspending to the original volume in de-ionized water and then repeating the centrifugation. To destroy vegetative cells, the recovered spores were mixed into 500 mL sterile phosphate buffered saline (PBS) ($137\ \text{mmol L}^{-1}\ \text{NaCl}$, $2.7\ \text{mmol L}^{-1}\ \text{KCl}$, $10\ \text{mmol L}^{-1}\ \text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4) and then heated at 80°C for 15 minutes. The spore suspension was washed 10 times with sterile de-ionized water followed by centrifugation each time at $2360\times g$ for 20 minutes to remove impurities, particularly vegetative cell debris.



Figure 3.3. Sterile filtration for separation of spores from cooked meat media particles.

A washed spore suspension can be seen in Figure 3.4. Finally, 50 mL sterile de-ionized water was added to re-suspend the spores of each strain. The suspended spores were distributed into 5 ml sterile bottles and were frozen at -80°C as a concentrated suspension until needed.

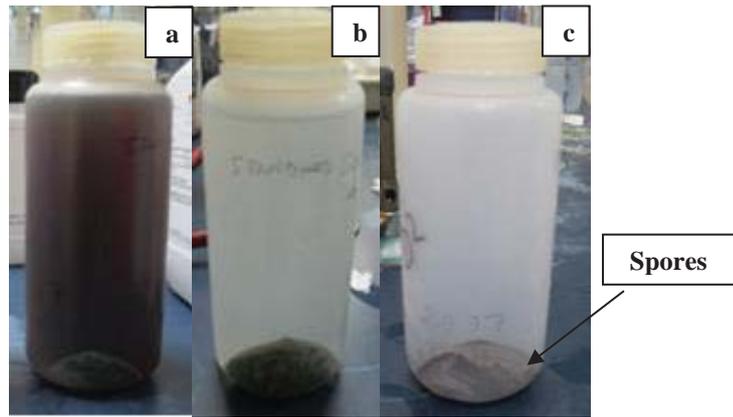


Figure 3.4. Washing steps to remove vegetative cell debris from spores. (a) after first washing step; (b) before last washing step (c) after last washing step.

3.2.3. Spore enumeration

Spore stocks were thawed before starting the experiment and were kept at 5°C during preparation of test samples. The population of spores was estimated by duplicate pour plating of 10-fold serial dilutions using reinforced clostridial agar (RCA). RCA and peptone water (buffered) were purchased from Merck. All media were autoclaved at 121°C for 15 minutes before use. Plates were incubated anaerobically at 37°C for 48 h. Plates with between 30 and 300 colonies were counted visually. All five spore stocks had a population higher than 10^6 CFU mL⁻¹. Confirmation of the purity for each strain was performed by incubating plates aerobically as well as anaerobically (Whiting & Oriente, 1997). Uniform colonies were observed on anaerobic plates for each of the five spore stocks produced and no colonies were detected on aerobic plates, showing that all spore stocks were pure.

Equal spore numbers of each strain were used to inoculate prepared growth media to a total level of 10^6 CFU mL⁻¹. The population of spores was checked monthly during an experiment to confirm that they had not fallen during storage at 5°C. The post process inoculation method was selected for this study, as this method has a greater challenge for growth media than hot inoculation. In addition, the aim of this study was to observe post contamination of high moisture, low acid products.

3.3. Preparation and inoculation of growth media

Nutrient broth is commonly used in primary investigations of the effects of preservatives in order to understand the effects of these preservatives singly and in combination. Nutrient broth is easily and accurately adjusted and controlled in terms of composition and allows more rapid growth than that observed in foods (McClure et al., 1994b). As food products are complex materials various interactions between their components might modify the effect of preservatives on micro-organisms. Therefore, in this project, the inhibitory effects of the selected preservatives were first observed in the nutrient broth and then in processed cheese analogue (PCA) as a complex food system.

3.3.1. Nutrient broth

Half a litre of thioglycollate broth was made for each tested preservatives combination according to the Oxoid Ltd formulation: tryptone (15 g L⁻¹), yeast extract (5 g L⁻¹), dextrose (5.5 g L⁻¹), L-cystine (0.5 g L⁻¹), sodium thioglycollate (0.55 g L⁻¹) and agar (0.755g L⁻¹). All selected preservatives were added to the prepared broth at different stages, which will be detailed in the materials and methods sections of the relevant chapters (Chapters 4 and 5). The pH of each prepared broth was adjusted to the required value with hydrochloric acid (1 M) or sodium hydroxide (0.1 M) using a pH meter (Orion model 250A/610, HACH LANGE GMBH, Dusseldorf, Germany).

Prepared broth (450 mL) was inoculated to a total level of 10⁶ CFU mL⁻¹ and the inoculated broth then distributed into 90 sterile screw cap bottles (5 mL). The residual un-inoculated broth (50 mL), containing preservatives, was distributed into nine sterile screw cap bottles (5 mL) to act as negative controls. A negative control was prepared for each preservative concentration combination to check that no microbiological contamination had occurred during the preparation of broth samples and to observe the stability of un-inoculated broth samples over the eight week storage period.

A batch of inoculated thioglycollate broth (50 mL) containing no preservatives was prepared once as a positive control. This was distributed into nine sterile screw cap bottles (5 mL) which were then stored anaerobically at 37°C. The aim of having a

positive control was to observe how broth components, other than the test preservatives, affected the growth of *C. sporogenes*.

3.3.2. Processed cheese analogue

PCA samples were prepared as described below in a temperature controlled blender (Vorwerk Thermomix TM 21 (Figure 3.5), Wuppertal, Germany) according to the formulation shown in Table 3.1. Rennet casein and skim milk powder (SMP) were provided by the Fonterra Co-operative Group Limited (Auckland, New Zealand) and their specifications are in the appendix. Soya oil and salt (sodium chloride) were purchased from a local supermarket. Potassium sorbate and polyphosphate emulsifier (JOHA S9) were obtained from Hawkins Watts New Zealand (Auckland, New Zealand) and Fibrisol Ltd (Heatherton, Australia) respectively. Nisaplin was purchased from Danisco Ltd. (Nisaplin 114373, Beaminster, United Kingdom). Table 3.1 shows the general formulation of the PCA used in this study (Azusa Sonoda, 2008, personal communication). Variations in PCA composition, due to the use of different levels of salt, potassium sorbate and nisin, were compensated for, by varying the vegetable oil content, in order to maintain the concentrations of the base components at constant percentages.



Figure 3.5. Vorwerk Thermomix TM 21 blender for preparing PCA

Table 3.1. Composition of PCA.

Ingredient	Percentage (% w/w)
Rennet casein	8.5
Emulsifier JOHA S9	2
Water	60
SMP	3.5
Vegetable oil	21.8 - 26
Salt*	-
Nisin*	-
Potassium sorbate*	-

*Details are given in Chapters 6 and 7

The preparation of 1500 g of PCA for each preservatives combination to be tested was carried out under aseptic conditions. The Vorwerk Thermomix TM 21 was sprayed with 70% ethanol and allowed to air-dry in a laminar flow cabinet. Rennet casein and polyphosphate emulsifier were hydrated in the Thermomix at 35°C for 45 minutes at a low mixing speed of 200 rpm. Hydrochloric acid (0.1 N) was added to the mixture to adjust the pH to 5.5. All the other ingredients were added after hydration and then the mixing speed was increased to 400 rpm at 85°C for 6 minutes. After that, the mixing speed was increased to 1000 rpm at 85°C for another 5 minutes to ensure all vegetative cells were eliminated. The PCA sample was then poured into a sterile beaker in a laminar flow cabinet and allowed to cool to room temperature. A schematic of PCA preparation steps is shown in Figure 3.6.

PCA (1200 g) was inoculated with the cocktail of spores of *C. sporogenes* to a total level of 10^6 CFU mL⁻¹. The inoculated samples were placed in 45 air-tight sterile containers (25 mL) and kept in an anaerobic jar at 37°C for eight weeks. The residual un-inoculated PCA (300 g), containing preservatives, was used to provide negative PCA controls. The negative controls were put in ten air-tight sterile 25 mL containers and kept under the same storage conditions. A negative control was prepared for each preservative combination to check that no contamination happened during the preparation of samples and to observe the stability of un-inoculated PCA samples over the eight weeks storage period.

A separate inoculated PCA sample (250 g), containing no preservatives, was prepared once without adding any of the test preservatives as a positive PCA control. These were put in ten air-tight sterile containers (25 mL) and were stored anaerobically at 37°C. The aim of having a positive control was to observe how PCA components other than the test preservatives, affected the growth of *C. sporogenes*.

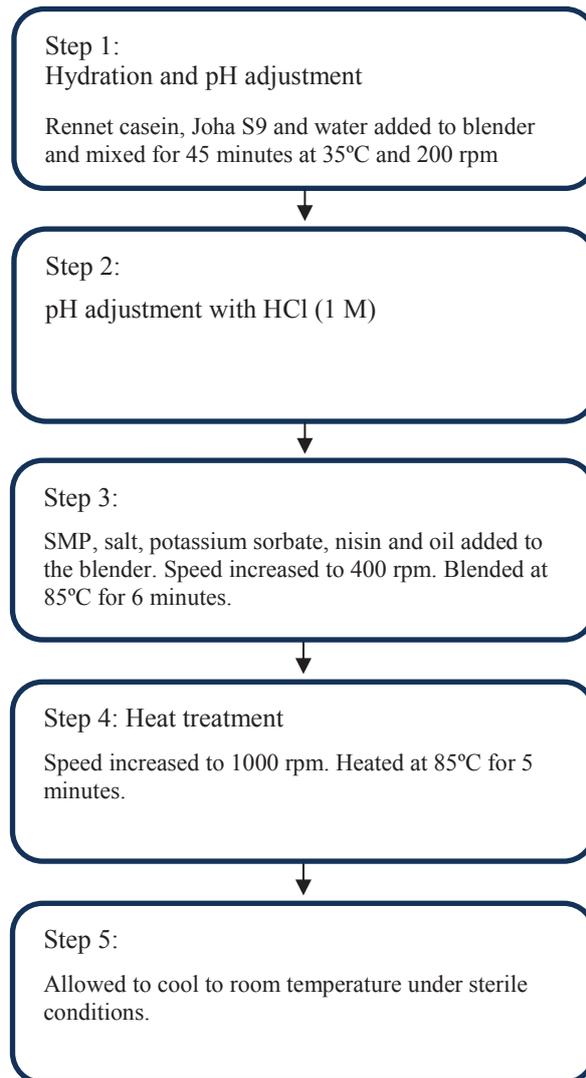


Figure 3.6. Preparation of PCA.

3.3.3. Incubation of inoculated samples

All prepared samples in this project were stored anaerobically at an optimum growth temperature for *C. sporogenes* (37°C) for eight weeks. Although common practice is incubation at 25°C, the optimum growth temperature of the target micro-organism was used in order to be able to complete experiments within a reasonable time-frame. BD GasPak EZ sachets (BD 260683, Maryland, U.S.A.) were used to create an anaerobic environment. An anaerobic test strip (Merck) was used to confirm anaerobic incubation (Yang, Crow-Willard, & Ponce, 2009).

3.4. Growth evaluation

3.4.1. Nutrient broth

The presence/absence of microbial growth was monitored weekly by measuring the optical density of the inoculated culture medium at 595 nm using an automated micro-plate reader (Spectro star nano, BMG LABTECH). At time zero and weekly, after manually shaking the bottles, the optical density (OD) of ten bottles for each preservative combination, one bottle of negative control for each preservative combination, and one bottle of positive control were measured. All bottles were then discarded. A bottle was considered as showing growth if the OD increased by at least 0.1 above the OD at time zero (Mertens et al., 2011). The results for each treatment were expressed as a probability of growth value ranging from 0 (none of the ten replicates showed growth) to 1 (all ten of the replicates showed growth).

The Gram stain was applied to the inoculated broth samples in which growth was detected to verify that growth was of a Gram positive micro-organism (*C. sporogenes*) rather than any contaminant.

3.4.2. Processed cheese analogue

OD measurement as used for broth cultures could not be used to check for growth in PCA samples, as food products are not transparent. Therefore, the PCA samples and the

negative and positive PCA control samples were subjected to plate counts at time zero, the second day and at one, two, four, six and eight weeks. For the microbiological plate counts, 10 g of sample was added to 90 mL of sterile peptone water (0.5%) and mixed in a peristaltic blender for 2 minutes. Serial 10-fold dilutions were prepared in 0.5% peptone water and 1 mL aliquots from each dilution were pour plated in duplicate using RCA. RCA and peptone water (buffered) were purchased from Merck. All media were autoclaved at 121°C for 15 minutes before use. Determination of growth or no growth was based on the colony count after 48 hours at 37°C under anaerobic conditions. An increase in cell numbers of more than 1 log (CFU mL⁻¹) was considered as indicating growth, whereas an increase in cell numbers of < 1 log (CFU mL⁻¹) was considered as indicating no growth.

3.5. Other analyses

Generally, moisture content, a_w and pH have important effects on microbial growth. Therefore, the moisture content, a_w and pH of the broth and PCA samples were measured at time zero and at the end of the eight week storage period to observe if these factors had any changes.

3.5.1. Moisture content

The moisture content of samples was determined according to AOAC 930.15 (AOAC, 1980). Dishes used for moisture content were dried in an oven for approximately 30 minutes. Each dish was weighed and 2-3 g of sample was placed on the dish and the total weight recorded. The dishes were put into an oven at 105°C for 6 hours. Then dishes were taken from the oven and allowed to cool down at room temperature in a desiccator. The cooled samples were weighed, and the loss in weight of each sample was calculated as the moisture content of the sample.

3.5.2. Water activity

A water activity meter (AquaLab 4TE, Pullman, United States) was used to measure the a_w of the broth and PCA samples. The sample cup was filled to half its depth and placed

in the sample chamber of the instrument. The measurement temperature was adjusted to 37°C, and the a_w measured using the standard procedure for the instrument.

3.5.3. pH

The pH of the broth and PCA samples was measured using a pH meter (Orion model 420A, HACH LANGE GMBH, Dusseldorf, Germany). The pH electrode was rinsed with distilled water prior to use and then placed into the sample. The pH measurements of the samples were conducted in triplicate.

3.6. Experimental design and statistical analyses

A central composite rotatable design (CCRD) (Box & Draper, 1986) was used for the preliminary experiment (see Chapter 4) to evaluate the effects of salt, nisin, lysozyme and sorbic acid on the probability of growth of a cocktail of spores of five species of *C. sporogenes* in the broth. The data were analysed using the response surface regression procedure of Minitab 15.0 (Minitap Inc., USA).

Full factorial experiments were designed and carried out to develop probability of growth models for *C. sporogenes* in broth and PCA that contained salt, nisin and sorbic acid as test preservatives (Chapters 5 and 7). The collected probability data were analyzed and modelled using ordinary (linear) logistic regression in SAS 9.1 (SAS Institute Inc., Cary, N.C., USA).



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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal 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: Elham Khanipour

Name/Title of Principal Supervisor: Professor Steve Flint

Name of Published Research Output and full reference:

Evaluation of the effects of salt, potassium sorbate, nisin and lysozyme on the probability of growth of *Clostridium sporogenes*, International Journal of Food Science and Technology, 2014, 49(6), 1506-1512.

In which Chapter is the Published Work: Chapter 4

Please indicate either:

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24/11/2014

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Chapter Four. Exploration of the relative preservative effects of salt, sorbic acid, nisin and lysozyme on the growth of *Clostridium sporogenes* in nutrient broth

4.1. Introduction

The growth of *C. botulinum* in low acid foods (pH > 4.5) is a hazard for consumers. The preservation of foods has to be effective to ensure a high degree of safety against the growth of this pathogenic micro-organism. The effectiveness of preservatives in preventing the growth of a target micro-organism can be expressed as the probability that not a single spore will grow. The probability of growth can be modelled to provide a useful tool in formulating foods to prevent the growth of *C. botulinum*.

Salt (sodium chloride) is a common preservative that is able to prevent the growth of *Clostridium* spp.; however growth can be prevented only with high concentrations of salt. This may not be desirable in many foods, as it affects palatability. In order to limit salt concentration, other preservatives can be used in combination with salt to ensure that no *C. botulinum* growth occurs (Lund, 1993). Potassium sorbate, an inhibitor of *C. botulinum*, is a preservative that is widely used in a range of foods. Its anti-botulinum efficacy increases at lower pH (Huhtanen et al., 1981; Ivey & Robach, 1976; Sofos, Busta, Bhothipaksa, & Allen, 1979). Salt acts synergistically with potassium sorbate against clostridia in meat slurries (Robach, 1980; Roberts et al., 1982; Sofos & Busta, 1981; Thomas, 1999). Furthermore, potassium sorbate at 4% w/w inhibited germination of spores of 20 species of *Clostridium*, while sorbic acid (3% w/w) at pH 6.7 was found to have an inhibitory effect on *C. sporogenes* (York & Vaughn, 1954).

The antibacterial effects of nisin and lysozyme have also been well documented in literature. Nisin is a natural bio-preservative that has been used for safeguarding minimally processed foods. Nisin inhibits the growth of a broad spectrum of Gram-positive micro-organisms such as *Clostridium* spp. and it is generally recognized as safe (GRAS) for use as a food additive in many countries. The antimicrobial effect of nisin is caused by its interaction with phospholipid components of the microbial cytoplasmic

membrane. Previous studies have shown that the presence of salt can enhance nisin activity against *L. monocytogenes* and *C. botulinum* (Harris et al., 1991; Parente et al., 1998; Thomas & Wimpenny, 1996). However, salt has been reported to reduce ability of nisin to prevent the outgrowth of *Bacillus* spores (Bell & Delacy, 1985; Bouttefroy et al., 2000).

Lysozyme is known to inhibit some Gram-positive bacteria and has been demonstrated to be active over the wide pH range of 4-10. However, high ionic strength (0.2M salt) was shown to have an inhibitory effect on lysozyme activity (Alderton, Chen, & Ito, 1974). A combination of nisin and lysozyme has synergy against Gram-positive spore forming bacteria such as *B. thermosphacta* (Chung & Hancock, 2000; Mastromatteo, Lucera, Sinigaglia, & Corbo, 2010a; Mastromatteo, Lucera, Sinigaglia, & Corbo, 2010b; Nattress et al., 2001).

The objective of the present study was to quantify the probability of growth of *C. sporogenes* in a laboratory culture medium as influenced by various combinations of the preservatives salt, sorbic acid, nisin and lysozyme. Such combinations have not been previously studied. A laboratory culture medium rather than a food was used as it provided a constant, homogeneous environment that it would allow the actions and interactions of these preservatives to be clearly identified.

4.2. Materials and methods

4.2.1. Experimental design

A central composite rotatable design (CCRD) was used (Box & Draper, 1986) in order to evaluate the effects of the combinations of salt (sodium chloride) (0-8% w/w), nisin (0-500 ppm) (Nisaplin 114373, Danisco Ltd., Beaminster, United Kingdom) (concentrations of Nisaplin, which contains 2.5% nisin, were set to give the required nisin concentrations), lysozyme (0-500 ppm) and potassium sorbate (0-4.5% w/w) on the growth of a cocktail of spores of *C. sporogenes* in thioglycollate broth at pH 7 (the optimum pH for growth of *C. sporogenes*). In order to observe the inhibitory interaction of these preservatives, a broad range of preservative concentrations was used without

considering the highest permitted level of them in food. The preservative concentrations shown in Table 4.1 were tested in the combinations listed as treatments in Table 4.3.

Table 4.1. The factors and the levels in the central composite design.

Independent variables	Number of levels	Concentrations
x ₁ : Salt (% w/w)	5	0, 2, 4, 6, 8
x ₂ : Nisin (ppm)	5	0, 125, 250, 375, 500
x ₃ : Potassium sorbate (% w/w)	5	0, 1.12, 2.25, 3.37, 4.50
x ₄ : Lysozyme (ppm)	5	0, 125, 250, 375, 500

4.2.2. Growth medium preparation and inoculation

Half a litre of thioglycollate broth was made (see Chapter 3) and then different levels of salt and potassium sorbate (Hawkins Watts New Zealand, Auckland, New Zealand) were added. The prepared broth were autoclaved at 121°C for 15 minutes, and then cooled to room temperature. Then stock solutions of nisin and lysozyme (Sigma Aldrich Co. LLC., L6876, St. Louis, United States) were separately added to the sterile nutrient broth through a sterile 0.20 µm pore filter (Minisart, Sartorius AG, Goettingen, Germany) (Bell & Delacy, 1985). The pH of each prepared nutrient broth was adjusted to 7 with hydrochloric acid (1 M) or sodium hydroxide (0.1 N). Equal spore numbers of each strain were used to inoculate the prepared broth aliquots and the positive control to a total level of 10⁶ CFU mL⁻¹. Finally the prepared nutrient broth samples, negative controls and positive control (see Chapter 3) were distributed into 5 mL sterile screw-cap bottles and then anaerobically stored at 37°C for eight weeks.

4.2.3. Growth evaluation

At time zero and weekly, after manually shaking the bottles, the OD of five bottles of test broth and one bottle of negative control for each preservatives combination, and one bottle of positive control, was measured. All bottles were then discarded. The high number of replicates (five bottles) allowed an accurate measurement of the probability of growth. To achieve an accurate determination of the growth/no growth interface,

numerous replicates are required (Masana & Baranyi, 2000; Salter et al., 2000). Each bottle was considered as showing growth if the OD had increased by at least 0.1 above the OD at time zero (Mertens et al., 2011). The result for each treatment was expressed as a probability of growth value ranging from 0 (none of the five replicates showed growth) to 1 (all five of the replicates showed growth).

4.2.4. Model development

The probability of growth of *C. sporogenes* was measured as the dependent variable. A CCRD with 31 treatments (Table 4.3) was used to determine whether the main effects of the independent variables (the selected preservatives) and their interaction effects affected the probability of growth of the target micro-organism. All the experiments were repeated twice. A full quadratic model, including interaction terms, was assumed to describe relationships between variables. The data were analysed using the response surface regression procedure of Minitab 15.0 (Minitab Inc., USA) to fit the following quadratic polynomial equation (equation (4.1)):

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_4 + \alpha_5 X_1 X_2 + \alpha_6 X_1 X_3 + \alpha_7 X_1 X_4 + \alpha_8 X_2 X_3 + \alpha_9 X_2 X_4 + \alpha_{10} X_3 X_4 + \alpha_{11} X_1^2 + \alpha_{12} X_2^2 + \alpha_{13} X_3^2 + \alpha_{14} X_4^2 \quad (4.1)$$

where Y is the dependent variable (the probability of growth), α_i and X_i are coefficients and independent variables (concentration of selected preservatives) respectively. The adequacy and fitness of this model was tested by analysis of variance (ANOVA).

4.2.5. Model validation

The adequacy of the developed model to predict the probability of growth was tested using three different combinations of preservative concentrations which had not been used in the main experiment. These three different combinations of preservatives were anaerobically stored at 37°C for eight weeks. These combinations are shown in Table 4.2.

Table 4.2. Combination of preservatives used for the model validation.

Treatments	Salt (% w/w)	Nisin (ppm)	Potassium sorbate (% w/w)	Lysozyme (ppm)
1	1	400	3	200
2	3	100	2	400
3	5	200	1	100

4.3. Results

The observed probabilities of growth for the 31 treatments tested are shown in Table 4.3. Regression of these data yielded the coefficients and *p*-values shown in Table 4.4 and Table 4.5.

The OD of negative controls for all treatments did not increase during the eight weeks storage time. This indicated that no microbial contamination had occurred during the preparation of treatments. As expected, the OD of the positive control increased dramatically, indicating that growth occurred in the absence of the test preservatives. These findings support confidence in the probability of growth data as presented in Table 4.3.

Table 4.3. Treatments (preservative combinations) and observed probabilities of growth.

Treatment	Salt (% w/w)	Nisin (ppm)	Potassium sorbate (% w/w)	Lysozyme (ppm)	Growth probability (mean of duplicate)
1	6	125	3.37	125	0.2
2	4	250	4.50	250	0
3	2	125	3.37	375	0.2
4	4	250	2.25	250	0
5	4	250	2.25	250	0
6	2	375	3.37	125	0
7	2	125	3.37	125	0
8	6	125	1.12	125	0.1
9	6	375	3.37	375	0
10	2	375	1.12	125	1
11	6	125	3.37	375	0
12	6	375	3.37	125	0
13	4	250	2.25	250	0.1
14	4	250	2.25	250	0
15	8	250	2.25	250	0
16	6	375	1.12	375	0
17	4	0	2.25	250	0
18	4	250	2.25	250	0.2
19	0	250	2.25	250	1
20	4	250	2.25	500	0
21	6	125	1.12	375	0
22	4	250	2.25	250	0.1
23	2	375	1.12	375	1
24	2	125	1.12	375	1
25	6	375	1.12	125	0.3
26	4	250	2.25	0	0
27	4	250	0	250	1
28	2	375	3.37	375	0
29	2	125	1.12	125	1
30	4	500	2.25	250	0.1
31	4	250	2.25	250	0

Table 4.4. Analysis of variance for the probability of growth model showing the effects of independent variables as a linear, quadratic and interaction terms on the response variables.

Source	DF*	SS**	MS***	F-Value	p-Value
Model	14		0.317297	57.16	0
Residual					
Lack of fit	10	0.05164	0.005167	0.83	0.619
Pure error	6	0.07314	0.006190		
Total error	16	0.08881	0.005551		
Total	30	4.5309			

* Degrees of Freedom

** Sum of Squares

*** Mean Square

Table 4.5. Coefficients and p-value given by regression of the data in Table 4.3.

Variables	Coefficient	Standard error	p-Value
Intercept	2.6258	0.1595	0
Salt	-0.5381	0.0315	0
Nisin	*	0.031586	0.37
Potassium sorbate	-1.01587	0.05615	0
Lysozyme	-	0.000505	0.159
Salt ²	0.0292	0.0025	0
Nisin ²	-	0.000001	0.662
Potassium sorbate ²	0.09242	0.008	0
Lysozyme ²	-	0.000001	0.433
Salt× potassium sorbate	0.1	0.006	0
Salt× lysozyme	-0.0002	0.0005	0.001
Nisin× potassium sorbate	-0.0002	0.0005	0.008
Salt× nisin	-	0.000054	0.362
Potassium sorbate× lysozyme	-	0.000097	0.072
Nisin× lysozyme	-	0.0000001	0.362

* Not significant (p -value > 0.05)

The linear and quadratic terms for salt and potassium sorbate were significant (p -value < 0.05). The values of the linear coefficients for salt (-0.5381) and potassium sorbate (-1.01587) showed that increasing the concentrations of these preservatives significantly

(p -value < 0.05) decreased the probability of growth of *C. sporogenes*. The effects of the salt- potassium sorbate, nisin-potassium sorbate, and salt-lysozyme interactions were all significant (p -value < 0.05). The coefficient values for the interaction effects showed that the salt-potassium sorbate interaction (0.1) had a greater influence on the probability of growth than the nisin-potassium sorbate (-0.0002) and salt-lysozyme (-0.0002) interactions.

The regression indicated that the developed model possessed a non-significant lack of fit (p -value > 0.05) and showed a high value of the coefficient of determination (R^2) 0.9804 and adjusted R^2 value of 0.9632. This means the derived model fitted the observed growth probability data well and is therefore a good predictor of probability of growth.

The probability of growth can be modelled using a specific form of equation (4.2) containing only the terms that were found to be significant (p < 0.05).

$$Y = 2.6285 - (0.5381 \times \text{Salt}) - (1.01587 \times \text{PS}) + (0.0292 \times \text{Salt}^2) + (0.09242 \times \text{PS}^2) + (0.1 \times \text{Salt} \times \text{PS}) - (0.0002 \times \text{Salt} \times \text{Lysozyme}) - (0.0002 \times \text{Nisin} \times \text{PS}) \quad (4.2)$$

where Y is the probability of growth, Salt is the sodium chloride concentration (% w/w), PS is the potassium sorbate concentration (% w/w), Nisin is the nisin concentration (ppm) and Lysozyme is the lysozyme concentration (ppm).

Equation (4.2) has been used to generate Figures 4.1, 4.2 and 4.3. The combinations of two preservatives when the two other preservatives are at median levels are shown in each of these figures.

The combined effect of salt and potassium sorbate on the probability of growth is shown in Figure 4.1. At higher concentrations of potassium sorbate, lower concentrations of salt were required for a given probability of growth. At a given salt concentration, the probability of growth decreased with increasing potassium sorbate concentration. The opposite effect, shown in the top right hand corner of the plot in

Figure 4.1, is probably an artefact resulting from the relatively small quantity of experimental data.

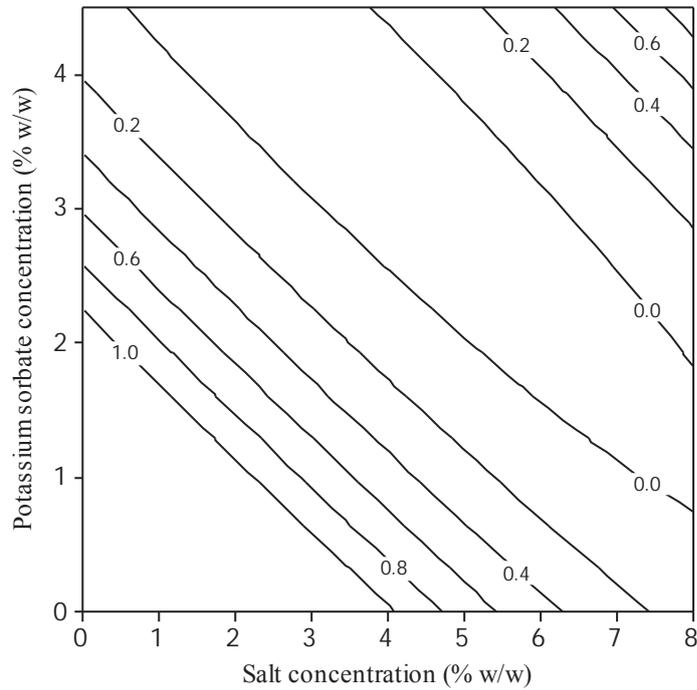


Figure 4.1. Probability of growth as a function of salt and potassium sorbate concentrations when nisin and lysozyme were at their median concentrations of 250 ppm.

The effect of nisin in combination with potassium sorbate on the probability of growth is shown in Figure 4.2. The probability of growth approached zero at potassium sorbate concentrations higher than approximately 2.5% w/w. Nisin concentration had a very small inhibitory effect on the probability of growth at potassium sorbate concentrations of < 2.5% w/w.

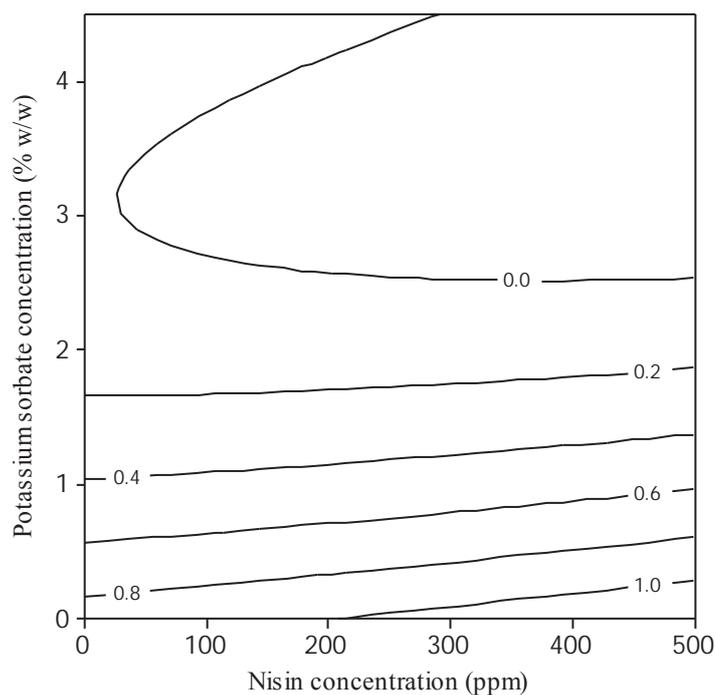


Figure 4.2. Probability of growth as a function of potassium sorbate and nisin concentrations when salt and lysozyme were at their median concentrations of 4% and 250 ppm, respectively.

The effect of the salt and lysozyme interaction on the probability of growth is illustrated in Figure 4.3. At a given salt concentration in the range of 1-2% the probability of growth increased slightly as lysozyme concentration increased. The probability of growth became zero at 4-5% salt, higher concentrations of salt being required at lower lysozyme concentrations. It is noted that while the effects of the potassium sorbate-nisin and salt-lysozyme interactions were significant, they are small as shown by the relatively low values of the corresponding coefficients in equation (4.2). Once again, the anomalous results for salt concentrations above 5% were probably artefacts.

The effects of the salt-nisin and potassium sorbate-lysozyme interactions were insignificant.

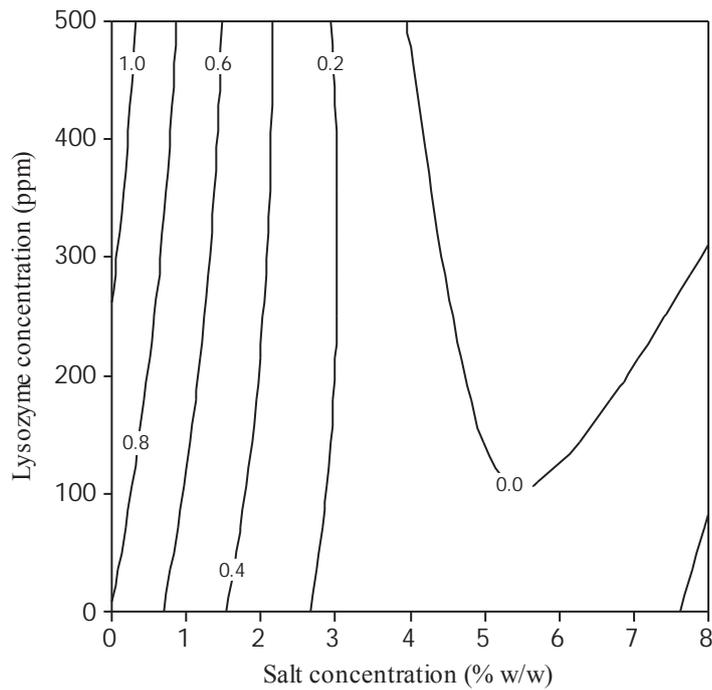


Figure 4.3. Probability of growth as a function of salt and lysozyme concentrations when nisin and potassium sorbate were at their median concentrations of 250 ppm and 2.25%, respectively.

4.3.1. Model validation

The adequacy of the developed model (equation (4.2)) to predict the probability of growth was tested using three different combinations of preservatives (Table 4.6) which were not used as treatments in the original experiment (Table 4.3). There is a close agreement between the observed and predicted values of the probability of growth. Hence, the model was found to be satisfactory.

Table 4.6. Validation of model: comparison between observed and predicted probabilities of growth

Salt (% w/w)	Nisin (ppm)	Potassium sorbate (% w/w)	Lysozyme (ppm)	Probability of growth	
				Experimental value	Predicted by the developed model
1	400	3	200	0	0 (actual value=-0.076)
3	100	2	400	0	0 (actual value=-0.065)
5	200	1	100	0.2	0.105

4.4. Discussion

The CCRD used in the current study has several advantages compared with classical experimental designs. Firstly, it allows a large amount of information to be gained from a small number of experiments; classical designs are time consuming and a large number of experiments are needed to explain the behaviour of a system. Secondly, with CCRD it is possible to observe easily the effects of the independent variables and their interactions on the response. The model easily clarifies these. The only drawback of this design in the current work is the generation of some anomalous results at high concentrations of preservatives, probably as a result of the relatively small quantity of experimental data collected.

The results of this study indicate that increasing potassium sorbate level from 2 to 4.5% w/w could reduce the amount of required salt to achieve the same probability of growth. Salt is used as an inhibitor of clostridial outgrowth and it has been reported that levels of 8.2-10.5% w/w salt used alone are necessary to inhibit spore outgrowth. Salt acts synergistically in combination with potassium sorbate against *C. botulinum* in meat slurries (Thomas, 1999). A level of 10% w/w salt can be considered as only marginal protection against *C. botulinum* spore outgrowth. However, this high level of salt is not desirable for palatable food products. The addition of other preservatives might allow the salt concentration to be reduced while preventing *C. botulinum* growth. Potassium sorbate was found to be an effective inhibitor of *C. botulinum* in experimental canned comminuted pork (Ivey & Robach, 1976). Robach (1980) reported that the effectiveness of potassium sorbate on the outgrowth of *C. sporogenes* PA 3679 spores might be due

to an interaction between potassium sorbate and salt. Robach (1980) found that increasing salt levels in the range 1% to 5% w/w enhanced inhibition by potassium sorbate (0.1-0.3% w/w) on the outgrowth of *C. sporogenes*. The results of the present study show that combining salt and potassium sorbate allows lower levels of salt to be used for a given probability of growth.

The presence of salt can enhance the preservative effect of nisin on *L. monocytogenes* and *C. botulinum* (Harris et al., 1991; Mazzotta et al., 1997; Parente et al., 1998; Thomas & Wimpenny, 1996). However, salt has been observed to reduce the ability of nisin to prevent the outgrowth of *Bacillus* spores and also to decrease the inhibitory effect of nisin on *L. monocytogenes*, with maximal antagonism at concentrations of 2-4% w/w of salt (Bell & Delacy, 1985; Bouttefroy et al., 2000).

The results show that nisin was not effective in lowering the probability of growth of *C. sporogenes* alone but had a significant inhibitory effect in combination with potassium sorbate. Rayman et al. (1981) showed that nisin sensitivity varies among *Clostridium* strains, and conflicting results on nisin effectiveness could be the result of resistance of some strains of *C. sporogenes* to nisin. A cocktail of *C. sporogenes* may be less sensitive than single strains to nisin at certain pH values and this has important practical implications (Rayman et al., 1981). Nisin resistance in *Clostridium* spores can be reduced by lowering the pH. The effectiveness of nisin increases with increasing acidity, and nisin is considerably more effective at lower pH than at either pH 7 or pH 8 (Scott & Taylor, 1981b). The high pH of the culture medium used in his work may explain the low inhibitory effect of nisin.

Lysozyme and nisin in combination are more effective in preventing the growth of Gram-positive spore-forming bacteria such as *B. thermosphacta* (Cannarsi et al., 2008). A mixture of nisin and lysozyme is more effective against food spoilage bacteria than using each of them individually (Chung & Hancock, 2000; Mastromatteo et al., 2010a; Mastromatteo et al., 2010b; Nattress et al., 2001). In the current study lysozyme alone and in combination with nisin had no inhibitory effect on *C. sporogenes*. This might be due to lysozyme losing its activity quickly when studied using pyrex, polypropylene or polyethylene containers, as it adheres to surfaces made of these materials (Nattress et

al., 2001). In this study, the samples were kept in polyethylene bottles. Lysozyme also can be inactivated by the presence of peptone, beef liver extract and boiled soybean, ingredients found in bacteriological media. However, none of these ingredients were not present in the thioglycollate broth used.

4.5. Conclusion

This study indicated that salt and potassium sorbate are the most effective preservatives against *C. sporogenes* in high moisture and low acid (pH 7) conditions. A combination of salt and potassium sorbate is more effective than using each of these preservatives individually at reducing the probability of growth of *C. sporogenes*. Although the effects of the nisin-potassium sorbate and lysozyme-salt interactions on the probability of growth were significant (p -value < 0.05), these effects were negligibly small. The use of nisin and lysozyme individually and in combination did not have significant inhibitory effects. The inhibitory effects of potassium sorbate and nisin are pH-dependent, and therefore expected to change under pH variation. On the basis of the results of this study, nisin was selected for further study along with salt and potassium sorbate. Lysozyme was eliminated because it appears to amplify the heat resistance of spore and may cause allergic reactions (Alderton et al., 1974). The inhibitory effect of salt, potassium sorbate and nisin will be evaluated in the nutrient broth in ongoing work (Chapter 5).

Chapter Five. Modelling the combined effects of salt, sorbic acid and nisin on the probability of growth of *Clostridium sporogenes* in nutrient broth

5.1. Introduction

Mathematical modelling has been used to describe quantitatively the growth response of micro-organisms to combinations of preservatives (Baker & Genigeorgis, 1993; McClure et al., 1994a; McMeekin, Olley, Ratkowsky, Corkrey, & Ross, 2013). Modelling provides information on the effects of inhibitory factors and their interactions, and it is useful in reducing the extent of challenge testing required to ensure product safety. Probability models are useful in measuring the microbial safety of food, particularly by providing a quantitative means of applying hurdle technology. The prediction of whether or not micro-organisms could grow or could produce toxin is preferred to kinetic models (which describe the growth rate) because any growth implies a possibility of food-borne disease, food intoxication or food spoilage. It is of economic interest to the food industry to be able to predict (without a need for time consuming and expensive challenge testing) the potential for growth of specific micro-organisms in a food or to establish product formulations excluding growth.

Probability of growth values can be calculated as functions of selected preservation factors and conversely can provide a means of setting combinations of preservative levels resulting in a given probability of growth (McKellar & Lu, 2001; Salter et al., 2000; Schaffner, Ross, & Montville, 1998). This approach enables the identification of specific combinations of preservative factors preventing the growth of a target micro-organism. There are several previously published models describing the combined effect of two or more factors on the probability of growth of *C. botulinum*. The effects of temperature, pH and sorbic acid on the probability of growth of *C. botulinum* vegetative cells were modelled by Lund et al. (1990) . The effects of temperature, salt and lactate on the growth of *C. botulinum* in turkey meat were modelled using probability models (Genigeorgis, Meng, & Baker, 1991; Meng & Genigeorgis, 1993). Tanaka et al. (1986) applied a logistic regression approach to model the effect of

sodium chloride, sodium phosphate, pH and moisture individually and in combination, on toxin production by *C. botulinum*. Their results showed that a high moisture product can be produced safely if the pH of the product is 6 and the salt plus sodium phosphate concentration is at 4.64% (Tanaka et al., 1986). No study has focused on or modelled the inhibitory effects of salt, sorbic acid (in the form of potassium sorbate) and nisin on *Clostridium* spores. In the current work, the combined effects of salt, potassium sorbate and nisin on *C. sporogenes* were determined. The effectiveness of these preservatives in preventing the growth of *C. sporogenes* was to be expressed as the probability of growth of this micro-organism. In the present study, *C. sporogenes* was used, as it is considered to be a non-toxicogenic equivalent of *C. botulinum* (Baird-Parker, Gould, & Lund, 2000; Goldoni et al., 1980). The aims of this study were:

- 1) To develop models as a function of the concentrations of the selected preservatives that can be used to predict the probability of growth of *C. sporogenes*.
- 2) To validate the developed models with fresh experimental data within the interpolation ranges of the independent variables.

5.2. Materials and methods

5.2.1. Experimental design

A full factorial experimental design with 100 treatments (combinations of preservative concentrations) was performed for each of two pHs in order to establish probability-of-growth-dependence on preservative concentrations for *C. sporogenes*. The response variable was probability of growth, assessed as the fraction of ten replicates exhibiting growth. The inhibitory effects of salt (sodium chloride) (0, 1, 2, 3, 4% w/w), potassium sorbate (0, 1, 2, 3, 4% w/w) (Hawkins Watts New Zealand) and nisin (0, 100, 200, 300 ppm) (Nisaplin 114373, Danisco Ltd., Beaminster, United Kingdom) (concentrations of Nisaplin, which contains 2.5% nisin, were set to give the required nisin concentrations) at pH 5.5 (the pH of processed cheese) and pH 7 (the optimum condition for the spore of *C. sporogenes* to germinate) were investigated. The 100 experimental treatments were carried out randomly and are shown in Table 5.1.

Table 5.1. Experimental treatments (combinations of preservative concentrations).

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Number of replicates
1	0	0	1	10
2	4	0	1	10
3	1	0	0	10
4	4	300	1	10
5	1	200	4	10
6	0	300	1	10
7	4	200	2	10
8	1	0	4	10
9	2	200	2	10
10	3	200	1	10
11	2	100	3	10
12	0	100	2	10
13	3	300	2	10
14	3	300	1	10
15	4	100	0	10
16	1	200	1	10
17	2	100	2	10
18	1	0	1	10
19	4	0	3	10
20	3	0	3	10
21	4	200	0	10
22	1	300	0	10
23	2	300	4	10
24	1	100	4	10
25	0	200	1	10
26	3	0	0	10
27	4	0	4	10
28	4	300	0	10
29	2	0	4	10
30	3	200	0	10
31	3	100	2	10
32	0	200	2	10

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Table 5.1. Continued.

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Number of replicates
33	3	300	4	10
34	1	300	2	10
35	3	300	3	10
36	4	100	1	10
37	0	0	2	10
38	1	100	0	10
39	4	300	3	10
40	3	200	2	10
41	4	100	4	10
42	2	300	1	10
43	1	200	2	10
44	4	200	4	10
45	3	0	2	10
46	0	100	3	10
47	0	300	2	10
48	0	300	4	10
49	4	100	2	10
50	1	0	3	10
51	3	200	4	10
52	4	300	4	10
53	2	300	2	10
54	3	100	1	10
55	2	300	3	10
56	3	200	3	10
57	2	100	4	10
58	2	100	0	10
59	3	100	4	10
60	1	100	3	10
61	4	300	2	10
62	0	100	1	10
63	2	200	4	10
64	0	200	4	10
65	2	300	0	10
66	0	300	3	10

Continued on next page

Table 5.1. Continued.

Treatment	Salt (% w/w) *	Nisin (ppm) *	Potassium sorbate (% w/w) *	Number of replicates
67	1	300	4	10
68	1	200	0	10
69	3	0	4	10
70	0	0	0	10
71	1	300	3	10
72	1	300	1	10
73	0	0	3	10
74	2	0	0	10
75	2	200	1	10
76	0	0	4	10
77	2	0	3	10
78	1	200	3	10
79	0	200	0	10
80	2	0	1	10
81	0	200	3	10
82	3	100	0	10
83	0	100	0	10
84	4	0	0	10
85	0	300	0	10
86	0	100	4	10
87	4	100	3	10
88	1	0	2	10
89	3	300	0	10
90	4	200	3	10
91	3	100	3	10
92	1	100	1	10
93	2	0	2	10
94	4	200	1	10
95	2	100	1	10
96	1	100	2	10
97	2	200	3	10
98	4	0	2	10
98	4	0	2	10
99	3	0	1	10
100	2	200	0	10

* As a proportion of total weight of broth

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5.2.2. Preparation and inoculation of nutrient broth

For each combination of preservatives, half a litre of thioglycollate broth was made (see section 3.4.1) and then different levels of salt and potassium sorbate (Hawkins Watts New Zealand, Auckland, New Zealand) were added. The prepared nutrient broth was autoclaved at 121°C for 15 minutes, and then cooled to room temperature. Then, a stock solution of nisin (Nisaplin 114373, Danisco Ltd., Beaminster, United Kingdom) in 0.02 N HCl were prepared and added to the sterile broth through a sterile 0.20 µm pore filter (Minisart, Sartorius AG, Goettingen, Germany) (Bell & Delacy, 1985). The pH of each prepared nutrient broth was adjusted with hydrochloric acid (1 M) or sodium hydroxide (0.1 N), aseptically. Equal spore numbers of each strain were used to inoculate the prepared broth and positive controls to a total level of 10^6 CFU mL⁻¹. Combinations of hurdles which prevent growth vary with inoculum size (Koutsoumanis & Sofos, 2005). Growth is more likely when a high inoculum is used. In this study, a high inoculum size (10^6 CFU mL⁻¹) was used to ensure a conservative approach in reducing the risk of *C. sporogenes* growth. However, this large inoculum size is greater than the expected contamination in food products. Therefore, the probability of growth as predicted by the developed model would be expected to be less under realistic conditions and the model tend to be ‘fail-safe’.

The inoculated broth, negative controls and positive control (see section 3.4.1) were distributed into 5 mL sterile screw cap bottles which were then anaerobically stored at 37°C for eight weeks. Common practice is storage at 25°C but 37°C was used to perform all experiments within a reasonable time frame. Aliquots were taken from the 5 mL bottles and transferred into a micro-plate reader. The presence/absence of microbial growth was monitored weekly by measuring the optical density of the inoculated culture medium at 595 nm using an automated micro-plate reader (Spectro star nano, BMG LABTECH). Each treatment had 10 replicates which were observed for growth weekly. Gram staining was used to check that for samples in which growth was detected, growth was due to *C. sporogenes* (which is Gram positive) and not a contaminant.

5.2.3. Growth evaluation

At time zero, and weekly, after manually shaking the bottles, the optical density (OD) of ten bottles for each preservative combination, one bottle of negative control for each preservative combination, and one bottle of positive control, were measured. All bottles were then discarded. A bottle was considered as showing growth if the OD increased by at least 0.1 above the OD at time zero (Mertens et al., 2011). The OD method of detecting growth has become established as a convenient and cost-effective way to generate large data sets in model development and was used in this study. The results for each treatment were expressed as a probability of growth value ranging from 0 (none of the ten replicates showed growth) to 1 (all ten of the replicates showed growth). The high number of replicates (ten bottles) allowed an accurate measurement of the probability of growth.

5.2.4. Development of probability of growth models

The probability of growth data were statistically analyzed and modelled using ordinary (linear) logistic regression in SAS 9.1 (SAS Intitute Inc., Cary, N.C., USA). The main effects were forced to stay in the final model equation regardless of their p -value. Models were derived using the automatic variable selection option (p -value < 0.05) with a stepwise selection method to determine estimates of significant parameters (Koutsoumanis, Kendall, & Sofos, 2004a; Koutsoumanis et al., 2004b; McKellar & Lu, 2001; Ratkowsky & Ross, 1995). The potential model form is shown as equation (5.1).

$$\begin{aligned} \text{Logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\ &= \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_1 X_2 + \alpha_5 X_1 X_3 + \alpha_6 X_2 X_3 + \alpha_7 X_1^2 \\ &\quad + \alpha_8 X_2^2 + \alpha_9 X_3^2 \end{aligned} \quad (5.1)$$

where $\text{logit}(p)$ is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), α_i and X_i are coefficients and independent variables (concentrations of

preservatives), respectively. Terms found to be not significant were omitted from each of the final models described in the following.

Five models were developed. For models A₁ (pH 5.5) and A₂ (pH 7), sorbic acid concentration was expressed as (% w/w) potassium sorbate. For models B₁ (pH 5.5) and B₂ (pH 7), sorbic acid concentration was expressed as mol L⁻¹ of undissociated sorbic acid. For model C (containing the data for both pHs), sorbic acid concentration was expressed as mol L⁻¹ of undissociated sorbic acid. It is undissociated sorbic acid that has the greatest preservative effect (Gysemans et al., 2007).

The undissociated sorbic acid concentration in mol L⁻¹ was calculated according to the Henderson-Hasselbalch equation (equation (5.2)):

$$\text{Undissociated sorbic acid (mol L}^{-1}\text{)} = \frac{\text{total sorbic acid (mol L}^{-1}\text{)}}{1 + 10^{(\text{pH}-\text{pK}_a)}} \quad (5.2)$$

where pK_a = 4.74. This is the pH at which the concentrations of dissociated and undissociated sorbic acid are equal.

Finally, the performances of the five models were compared and the best models for predicting the probability of growth of *C. sporogenes* in broth were selected.

5.2.5. Assessment of model performance

For each model, goodness of fit statistics and measures of predictive power were calculated in SAS. Goodness of fit was determined by:

- 1) Likelihood ratio test (-2lnL), where L is the likelihood at its optimum.
- 2) Akaike's Information Criterion (AIC = -2lnL+2k, where k is the number of parameters in the model).
- 3) The Hosmer-Lemeshow statistic (HL).

The -2lnL and the AIC can be used to rank models based on the same data set, where lower values indicate better fitting models. However, these statistics do not indicate if a

model fits the data adequately. The HL statistic indicates if there is a lack of fit (Gysemans et al., 2007). If the HL statistic takes a small value (or a high corresponding p -value) then the model fits the data well. The model will be rejected if the p -value is below 0.05. The disadvantage of the HL statistic is the fact that it can be heavily influenced by a single bad prediction. Consequently, this statistic must be interpreted with caution (Agresti, 2007).

The predictive power of the models was measured by the c -value (the concordance index) or percentage of correct prediction. The predictive power is perfect if the c -value equals 1 or the percentage of correct prediction is 100%. The percentage of correct prediction shows to what extent the predicted values match the observed values. Both indicate the extent of agreement between the predicted probability of growth and the observed growth (Dang et al., 2010; Gysemans et al., 2007).

In order to compare observed growth/no growth data with model predictions, predicted probabilities at 0.1, 0.5 and 0.9 levels were calculated as functions of preservative concentrations and plotted in Microsoft Excel (Microsoft Corporation) together with experimentally determined probabilities.

5.2.6. Model validation

In order to validate a developed model it is appropriate to use data derived under well controlled conditions, so that the model's performance is not unfairly biased by comparison with unrepresentative data or data collected under inadequately controlled or defined conditions. Therefore, in the present study, validation conditions were selected as new combinations of factors (preservative concentrations) which had not been previously tested but were within the ranges used in developing the models. Extrapolation beyond these ranges was not used, owing to the uncertainty that would exist (Roberts, Gibson, & Robinson, 1981). Validation at pH 5.5 and pH 7 was carried out using the preservative combinations shown in Table 5.2.

One of the most common methods of validation is to compare model predictions with high quality literature data. This is a reasonable approach when literature data are

obtained under defined conditions that do not differ markedly from those used to develop the model. In this case the model predictions will be reasonably reflected in the published data if the model is satisfactory. There is a dearth of published data relating both to *C. botulinum* and to the independent variables studied in the work reported here; the only relevant data are those in Roberts et al. (1982) and Whiting and Call (1993). These data were used for validation purposes.

Table 5.2. Combinations of preservatives used in testing model predictions at pH 5.5 and 7.

Treatments	Salt (% w/w) *	Nisin (ppm) *	Potassium sorbate (% w/w) *
1	0.5	250	2.5
2	1.5	50	1.5
3	2.5	150	0.5

*As a proportion of total weight of broth

5.2.7. Checking growth at room temperature (25°C)

Though the optimum temperature for growth rate of *C. sporogenes* is 37°C, there is evidence that resistance to stress may be greater, and therefore the probability of growth might be greater, at more moderate temperatures (Corkrey et al., 2014; Presser et al., 1998). This observation is explained in Corkrey et al. (2014) in the differentiation between T_{opt} , the temperature at which growth rate is fastest and T_{mes} , the temperature at which growth is most efficient. However, given the typically low contamination level of *C. botulinum* in foods and the need for extensive growth before toxigenic levels are reached, we chose to assess probability of growth at 37°C as a trade-off between absolute probability of growth and probability of growth to dangerous levels. Nevertheless, to test the validity of this approach, the combinations for which growth did not occur at 37°C were prepared at both pHs. The OD of samples was measured weekly for eight weeks in order to record the probability of growth at 25°C compared to that at 37°C. The samples are listed in Table 5.3.

Table 5.3. Combinations of different preservatives at both pH 5.5 and pH 7 that showed no growth at 37°C, and which were tested again at 25°C.

Treatments	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Number of replicates
5	1	200	4	10
7	4	200	2	10
9	2	200	2	10
13	3	300	2	10
17	2	100	2	10
19	4	0	3	10
23	2	300	4	10
31	3	100	2	10
33	3	300	4	10
35	3	300	3	10
39	4	300	3	10
40	3	200	2	10
41	4	100	4	10
44	4	200	4	10
48	0	300	4	10
49	4	100	2	10
51	3	200	4	10
52	4	300	4	10
53	2	300	2	10
55	2	300	3	10

*As a percentage of total weight of broth

5.3. Results

The observed probability of growth data at different combinations of salt, potassium sorbate and nisin at pHs 5.5 and 7 are shown in Table 5.4. The OD of negative controls for all treatments did not increase during the eight week storage period. This indicated that no microbial contamination had occurred during the preparation of the test broths. As expected, the OD of the positive control increased, indicating that growth occurred in the absence of the test preservatives, as would be expected. These findings allow confidence to be placed in the probability of growth data presented in Table 5.4.

Table 5.4. Observed probabilities of growth of *C. sporogenes* in broth at pHs 5.5 and 7.

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
1	0	0	1	0.068	0.01	0.0003	0.2	1.0
2	4	0	1	0.068	0.01	0.0003	0	1.0
3	1	0	0	0	0	0	1.0	1.0
4	4	300	1	0.068	0.01	0.0003	0	1.0
5	1	200	4	0.275	0.04	0.0015	0	0
6	0	300	1	0.068	0.01	0.0003	0	1.0
7	4	200	2	0.137	0.02	0.0007	0	0
8	1	0	4	0.275	0.04	0.0015	0	0.2
9	2	200	2	0.137	0.02	0.0007	0	0
10	3	200	1	0.068	0.01	0.0003	0	1.0
11	2	100	3	0.206	0.03	0.0011	0	0.1
12	0	100	2	0.137	0.02	0.0007	0	0.9
13	3	300	2	0.137	0.02	0.0007	0	0
14	3	300	1	0.068	0.01	0.0003	0	0.6
15	4	100	0	0	0	0	0	1.0
16	1	200	1	0.068	0.01	0.0003	0	1.0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
17	2	100	2	0.137	0.02	0.0007	0	0
18	1	0	1	0.068	0.01	0.0003	0	1.0
19	4	0	3	0.206	0.03	0.0011	0	0
20	3	0	3	0.206	0.03	0.0011	0	0.8
21	4	200	0	0	0	0	0	1.0
22	1	300	0	0	0	0	1.0	1.0
23	2	300	4	0.275	0.04	0.0015	0	0
24	1	100	4	0.275	0.04	0.0015	0	0.2
25	0	200	1	0.068	0.01	0.0003	0	1.0
26	3	0	0	0	0	0	1.0	1.0
27	4	0	4	0.275	0.04	0.0015	0	0.6
28	4	300	0	0	0	0	0	1.0
29	2	0	4	0.275	0.04	0.0015	0	0.2
30	3	200	0	0	0	0	0	1.0
31	3	100	2	0.137	0.02	0.0007	0	0
32	0	200	2	0.137	0.02	0.0007	0	1.0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
33	3	300	4	0.275	0.04	0.0015	0	0
34	1	300	2	0.137	0.02	0.0007	0	0.2
35	3	300	3	0.206	0.03	0.0011	0	0
36	4	100	1	0.068	0.01	0.0003	0	0.2
37	0	0	2	0.137	0.02	0.0007	0.1	1.0
38	1	100	0	0	0	0	0.1	1.0
39	4	300	3	0.206	0.03	0.0011	0	0
40	3	200	2	0.137	0.02	0.0007	0	0
41	4	100	4	0.275	0.04	0.0015	0	0
42	2	300	1	0.068	0.01	0.0003	0	1.0
43	1	200	2	0.137	0.02	0.0007	0	1.0
44	4	200	4	0.275	0.04	0.0015	0	0
45	3	0	2	0.137	0.02	0.0007	0	1.0
46	0	100	3	0.206	0.03	0.0011	0	0.1
47	0	300	2	0.137	0.02	0.0007	0	0.8
48	0	300	4	0.275	0.04	0.0015	0	0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
49	4	100	2	0.137	0.02	0.0007	0	0
50	1	0	3	0.206	0.03	0.0011	0	0.2
51	3	200	4	0.275	0.04	0.0015	0	0
52	4	300	4	0.275	0.04	0.0015	0	0
53	2	300	2	0.137	0.02	0.0007	0	0
54	3	100	1	0.068	0.01	0.0003	0	0.9
55	2	300	3	0.206	0.03	0.0011	0	0
56	3	200	3	0.206	0.03	0.0011	0	0
57	2	100	4	0.275	0.04	0.0015	0	0.7
58	2	100	0	0	0	0	0.4	0.9
59	3	100	4	0.275	0.04	0.0015	0	0
60	1	100	3	0.206	0.03	0.0011	0	0.1
61	4	300	2	0.137	0.02	0.0007	0	0
62	0	100	1	0.068	0.01	0.0003	0	1.0
63	2	200	4	0.275	0.04	0.0015	0	0
64	0	200	4	0.275	0.04	0.0015	0	0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
65	2	300	0	0	0	0	1.0	1.0
66	0	300	3	0.206	0.03	0.0011	0	0.2
67	1	300	4	0.275	0.04	0.0015	0	0
68	1	200	0	0	0	0	1.0	1.0
69	3	0	4	0.275	0.04	0.0015	0	1.0
70	0	0	0	0	0	0	1.0	1.0
71	1	300	3	0.206	0.03	0.0011	0	0
72	1	300	1	0.068	0.01	0.0003	0	1.0
73	0	0	3	0.206	0.03	0.0011	0	1.0
74	2	0	0	0	0	0	1.0	1.0
75	2	200	1	0.068	0.01	0.0003	0	1.0
76	0	0	4	0.275	0.04	0.0015	0	0.6
77	2	0	3	0.206	0.03	0.0011	0	1.0
78	1	200	3	0.206	0.03	0.0011	0	0.4
79	0	200	0	0	0	0	1.0	1.0
80	2	0	1	0.068	0.01	0.0003	0.9	1.0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
81	0	200	3	0.206	0.03	0.0011	0	0.8
82	3	100	0	0	0	0	0.5	0.7
83	0	100	0	0	0	0	1.0	1.0
84	4	0	0	0	0	0	1.0	1.0
85	0	300	0	0	0	0	1.0	1.0
86	0	100	4	0.275	0.04	0.0015	0	0.4
87	4	100	3	0.206	0.03	0.0011	0	0
88	1	0	2	0.137	0.02	0.0007	0	1.0
89	3	300	0	0	0	0	0.5	1.0
90	4	200	3	0.206	0.03	0.0011	0	0
91	3	100	3	0.206	0.03	0.0011	0	0
92	1	100	1	0.068	0.01	0.0003	0.2	0.7
93	2	0	2	0.137	0.02	0.0007	0	1.0
94	4	200	1	0.068	0.01	0.0003	0	0
95	2	100	1	0.068	0.01	0.0003	0.1	0.6
96	1	100	2	0.137	0.02	0.0007	0	0.2
97	2	200	3	0.206	0.03	0.0011	0	0

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Table 5.4. Continued

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*	Sorbic acid (mol L ⁻¹)*	Undissociated sorbic acid (mol L ⁻¹)*		Observed probability of growth	
					pH 5.5	pH 7	pH 5.5	pH 7
98	4	0	2	0.137	0.02	0.0007	0	0.6
99	3	0	1	0.068	0.01	0.0003	0	1.0
100	2	200	0	0	0	0	0.6	1.0

* As a proportion of total weight of the broth

5.3.1. Development of probability of growth models

The five models and their performances are detailed in the following sections.

5.3.1.1. Model A₁ (pH 5.5) and Model A₂ (pH 7): sorbic acid concentration expressed as potassium sorbate concentration (% w/w)

The application of logistic regression to the relevant data in Table 1 yielded the models A₁ (pH 5.5) and A₂ (pH 7). Model coefficients and their corresponding *p*-values, goodness-of-fit criteria and predictive power criteria are presented in Table 5.5.

Table 5.5. Coefficients of, and statistics for, Models A₁ and A₂, for which sorbic acid concentration is expressed as potassium sorbate concentration (% w/w).

Parameter	Model A ₁		Model A ₂	
	pH 5.5		pH 7	
	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value
Constant	2.8658	<0.0001	7.7998	<0.0001
Salt	0.8847	<0.0001	-0.7818	0.0067
Potassium sorbate	-3.9725	<0.0001	-3.9584	<0.0001
Nisin	-0.0302	0.0004	-0.00310	0.6304
Salt ²	-0.2795	0.0317	N.S.*	N.S.
Potassium sorbate ²	N.S.	N.S.	0.5506	<0.0001
Nisin ²	0.000155	<0.0001	0.000041	<0.0005
Salt× potassium sorbate	N.S.	N.S.	0.1827	0.0410
Salt× nisin	-0.00889	<0.0001	-0.00424	<0.0001
Potassium sorbate× nisin	-0.0210	0.0007	-0.00529	0.0002
Goodness of fit /predictive power				
-2lnL	815.415		1365.589	
AIC	831.415		1383.589	
HL	4.8		12.3	
	<i>p</i> -value = 0.490		<i>p</i> -value = 0.139	
c-value	0.987		0.951	
Correct prediction %	98.6		95.7	

* Not significant (*p*-value > 0.05)

The significant terms (*p*-value < 0.05) for each pH (5.5 and 7) were retained in the models, shown as equation (5.3) (Model A1) and equation (5.4) (Model A2).

$$\begin{aligned}
\text{Logit (p)} &= \ln\left(\frac{p}{1-p}\right) \\
&= 2.8658 + (0.8847 \times \text{Salt}) - (3.9725 \times \text{PS}) - (0.0302 \times \text{Nisin}) \\
&\quad - (0.2795 \times \text{Salt}^2) + (0.000155 \times \text{Nisin}^2) \\
&\quad - (0.00889 \times \text{Salt} \times \text{Nisin}) - (0.0210 \times \text{PS} \times \text{Nisin}) \quad (5.3)
\end{aligned}$$

$$\begin{aligned}
\text{Logit (p)} &= \ln\left(\frac{p}{1-p}\right) \\
&= 7.7998 - (0.7818 \times \text{Salt}) - (3.9584 \times \text{PS}) - (0.0031 \times \text{Nisin}) \\
&\quad + (0.5506 \times \text{PS}^2) + (0.000041 \times \text{Nisin}^2) + (0.1827 \times \text{Salt} \times \text{PS}) \\
&\quad - (0.00424 \times \text{Salt} \times \text{Nisin}) - (0.00529 \times \text{PS} \times \text{Nisin}) \quad (5.4)
\end{aligned}$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), Salt is sodium chloride concentration (% w/w), PS is potassium sorbate concentration (% w/w) and Nisin is nisin concentration (ppm).

The HL statistics for models A₁ and A₂ were small values (4.8 and 12.3 respectively) and their corresponding p-values were higher than 0.05. This indicates that the models fitted the data adequately. The c-values were close to 1 for both models, and the percentages of correct prediction were 98.6% and 95.7% for models A₁ and A₂ respectively. This indicates that the performance of the models would be satisfactory. The -2lnL and AIC criteria were used for model ranking in order to be able to select the best developed model (see section 5.3.1.2 below).

5.3.1.2. Model B₁ (pH 5.5) and Model B₂ (pH 7): sorbic acid concentration expressed as undissociated sorbic acid concentration (mol L⁻¹)

Coefficients and their p-values, goodness of fit criteria and predictive power criteria for models B₁ and B₂ are presented in Table 5.6.

Table 5.6. Coefficients of, and statistics for, Models B₁ and B₂, for which sorbic acid concentration is expressed as undissociated sorbic acid concentration (mol L⁻¹).

Parameter	Model B ₁		Model B ₂	
	pH 5.5		pH 7	
	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value
Constant	2.8658	<0.0001	7.4206	<0.0001
Salt	0.8847	<0.0001	-0.7381	0.0066
Undissociated sorbic acid	-397.2	<0.0001	-10949	<0.0001
Nisin	-0.0302	0.0004	-0.00499	0.4249
Salt ²	-0.2795	0.0317	N.S.	N.S.
Undissociated sorbic acid ²	N.S.*	N.S.	4085704	<0.0001
Nisin ²	0.000155	<0.0001	0.000042	0.0003
Salt × undissociated sorbic acid	N.S.	N.S.	446.2	0.048
Salt × nisin	-0.00889	<0.0001	-0.00425	<0.0001
Undissociated sorbic acid × nisin	-2.0994	<0.0001	-13.1158	0.0002
Goodness of fit /predictive power				
-2lnL	831.415		1383.589	
AIC	847.15		1401.589	
HL	9.2588		26.7090	
	<i>p</i> -value = 0.0992		<i>p</i> -value = 0.0008	
c-value	0.987		0.948	
Correct prediction %	98.6		94.7	

* Not significant (*p*-value > 0.05)

The significant terms (*p*-value < 0.05) were retained in the models, which are shown as equation (5.5) (Model B1) and equation (5.6) (Model B2) for pH 5.5 and 7, respectively.

$$\begin{aligned}
 \text{Logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\
 &= 2.8658 + (0.8847 \times \text{Salt}) - (397.2 \times \text{US}) - (0.0302 \times \text{Nisin}) \\
 &\quad - (0.2795 \times \text{Salt}^2) + (0.000155 \times \text{Nisin}^2) \\
 &\quad - (0.00889 \times \text{Salt} \times \text{Nisin}) - (2.0994 \times \text{US} \times \text{Nisin}) \quad (5.5)
 \end{aligned}$$

$$\begin{aligned}
 \text{Logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\
 &= 7.4206 - (0.7381 \times \text{Salt}) - (10490 \times \text{US}) - (0.00499 \times \text{Nisin}) \\
 &\quad + (4085704 \times \text{US}^2) + (0.000042 \times \text{Nisin}^2) + (446.2 \times \text{Salt} \times \text{US}) \\
 &\quad - (0.00425 \times \text{Salt} \times \text{Nisin}) - (13.1158 \times \text{US} \times \text{Nisin}) \quad (5.6)
 \end{aligned}$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), Salt is sodium chloride concentration (% w/w), US is undissociated sorbic acid concentration (mol L^{-1}) and Nisin is nisin concentration (ppm).

The HL statistics of models B_1 and B_2 were small values (9.25 and 26.7 respectively) but the corresponding p -value for Model B_2 was less than 0.05, which indicates a poor fit. The c -values were close to 1 for both models, indicating good predictive power. The percentages of correct prediction were 98.6 and 94.7 for models B_1 and B_2 respectively. The $-2\ln L$ and the AIC criteria were similar to those for models A_1 and A_2 . This indicates there is not much difference between models A_1 and B_1 and between models A_2 and B_2 . Models A_1 and B_1 can be used for the prediction of probability of growth at pH 5.5. However, Model B_2 fitted the data less well and thus Model A_2 is preferable for pH 7.

5.3.1.3. Model C (data for pH 5.5 and pH 7 continued): sorbic acid concentration expressed as the concentration of undissociated sorbic acid (mol L^{-1})

The undissociated sorbic acid concentration increases as pH decreases. Therefore, a data set of 200 data points, made up by combining the 100 data points for pH 5.5 with the 100 data points for pH 7, was used to develop a model based on salt, nisin and undissociated sorbic acid, allowing the effect of pH to express itself partly by its effect on undissociated sorbic acid concentration. Coefficients and their corresponding p -values, goodness of fit criteria and predictive power criteria are presented in Table 5.7.

Table 5.7. Coefficients of, and statistics for, Model C, for which sorbic acid concentration is expressed as mol L⁻¹ of undissociated sorbic acid.

Parameter	Model C	
	Coefficient	<i>p</i> -value
Constant	2.0683	<0.0001
Salt	-0.3301	0.0002
Undissociated sorbic acid	-134.1	<0.0001
Nisin	0.000457	0.8737
Salt ²	N.S.*	N.S.
Undissociated sorbic acid ²	N.S.	N.S.
Nisin ²	0.000024	0.0018
Salt× undissociated sorbic acid	-60.9797	0.0018
Salt× nisin	-0.00281	<0.0001
Undissociated sorbic acid × nisin	-15.2236	<0.0001
Goodness of fit /predictive power		
-2lnL	2540.943	
AIC	2556.943	
HL	21.4128	
	<i>p</i> -value = 0.0061	
c-value	0.725	
Correct prediction %	72.3	

* Not significant (*p*-value > 0.05)

Significant terms (*p*-value < 0.05) were retained in the model, which is shown as equation (5.7) (Model C).

$$\begin{aligned}
 \text{Logit (p)} &= \ln\left(\frac{p}{1-p}\right) \\
 &= 2.0683 - (0.3301 \times \text{Salt}) - (134.1 \times \text{US}) + (0.000457 \times \text{Nisin}) \\
 &\quad + (0.000024 \times \text{Nisin}^2) - (60.9797 \times \text{Salt} \times \text{US}) \\
 &\quad - (0.00281 \times \text{Salt} \times \text{Nisin}) - (15.2236 \times \text{US} \times \text{Nisin}) \quad (5.7)
 \end{aligned}$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), Salt is sodium chloride concentration (% w/w), US is the undissociated sorbic acid concentration (mol L⁻¹) and Nisin is nisin concentration (ppm).

The HL value for Model C was 21.4 and the corresponding p -value was less than 0.05, indicating that Model C did not fit the data well. Also, the c -value and the percentage of correct prediction were only 0.725 and 72.3%, respectively. This indicates that when pH is not explicitly included as a variable, or is allowed for only by its effect on sorbic acid, a poor model results. This is probably because the effect of pH on the inhibitory efficacy of nisin is not taken into account (Scott & Taylor, 1981b). The $-2\ln L$ and the AIC of Model C were higher than for models A_1 , A_2 , B_1 and B_2 , again indicating relatively poor model performance.

The $-2\ln L$ and the AIC of models A_1 and B_1 are similar to those of A_2 and B_2 . Models A_1 and A_2 are recommended as, generally, sorbic acid concentration is expressed as potassium sorbate concentration for convenience.

5.3.2. Validation of Models A_1 and A_2

To assess the reliability of the models a comparison was made between the probabilities of growth predicted by the best developed models (A_1 and A_2) and those observed after eight weeks in fresh experiments. The results are shown in Table 5.8.

Table 5.8. Models A_1 and A_2 validation: observed and predicted probabilities at pH 5.5 and 7.

Salt (%w/w)	Nisin (ppm)*	Potassium sorbate (% w/w)	Probability of growth			
			Observed at pH 5.5	Predicted by Model A_1	Observed at pH 7	Predicted by Model A_2
0.5	250	2.5	0	9.1×10^{-6}	0.1	0.17
1.5	50	1.5	0	0.12	0.7	0.96
2.5	150	0.5	0	0.03	0.5	0.67

* As a proportion of total weight of the broth

There is a reasonably close agreement between predicted and observed values of probability of growth, indicating that models A_1 and A_2 perform reasonably well.

Probabilities of growth predicted by Model A₁ and Model A₂ are compared with literature data from Roberts et al. (1982) and Whiting and Call (1993) in Table 5.9.

Table 5.9. Test of Models A₁ and A₂ using literature data.

Salt (% w/w)	Potassium sorbate (% w/w)	Probability of growth			
		Observed pH 5.5	Predicted by Model A ₁	Observed pH 7	Predicted by Model A ₂
2.5*	0*	100*	91.8	100*	99.79
2.5*	0.26*	100*	91.8	100*	99.53
3.5*	0*	100*	96.54	100*	99.79
3.5*	0.26*	60*	86.37	100*	99.03
4.5*	0*	100*	91.72	100*	98.92
4.5*	0.26*	80*	86.87	100*	98.41
0.75**	0**	93**	96.68	-	-
0**	0**	84**	94.61	91**	99.95
1.5**	0**	84**	97.24	-	-
3**	0**	-	-	91**	99.57

*Data from Roberts et al. (1982) relating to the spoilage of cured meat by *C. botulinum*.

**Data from Whiting and Call (1993) relating to the growth of *C. botulinum* in broth.

The comparison shows that model A₁ mostly underestimate the probability of growth when tested against the data of Roberts et al. (1982), although the underestimation was slight in 50% of samples the case of model A₂. In contrast, the two models tended to over-estimate the probability of growth when tested against the data of Whiting and Call (1993), which is fail safe. Thus these two models lack some accuracy when applied to clostridial growth in o for which they were not developed.

5.3.3. Checking probability of growth at room temperature (25°C)

The observed probabilities of growth for 20 different treatments (Table 5.3) for each pH at 25°C were the same as at 37°C. The probability of growth of *C. sporogenes* at the optimum temperature for growth (37°C) did not change at the moderate temperature of 25°C which indicates the developed models at 37°C are also applicable at 25°C.

5.3.4. Exploration of the relative preservative effects of salt, nisin and sorbic acid using Models A₁ and A₂

Based on the results of this study, the use of models A₁ and A₂ for predicting the probability of growth of *C. sporogenes* is favoured. It is possible to predict the probability of growth using these models for any given combination of preservative concentrations within the ranges tested. Figures 5.1-5.6, which display comparisons between measured and predicted probabilities for various combinations of salt, nisin and sorbic acid concentrations, show that except for some slight, inevitable discordance, there is generally close agreement between the observed and predicted values.

An important feature of the models developed in this study is that they enable the level of probability to be set to give the level of stringency required. Models A₁ and A₂ show how combinations of different concentrations of the preservatives tested affect the probability of growth. Concentrations required to give a 'conservative' probability of growth of 0.1, a probability of 0.5, and a 'liberal' probability of growth of 0.9, were calculated using models A₁ and A₂. Lines of constant probability were then compared graphically with experimentally observed probabilities (Figures 5-6) to illustrate both the degree of accuracy of models A₁ and A₂, and interactions between the three preservatives investigated.

5.3.4.1. The inhibitory effect of salt

The inhibitory effects of potassium sorbate and nisin as influenced by different levels of salt at pH 7 and pH 5.5 are shown in Figures 5.1 and 5.2 respectively. Salt had a significant inhibitory effect on the probability of growth of *C. sporogenes*. Figures (5.1a- e) show that as salt concentration increased from 0 to 4% at pH 7, lower concentrations of potassium sorbate and nisin were required to achieve a given probability of growth. The no growth zone (red triangles) at 0% salt was achieved only at relatively high concentrations of nisin (200-300 ppm) and potassium sorbate (3.5-4%) (Figure 5.1a). By increasing salt concentration to 4%, lower and lower concentrations of nisin and potassium sorbate are required to achieve a given probability of growth (Figures 5.1b-e). Nevertheless, even salt at the highest concentration (4%) was not able

to prevent growth in the absence of potassium sorbate and nisin at pH 7 (Figure 5.1e).

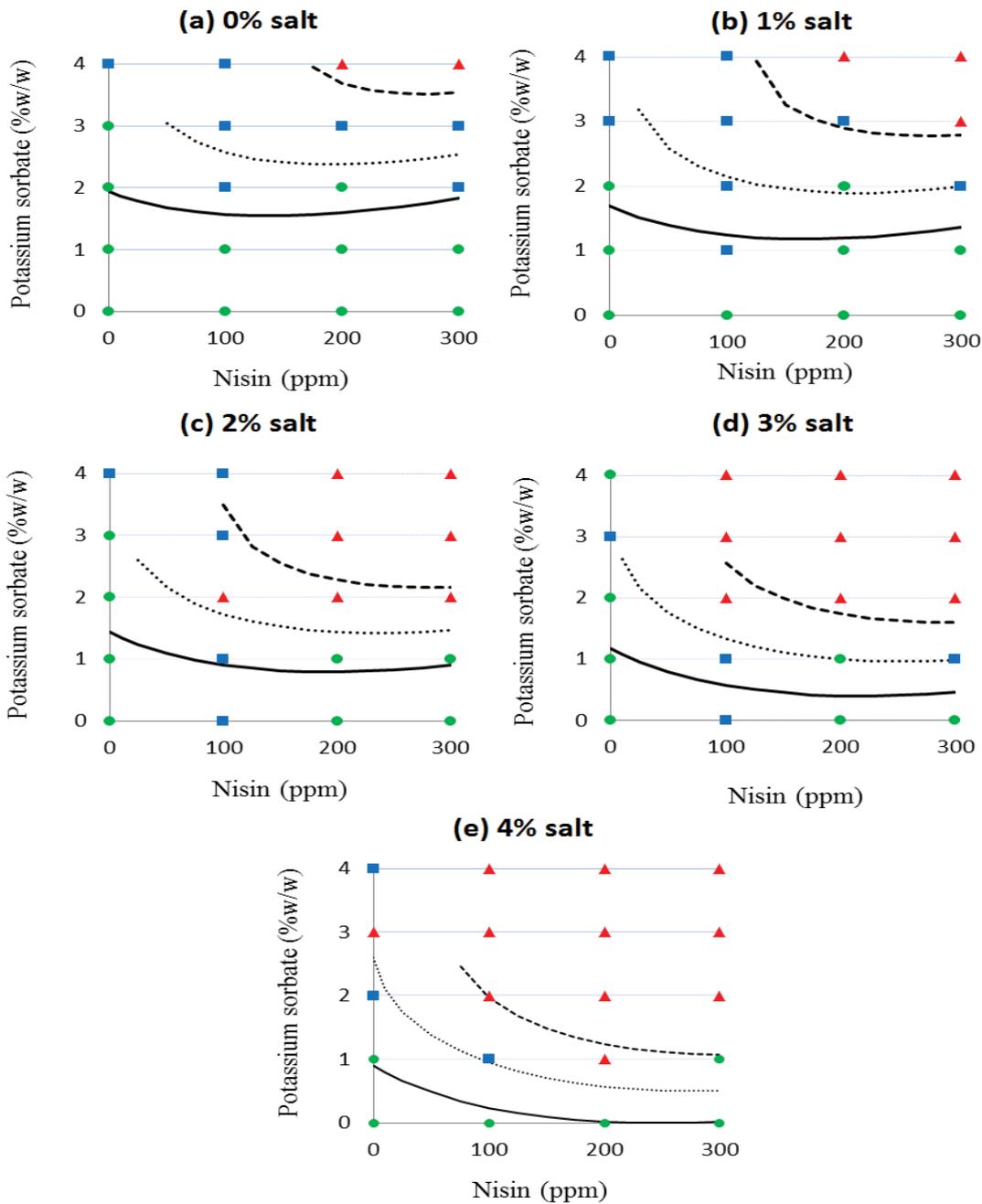


Figure 5.1. Comparisons, highlighting the effect of salt concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₂ (lines) at pH 7. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

The interaction of potassium sorbate and nisin was significant (Table 5.5, equation (5.2), Figures 5.1 a-e), i.e. by increasing the nisin concentration, less potassium sorbate was required to achieve a given probability of growth at pH 7. This is particularly evident in Fig 5.1 for nisin concentrations lower than 200 ppm; little additional change occurred by increasing nisin concentration above 200 ppm.

Figures 5.2 (a-e) show that by increasing the salt concentration from 0 to 4% at pH 5.5, the probability of growth lines shifted slightly to lower concentrations of potassium sorbate and nisin. The no growth area (red triangles) increased as salt concentration was increased from 0-4%. Salt at the highest concentration (4%) was unable to prevent growth in the absence of nisin and potassium sorbate; nisin concentration of ≥ 100 ppm and potassium sorbate concentration $\geq 2\%$ were required to halt growth (Figure 5.2e).

Figures 5.2 (a-e) illustrate that lower concentrations of potassium sorbate were required at nisin concentrations from 0-100 ppm at pH 5.5 than at pH 7 (Figures 5.1 a-e). The required potassium sorbate concentration at pH 5.5 was constant (or at least changed very little) at nisin concentrations from 100 to 200 ppm to achieve a given probability of growth. An apparent antagonistic effect occurred between potassium sorbate and nisin at 200-300 ppm. As a result of this effect, at nisin concentrations from 200 to 300 ppm, the required potassium sorbate concentrations slightly increased to achieve a given probability of growth at pH 5.5.

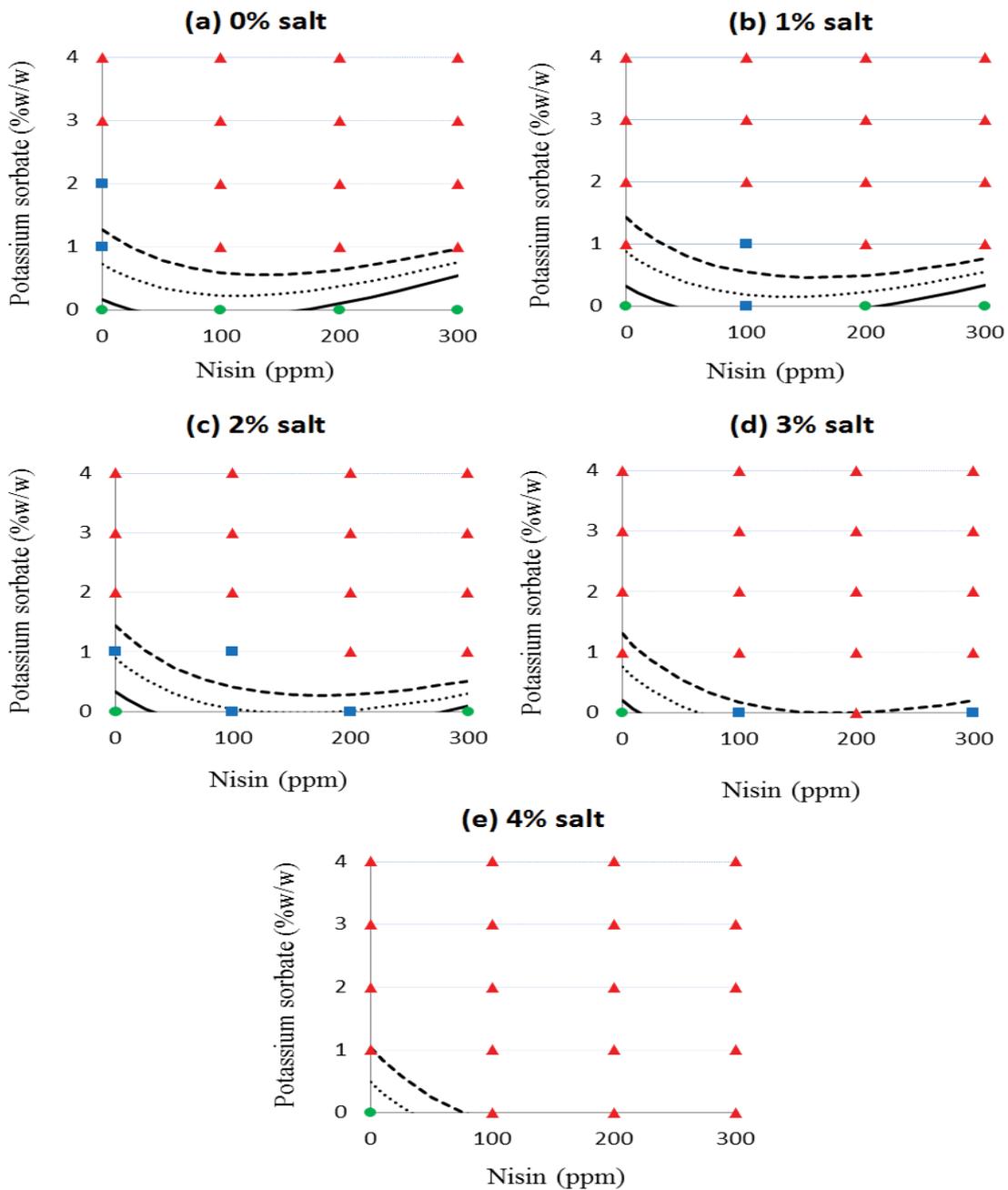


Figure 5.2. Comparison, highlighting the effect of salt concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₁ (lines) at pH 5.5. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

The no growth area (red triangles) was bigger at pH 5.5 (Figures 5.2 a-e) than at pH 7 (Figures 5.1 a-e). The potassium sorbate and nisin concentrations required to achieve a given probability of growth were lower at pH 5.5 (Figure 5.2) than at pH 7 (Figure 5.1), illustrating the fact that the preservative effects of these two preservatives is greater at lower pH.

5.3.4.2. The inhibitory effect of sorbic acid

The inhibitory effects of salt and nisin as influenced by potassium sorbate concentration at pH 7 and 5.5 are shown in Figures 5.3 and 5.4, respectively. These figures are based on the same two sets of 100 data points as shown in Figures 5.1 and 5.2, just displayed graphically in a different way.

Increasing the potassium sorbate concentration from 0 to 4% at pH 7 resulted in the probability of growth lines shifting to lower concentrations of salt and nisin (Figures 5.3 (a-e)), and the no growth area (red triangles) gradually increased in size. A systematic lack of fit is observed in Figure 5.3 d at 3% potassium sorbate concentration and the predicted probability lines cannot be seen in Figure 5.3e at 4% potassium sorbate. This probability reflects deficiencies in Model A_2 .

Lower concentrations of salt were needed with increasing nisin concentrations from 0-200 ppm in order to achieve a given probability of growth at a given potassium sorbate concentration at pH 7. The required salt concentration remained approximately constant as the nisin concentration increased above 200 ppm.

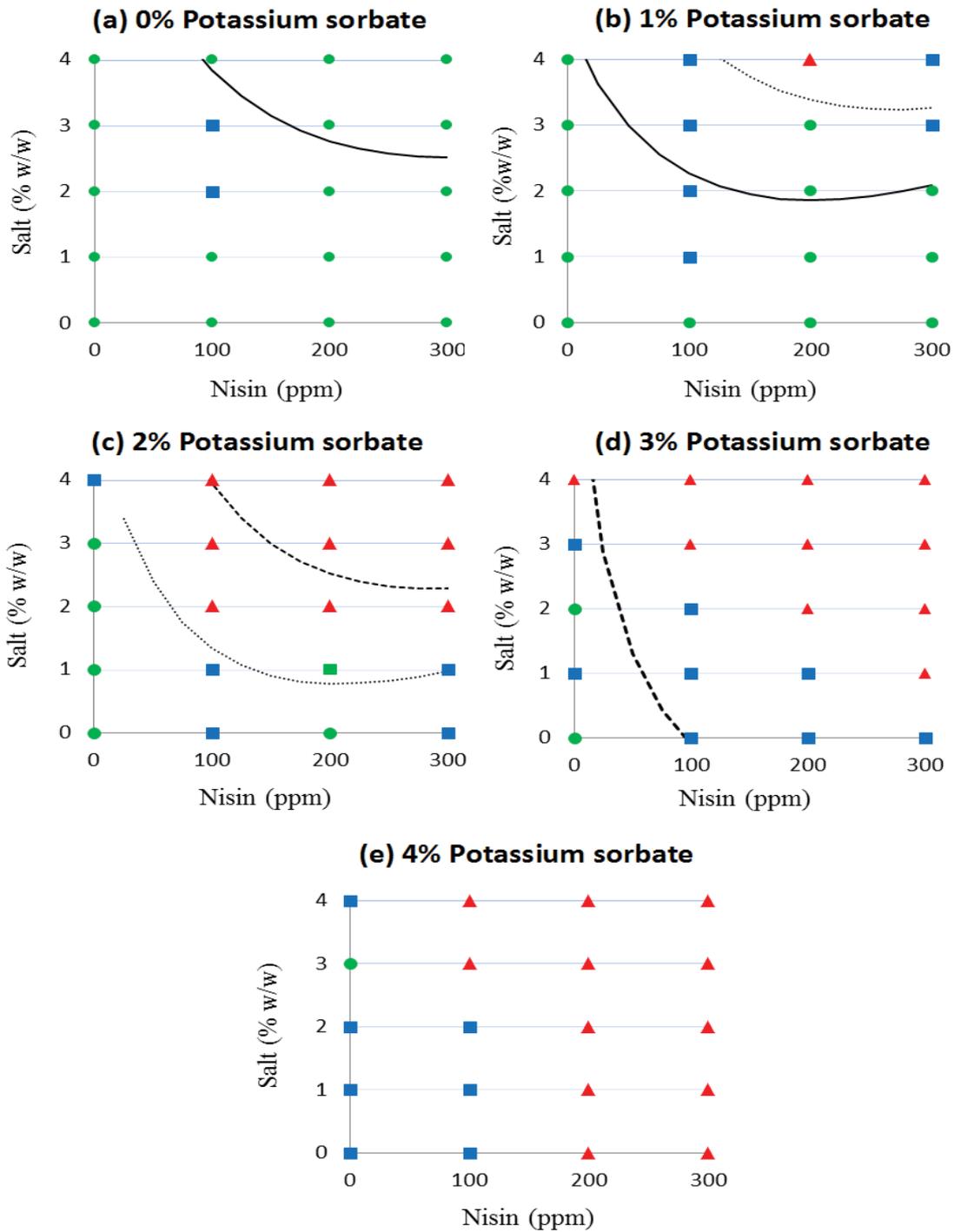


Figure 5.3. Comparison, highlighting the effect of potassium sorbate concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₂ (lines) at pH 7. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

Figures 5.4 (a-e) show that the same general effects occurred at pH 5.5. The interaction of salt and nisin was significant at pH 5.5 (Table 5.5, Equation 5.2, Figure 5.4). Nisin concentrations greater than 200 ppm had an apparent antagonistic effect on salt at pH 5.5, especially evident in the absence of potassium sorbate (Figure 5.4a). As a consequence of this effect, the required salt concentration had to increase with increasing nisin concentrations from 200 ppm to 300 ppm, to achieve a given probability of growth.

Potassium sorbate at concentrations of 3% or higher inhibited the growth of *C. sporogenes* in the absence of salt and nisin at pH 5.5 (Figures 5.4 d & e) whereas growth was observed in the same conditions at pH 7 (Figures 5.3 d & e). At a given potassium sorbate concentration the required salt and nisin concentrations to achieve a given probability of growth were higher at pH 7 compared with pH 5.5.

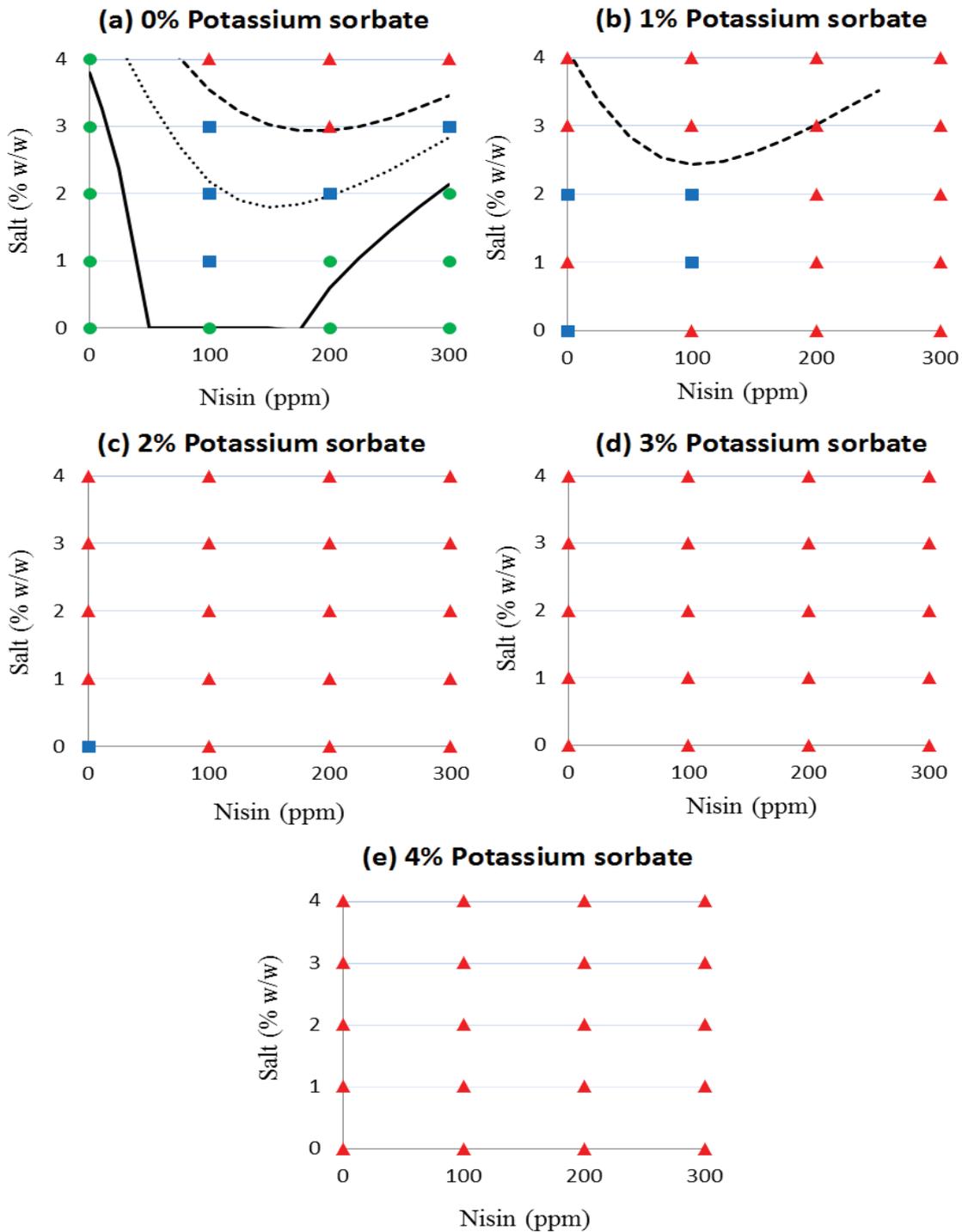


Figure 5.4. Comparison, highlighting the effect of potassium sorbate concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₁ (lines) at pH 5.5. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

5.3.4.3. The inhibitory effect of nisin

The inhibitory effects of salt and potassium sorbate as influenced by nisin concentration at pH 7 and 5.5 are shown in Figures 5.5 and 5.6 respectively.

At pH 7, nisin at 100 ppm had an inhibitory effect on *C. sporogenes* growth (Figure 5.5 b) but this effect increased only slightly at higher nisin concentrations (200 and 300 ppm).

Figures 5.5 a-d show that a combination of salt and potassium sorbate had a significant inhibitory effect at pH 7 and increasing the nisin concentration from 0 to 300 ppm did not change this trend. Lower concentrations of potassium sorbate were needed with increasing salt concentrations to achieve the same probability of growth.

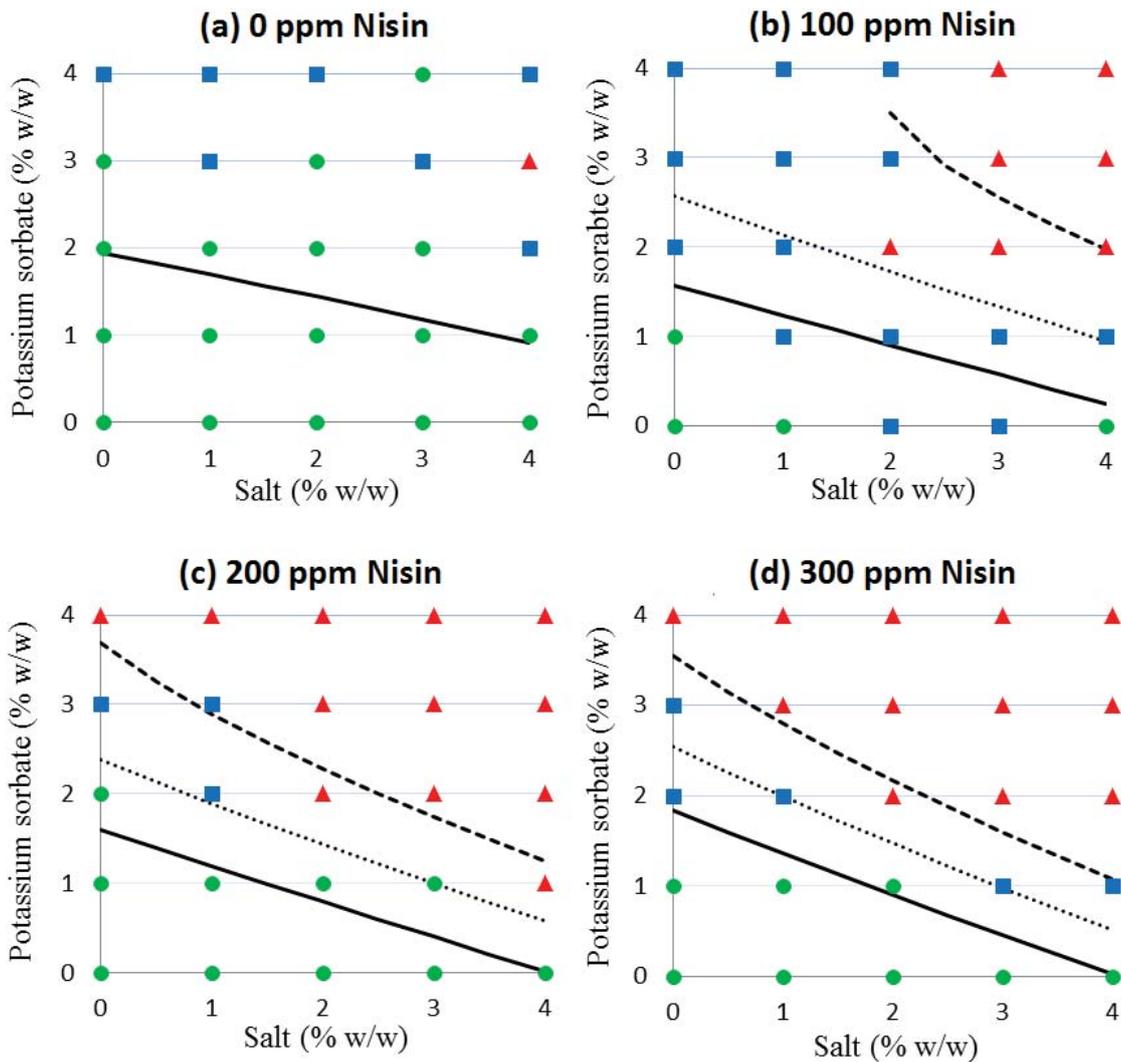


Figure 5.5. Comparison, highlighting the effect of nisin concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₂ (lines) at pH 7. Red triangle: observed $p = 0$; blue square: $0.1 \leq$ observed $p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

Figure 5.6 (a) shows that in the absence of nisin the probability of growth depended mainly on the potassium sorbate concentration and was largely independent of salt concentration. At 100 ppm nisin, the probability of growth became dependent on both the potassium sorbate and salt concentrations: slightly lower potassium sorbate concentrations were required at increasingly higher salt concentrations to achieve a given probability of growth (Figure 5.6 b). Higher nisin concentrations appear to have had an increasingly antagonistic effect on the preservative actions of potassium sorbate

and salt, as slightly higher concentrations of salt and potassium sorbate were required to achieve a given probability of growth at nisin concentrations higher than 100 ppm (Figures 5.6 (c & d)).

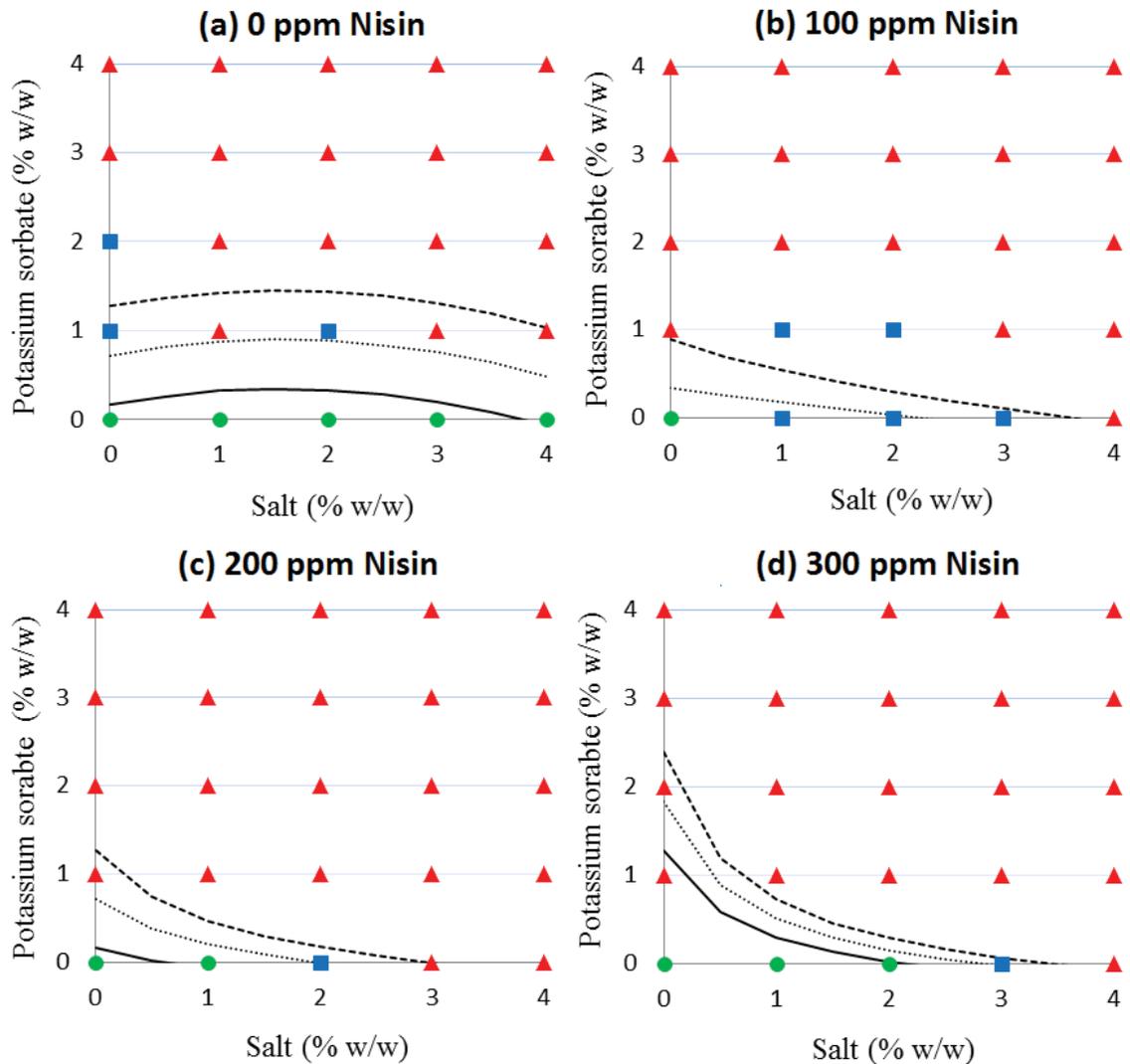


Figure 5.6. Comparison, highlighting the effect of nisin concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by Model A₁ (lines) at pH 5.5. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

The inhibitory effect of nisin was pH dependent: the no growth area (red triangles) was greater at pH 5.5 (Figures 5.6 a-d) compared with pH 7 (Figures 5.5 a-d): the salt and

potassium sorbate concentrations required to achieve a given probability of growth were higher at pH 7 than at pH 5.5.

5.4. Discussion

The magnitude of the predicted probability of growth depended on the combination of salt, potassium sorbate and nisin concentrations. Combining these preservatives allowed lower levels of each preservative to be used than would have been needed if each preservative was used on its own. If one of these selected preservatives is omitted, the other two preservatives must be used at higher concentrations to achieve a given probability of growth.

The results of the current study show that the inhibitory effects of the preservatives investigated were pH dependent. The no growth area (Figures 5.1-5.6) increased when the pH was reduced to 5.5 compared with pH 7. These results agree with the finding of a study on the effect of pH, sodium chloride, sucrose, potassium sorbate and benzoate on the growth of food spoilage yeasts (Praphailong & Fleet, 1997). At low pH, given probabilities of growth can be achieved at lower concentrations of preservatives. As a low pH acts as an additional stress factor, micro-organisms are already under stress even before the addition of preservatives.

The inhibitory effect of nisin is pH dependent and is greatest at acidic pHs (Huhtanen & Feinberg, 1980; Jack et al., 1995; Parente et al., 1998; Restaino et al., 1981; Sofos & Busta, 1981; Yigit & Korukluoglu, 2007). Nisin efficacy is greatly diminished at neutral pH (Glass & Doyle, 2005; Scott & Taylor, 1981b). This is linked to the solubility and stability of nisin in acidic environments. The results of the current study showed lower concentrations of nisin were required at pH 5.5 compared to pH 7 to achieve a given probability of growth, for given concentrations of salt and potassium sorbate. In contrast, at both pHs, the preservative effect of nisin was lower at 300 ppm than at lower concentrations. The results of the present study show that nisin used alone could not ensure the safety of foods with respect to clostridia. However, it could be used to reduce preservative use in extending the shelf life of food products when used in combination with other preservatives such as salt and potassium sorbate.

The inhibitory effect of potassium sorbate is also pH dependent. The results of this study showed that lower concentrations of potassium sorbate were required at pH 5.5 compared with pH 7 to achieve a given probability of growth. Decreasing the pH would allow the food developer to use less potassium sorbate to achieve a given probability of growth (Battey et al., 2002). There is a synergistic relationship between pH and potassium sorbate with a greater inhibiting effect at lower pH (Battey et al., 2001). As sorbic acid is a weak acid its molecules remain largely undissociated at low pH. Its inhibitory effect can be related to the undissociated form of the acid (Huhtanen & Feinberg, 1980). Lambert and Stratford (1999) showed the higher antimicrobial effect of the undissociated form of the acid is related to the lipophilic nature of this form, which allows a rapid diffusion through the plasma membrane. The undissociated form will dissociate into H⁺ and the anion in the bacterial cell. The increased concentration of protons leads to a drop in the internal pH, inhibiting many metabolic functions, while the accumulation of the anions may create a high turgor pressure inhibiting growth by disturbing cell metabolism (Lambert & Stratford, 1999; Stewart et al., 2001). However, it is possible that the increased microbial action of potassium sorbate at low pH may be due to the increased vulnerability of the micro-organism at low pH and not due to the increased activity of the undissociated form (Eklund, 1983). In the current work, the inhibitory effect of potassium sorbate was found to be more effective at pH 5.5 than at pH 7.

The salt-nisin interaction, which enhances the preservative effects of these two preservatives, was significant ($p < 0.05$) at pHs 5.5 and 7. Previous studies have shown that the presence of salt can enhance the preservative effect of nisin against *L. monocytogenes* (Harris et al., 1991; Mazzotta et al., 1997; Parente et al., 1998; Thomas & Wimpenny, 1996). On the other hand, salt can reduce the inhibitory effect of nisin in preventing the outgrowth of *Bacillus licheniformis* spores (Bell & Delacy, 1985). Bouttefroy et al. (2000) reported that salt reduced the inhibitory effect of nisin against *L. monocytogenes*. Conflicting results from previous studies on the effectiveness of salt and nisin in combination could be strain or species dependent.

Increasing salt concentration from 1 to 4% w/w has been reported to enhance inhibition by potassium sorbate (0.1-0.3% w/w) of the outgrowth of *C. sporogenes* spores at pH 6

(Robach, 1980). Robach (1980) reported that this might be due to a synergistic interaction between potassium sorbate and salt. It was reported that salt concentrations of 8.2 to 10% were necessary to inhibit the germination of spores when salt was used individually as an inhibitor of clostridial outgrowth (Lechowich, 1970).

5.5. Conclusion

The results of this study show that the probability of growth of *C. sporogenes* is reduced when salt, potassium sorbate and nisin are used simultaneously. The results also show that combinations of salt, sorbate and nisin were found to be more effective than the use of any single extreme treatment, e.g. 4% salt could not prevent the growth of *C. sporogenes* in the absence of potassium sorbate and nisin at either pH 5.5 or pH 7. The probability models presented here successfully describe the experimental data, and quantify the relationships between preservative concentrations and the probability of growth of *C. sporogenes* in broth. While the models are satisfactory, they do need to be validated for actual food products before they can be applied in practice. In general, models developed in broth should be validated in real food systems (Tienungoon et al., 2000).

Chapter Six. Applicability in processed cheese analogue of the developed broth probability of growth model for *Clostridium sporogenes*

6.1. Introduction

Most predictive microbiology models are based on data on the growth of micro-organisms in broth. It seems more efficient to build a model with data obtained from nutrient broth. This is a convenient and reproducible method for producing the data needed to develop a model. However, it may not represent the situation in another environment such as a food, where the physical structure and chemical composition differ from those of broth. In broth, micro-organisms are distributed homogeneously, and growth is often faster than in food. Therefore, the reliability of a developed laboratory media model needs to be assessed before being applied to a food, by measuring its ability to predict probability of growth in that food. It is important that the model gives realistic predictions without being fail-dangerous or excessively fail-safe (Cole, Franklin, & Keenan, 1987; Stewart et al., 2002; Valero et al., 2006; Vermeulen et al., 2008; Vermeulen, Devlieghere, Bernaerts, Van Impe, & Debevere, 2007).

To extend the study reported in Chapter 5, experiments were performed with eight selected combinations of salt, potassium sorbate and nisin in a processed cheese analogue (PCA). In order to use the developed broth model for predicting the probability of growth in the PCA, the observed probability of growth values for the product should be close to the probability of growth predicted by the developed broth model.

6.2. Materials and methods

6.2.1. Experimental approach

Eight random combinations of different concentrations of salt, potassium sorbate and nisin (concentrations of Nisaplin, which contains 2.5% nisin which produce probability of growth of 50% in broth were calculated from the broth Model A₁ (chapter 5) and were added to PCA samples in order to check the applicability of the probability of growth Model A₁ for *C. sporogenes*. Model A₁ was used in this study as the pH of PCA samples was adjusted to 5.5. The pH range of a good quality of PCA should be between 5.4 and 5.8.

In the previous study, the selected preservative concentrations were based on total broth weight, as the broth contained low dry matter content (less than 2%). The moisture content of the food needs to be considered in order to use the developed broth model in a food system. If the moisture content of a food is particularly high, then models for preservative efficacy based on broth are likely to be appropriate and convenient. If the moisture content of food is low, the amount of preservative required per gram of food is likely to be less, than that required in broth concentrations should be based on the water content of that food.

In this study, the moisture content of the PCA was much lower than the moisture content of the thioglycollate broth (> 98%) that was used for developing the models in the previous study (Chapter 5). In practice, concentrations of preservatives are often based on the moisture content of food products. Therefore, a small amount of PCA (see section 3.4.2) was prepared, and the average moisture content determined in order to calculate the necessary amounts of selected preservatives on the basis of water content.

Eight different combinations of preservative concentrations in this study were to a PCA moisture content basis. The moisture content of each of the eight PCA samples was considered 60% w/w. The eight different combinations are shown in Table 6.1.

Table 6.1. Experimental preservative treatments.

Treatment	Salt concentration (%) [*]	Nisin concentration (ppm) [*]	Potassium sorbate concentration (%) [*]
1	1.5	0	0.48
2	0.6	90	0
3	1.2	60	0
4	0.9	30	0.36
5	1.8	150	0.78
6	0.9	30	0.36
7	1.8	0	0.42
8	0	90	1.3

^{*}As percentage or ppm of water content of PCA

6.2.2. Preparation of samples

The PCA samples, negative controls and positive control were prepared as described in Chapter 3 (see section 3.4.2). The spores of five strains of *C. sporogenes* were prepared individually (see section 3.3) and equal spore numbers of each strain were used to inoculate the PCA samples to a total level of 10^6 CFU mL⁻¹. The PCA samples were then stored anaerobically for eight weeks at 37°C.

6.2.3. Measurements made during storage

For evaluating microbial growth in PCA samples, negative controls and positive control plate counts (see section 3.5.2) were carried out at time zero, on the second day and at one, two, four, six and eight weeks. Five individual containers as replicates were plate counted each time. Growth was considered to have occurred if there had been an increase in count of one or more log (CFU mL⁻¹) relative to the inoculum concentration. For each preservative treatment, a probability of growth was calculated by dividing the number of replicates exhibiting growth by the total number of replicates (five). The result was expressed as a probability value ranging from 0 (none of the five replicates showed growth) to 1 (all five of the replicates showed growth).

Finally, the observed probabilities of growth data were compared with the growth probability predicted by the broth model (0.5). The broth model was to be considered as a valid model to use for the prediction of probability of growth in the PCA if the observed probability of growth agreed with the predicted probability of growth. The moisture content, a_w and pH of the PCA samples were measured at time zero and at the end of eight weeks.

6.3. Results

The observed probability of growth for the eight different combinations of preservatives in the PCA samples and the predicted probability of growth shown in Table 6.2.

Table 6.2. Observed and predicted probabilities of growth.

Treatment	Salt (%) [*]	Nisin (ppm) [*]	Potassium sorbate (%) [*]	Probability of growth	
				Observed ¹	Predicted by Model A ₁ ²
1	1.5	0	0.48	100	50
2	0.6	90	0	100	50
3	1.2	60	0	100	50
4	0.9	30	0.36	100	50
5	1.8	150	0.78	100	50
6	0.9	30	0.36	60	50
7	1.8	0	0.42	80	50
8	0	90	1.3	100	50

^{*} As percentage or ppm of moisture content of PCA

¹ Observed probability of growth in the PCA samples at eight weeks

² probability of growth predicted by Model A₁

Table 6.2 shows that the observed probability of growth was always greater than the probability of growth predicted by the Model A₁. This means that the Model A₁ underestimated the probability of growth in the PCA. Therefore, Model A₁ is fail-dangerous.

The a_w and pH of the PCA samples remained constant during the eight week storage period. The moisture content of the samples decreased slightly, probably owing to water

evaporation. The moisture content and a_w of PCA samples at zero time and the end of the eight weeks of storage are shown in Table 6.3.

Table 6.3. Moisture content and a_w of the PCA samples at time zero and at the end of eight weeks.

Treatment	Salt (%) [*]	Nisin (ppm) [*]	Potassium sorbate (%) [*]	Moisture content		Water activity	
				M ₀ ¹ %	M ₈ ² %	a _{w0} ³	a _{w8} ⁴
1	1.5	0	0.48	60.78	60.38	0.978	0.978
2	0.6	90	0	60.91	59.43	0.992	0.998
3	1.2	60	0	60.89	59.68	0.989	0.998
4	0.9	30	0.36	60.73	56.65	0.952	0.962
5	1.8	150	0.78	60.22	59.22	0.976	0.986
6	0.9	30	0.36	60.7	59.41	0.957	0.966
7	1.8	0	0.42	60.21	59.23	0.976	0.986
8	0	90	1.3	60.98	59.99	0.959	0.965

^{*}As percentage or ppm of water content of PCA

¹Moisture content of the PCA at time zero

²Moisture content of the PCA at eight weeks

³a_w of the PCA at time zero

⁴a_w of the PCA at eight weeks

The plate counts of negative control samples of each tested PCA samples did not change during the eight weeks storage time. This indicated that no microbial contamination occurred during the preparation of the PCA samples and they were stable over the eight weeks storage period. As expected, the plate counts of the positive control increased dramatically, indicating growth occurrence in the inoculated PCA samples in the absence of preservatives.

6.4. Discussion

In any food formulation, the concentration of a preservative can be considered on a total product basis or on the product moisture content basis. If a preservative is largely water soluble, then it may be concentrated in the water phase of the product. This means the effective concentration of the preservative would be greater than if it were evenly

distributed in the food. It is in the water phase of the product where most of the bacteria will be present. Verrips et al. (1980) has established formulas to calculate the microbial stability of a water in oil emulsion (margarine). In these formulas, the concentration of glucose (being used as humectant) was expressed on a water content basis (Verrips, Smid, & Kerkhof, 1980). In another study, the concentrations of salt, citrate and lactic acid in cheese were based on the moisture content of this product in order to develop a model to predict the time to a 100 fold increase in the cell numbers of *C. botulinum* (Glass & Doyle, 2005). The selected preservatives (salt, potassium sorbate and nisin concentrations) were based on the moisture content of the PCA in this study. The preservative concentrations would have been effectively 1.6 times higher in the aqueous fraction of the product if the preservatives concentrations were based on the total PCA mass. Based on published data in the predictive microbiology area, it is common to calculate the preservatives concentrations on a moisture content basis (Bolton & Frank, 1999; Bozaris & Nychas, 2006; Evans, Everis, & Betts, 2004; Jamshidi, Kazerani, Seifi, & Moghaddas, 2008; Jenkins et al., 2000; McKellar & Lu, 2001; Presser et al., 1998; Ter Steeg et al., 1995; Vermeulen et al., 2008; Vermeulen, Devlieghere, et al., 2007).

In other studies in this area, preservative concentrations were based on the total weight of the products (Battey et al., 2001, 2002; Cole et al., 1987; Lanciotti et al., 2001; Lopez-Malo et al., 2000; Stewart et al., 2001; Stewart et al., 2002; Tienungoon et al., 2000; Vermeulen, Gysemans, et al., 2007). Concentrations depended on the dry matter of culture media used for model development. Preservative concentrations should be converted to a moisture content basis if the culture medium contains considerable amounts of dry matter; otherwise preservative concentrations based on the total weight of media culture are used.

It was hoped that the observed probabilities of growth for the eight combinations of preservative concentrations in PCA shown in Table 6.2 would be close to the value of 0.5 predicted by Model A₁. The results of this study showed that the broth model was unable to predict the probability of growth in the PCA. This result may be partly explained by considering the solubility of the preservatives and the distribution of spores in the product. The assumption that the preservatives and spores were both

totally in the aqueous phase may not be correct. It could also be partly explained by organic components of the product (proteins and fats) interfering with the activity of the preservatives. Food components and structure have important roles in the possible growth of micro-organisms and are responsible for variations between the predicted probability of growth from a broth model and observed probability of growth in food systems (Mertens et al., 2011; Skandamis, Stopforth, Yoon, Kendall, & Sofos, 2009; Wilson et al., 2002). Generally, food microstructure enhances the antimicrobial effect by providing physical constraints or limiting nutrient availability for microbial growth (Baird-Parker et al., 2000). Microbial growth can be influenced by the location of the micro-organisms in the food. There is a difference between growth of micro-organism in liquid foods and structured foods (emulsions, solids and gels) in terms of nutrients and metabolites. Liquid foods which are homogeneous and planktonic growth occurs, whereas in structured foods micro-organisms are immobilized and constrained to grow in colonies (Wilson et al., 2002). Generally, there is a need to gain more knowledge of the interaction between medium structure and micro-organisms at a microscopic level (Mertens et al., 2011).

As processed cheese can be described as a stable oil in water emulsion, there are no physical constraints on the growth of micro-organisms like *C. sporogenes*. Processed cheese structure essentially consists of a fat phase evenly dispersed (in the form of fat globules, approximately < 1 to about 5 µm in diameter) in a casein gel network (Shimp, 1985). The micro-organisms are likely to proliferate mainly in the aqueous phase, which constitutes about 60% of the processed cheese. It is likely that the influenced of PCA structure on the probability of growth of *C. sporogenes* was not significant.

On the other hand, there is often a quantity of material which may affect the efficacy of preservatives for non-structure reasons in food systems (Dang et al., 2011). The stability and activity of hurdles have been changed by this and other phenomena such as binding to food components (proteins and fats), chemical degradation, pH and temperature (Alzamora, Tapia, & Chanes, 1998). In the present study there might have been inhibition of preservative action by PCA constituents. An understanding of the product's composition and what influences the efficacy of preservatives is important in ensuring food safety. The effectiveness of many naturally occurring and added

preservatives may be decreased by certain food components. For example, several anions, like Cl^- , I^- , $(\text{PO}_4)^{3-}$ and proteolytic enzymes reduce the bactericidal property of bacteriocins, including nisin (Bhunja, Johnson, Ray, & Kalchayanand, 1991; Ray, 1994). The presence of cations such as Mg^{2+} and Ca^{2+} in foods may reduce the efficacy of nisin against Gram-positive bacteria. These cations interact with the negatively charged phospholipid groups in the cytoplasmic membrane of cell and prevent inhibitory interactions between nisin and these groups (Abee et al., 1994).

The amount of fat in a product may also influence the effect of some antimicrobials (Glass & Johnson, 2004a). Glass et al. (2004) reported that the anti-listerial activity of nisin is reduced in milk with 12.9% fat, compared with its activity in skim milk. Other lipophilic antimicrobials such as monolaurin, sorbic acid and certain fatty acids have shown reduced antimicrobial activity in high fat foods. Glass and Johnson's study (2004) showed that the addition of 20% of dairy or vegetable fat decreases the anti-botuinal activity of nisin and potassium sorbate. Emulsifiers and amount of fat have been shown to interfere with the action of nisin and reduce its antimicrobial efficacy (Mazzotta et al., 1997; Scott & Taylor, 1981a). Therefore, the presence of a polyphosphate emulsifier and fat in the PCA samples might have reduced the inhibitory efficacy of nisin in the experiments reported here.

6.5. Conclusion

The results of this study confirmed that the broth model (Chapter 5) was unable to predict accurately the probability of growth of *C. sporogenes* in the PCA. This may have been due to the influence of the PCA components. Therefore, developing a specific probability of growth model for the PCA was necessary in order to produce accurate prediction of this thesis.

Chapter Seven. Modelling the combined effects of salt, sorbic acid and nisin on the probability of growth of *Clostridium sporogenes* in high moisture processed cheese analogue

7.1. Introduction

Microbial probabilities of growth predictions from models based on data obtained by culturing in nutrient broth are not necessarily valid for a complex food system. Food systems incorporate many factors that may positively or negatively influence bacterial growth. Therefore, a probability of growth model for a particular application in the food industry needs to be developed in the actual food product. The results of the previous study (Chapter 6) showed that the developed broth model described in Chapter 5 was not able to predict the probability of growth of *C. sporogenes* in processed cheese analogue (PCA). Therefore, the use of PCA to develop a probability of growth model was necessary for accurate prediction of the probability of growth in this product. The probability of growth in PCA could be also set by appropriately selecting preservative concentration levels in developed models.

The objectives of this study were:

- 1) To develop a logistic regression model as a function of concentrations of the selected preservatives that enables the prediction of the probability of growth of *C. sporogenes* in PCA.
- 2) To validate the developed probability of growth model with new experimental data within the interpolation region.

7.2. Materials and methods

7.2.1. Experimental design

A full factorial experimental design with 48 treatments (Table 7.1) was used in order to evaluate the inhibitory effect of salt (sodium chloride) (0, 1, 2, 3% w/w), potassium

sorbate (0, 0.1, 0.2% w/w) (Hawkins Watts New Zealand) and nisin (0, 80, 160, 240 ppm) (Nisaplin 114373, Danisco Ltd., Beaminster, United Kingdom) (concentrations of Nisaplin, which contains 2.5% nisin in the PCA. In this experiment the highest permitted levels of potassium sorbate (0.2%) and nisin (240 ppm) in the PCA were selected and salt levels were kept to medium levels as the aim of this study was to be able to reduce salt concentration. Generally, in processed cheese 5-8% salt is used. Samples of PCA were prepared with the different combinations of preservative concentrations shown in Table 7.1.

Table 7.1. Experimental treatments.

Treatment	Salt* (% w/w)	Nisin* (ppm)	Potassium sorbate* (% w/w)
1	1	160	0.2
2	2	0	0.1
3	2	240	0.0
4	0	0	0.2
5	1	160	0.0
6	3	240	0.2
7	1	0	0.0
8	3	80	0.2
9	1	80	0.2
10	2	160	0.1
11	2	0	0.0
12	1	0	0.1
13	3	80	0.0
14	0	160	0.1
15	1	240	0.0
16	0	160	0.2
17	2	240	0.1
18	3	0	0.0
19	1	240	0.1
20	1	80	0.1
21	0	80	0.2

Continued on next page

Table 7.1. Continued.

Treatment	Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*
22	2	80	0.1
23	2	160	0.2
24	2	80	0.2
25	0	240	0.2
26	0	80	0.1
27	2	160	0.0
28	3	160	0.2
29	2	240	0.2
30	3	240	0.0
31	2	80	0.0
32	2	0	0.2
33	0	80	0.0
34	3	240	0.1
35	1	160	0.1
36	1	80	0.0
37	3	160	0.1
38	0	240	0.1
39	0	0	0.0
40	3	80	0.1
41	3	0	0.1
42	0	240	0.0
43	3	0	0.2
44	1	0	0.2
45	3	160	0.0
46	0	160	0.0
47	1	240	0.2
48	0	0	0.1

* As a proportion of the total mass of PCA

Preservative concentrations can be based either on total weight of product if the dry matter content of the product is negligible or on the moisture content of the product if the dry matter content cannot be neglected. Ter Steeg et al. (1995) used sodium chloride and other emulsifying salts based on the moisture content of a processed cheese product to develop a model for the growth of proteolytic *C. botulinum*. However, a study on the

growth/no growth interface for *L. monocytogenes* in Mexican-Style cheese used salt based on the total weight of the cheese (Bolton & Frank, 1999). In this experiment, selected preservative levels were based on the total weight of the PCA but their levels could also be calculated on the basis of the moisture content of the PCA. Therefore, the average moisture content of each PCA sample was calculated from the moisture contents at time zero and eight weeks. As described below, two models were constructed: 1) using the selected preservative concentrations based on the total weight of the PCA and 2) using selected preservative concentrations based on the moisture content of the PCA.

7.2.2. Preparation of PCA samples

The PCA samples, negative controls and the positive control were prepared as described in section 3.4.2. The spores of five strains of *C. sporogenes* were prepared individually (see section 3.3) and equal spore numbers of each strain were used to inoculate the samples to a total level of 10^6 CFU mL⁻¹. The samples were stored anaerobically for eight weeks at 37°C.

7.2.3. Measurements made during sample storage

For evaluating microbial growth in the PCA samples, negative controls and the positive control plate counts were carried out at time zero, day two, and one, two, four, six and eight weeks (see section 3.5.2). Five individual containers for each treatment were plate counted each time. Growth was considered to have occurred if there had been an increase in count of one or more log (CFU mL⁻¹) relative to the inoculum concentration. For each sample, a frequency of growth was calculated by dividing the number of replicates exhibiting growth by the total number of replicates (five). The result was expressed as a probability value ranging from 0 (none of the five replicates showed growth) to 1 (all five of the replicates showed growth). In addition, the moisture content, a_w and pH of the PCA samples were measured at time zero and at the end of eight weeks.

7.2.4. The development of the probability of growth model

The probability of growth data were statistically analyzed and modelled using ordinary (linear) logistic regression in SAS 9.1 (SAS Intitute Inc., Cary, N.C., USA). Models were derived using the automatic variable selection option (p -value < 0.05) with a stepwise selection method to determine estimates of significant parameters (Koutsoumanis et al., 2004a, 2004b; McKellar & Lu, 2001; Ratkowsky & Ross, 1995). The potential model form is shown as equation (7.1).

$$\begin{aligned}\text{Logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\ &= \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_1 X_2 + \alpha_5 X_1 X_3 + \alpha_6 X_2 X_3 + \alpha_7 X_1^2 \\ &\quad + \alpha_8 X_2^2 + \alpha_9 X_3^2\end{aligned}\quad (7.1)$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range of 0-1), and α_i and X_i are coefficients and independent variables (concentrations of preservatives), respectively. Terms found to be not significant were omitted from each of the final models described in the following.

7.2.5. Assessment of model performance

For each model, goodness of fit statistics and measures of predictive power were calculated in SAS. Goodness of fit was determined by:

- 1) Likelihood ratio test ($-2\ln L$), where L is the likelihood at its optimum.
- 2) Akaike's Information Criterion ($AIC = -2\ln L + 2k$, where k is the number of parameters in the model).
- 3) The Hosmer-Lemeshow statistic (HL).

The $-2\ln L$ and the AIC can be used to rank models based on the same data set, where lower values indicate better fitting models. However, these statistics do not indicate if a model fits the data adequately. The HL statistic indicates if there is a lack of fit (Gysemans et al., 2007). If the HL statistic takes a small value (or a high corresponding p -value) then the model fits the data well. The model will be rejected if the p -value is

below 0.05. The disadvantage of the HL statistic is the fact that it can be heavily influenced by a single bad prediction. Consequently, this statistic must be interpreted with caution (Agresti, 2007).

The predictive power of the models was measured by the c-value (the concordance index) or percentage of correct prediction. The predictive power is perfect if the c-value equals 1 or the percentage of correct prediction is 100%. The percentage of correct prediction shows to what extent the predicted values match the observed values. Both indicate the extent of agreement between the predicted probability of growth and the observed probability of growth. Lemeshow (1994) pointed out that the model developer should not be satisfied unless the c-value was > 0.7 . A value of c-value = 0.5 indicates that predictions will not be more accurate than random guessing (Dang et al., 2010; Gysemans et al., 2007).

In order to compare observed growth/no growth data with model predictions, combinations of preservative concentrations giving set probabilities of growth 0.1, 0.5 and 0.9 were calculated using one of the developed models and plotted in Microsoft Excel (Microsoft Corporation) together with experimentally determined probabilities of growth.

7.2.6. Validation of the models

Model can be validated via the use of interpolation and/or extrapolation but extrapolation beyond the limits of the experiments will result in loss of precision (Dodds, 1993; Roberts et al., 1981). Here, validation was carried out using preservative concentrations within the ranges used in the main experimental design in fresh experiments. The preservative concentration combinations used in this validation experiment are shown in Table 7.2.

Table 7.2. Combinations of preservative used for validation of the models.

Salt (% w/w)*	Nisin (ppm)*	Potassium sorbate (% w/w)*
0.5	100	0.05
1.5	150	0.15
2.5	40	0.09
1.7	200	0.16
1.2	40	0.07
2.1	60	0.02
0.7	220	0.18
1.4	100	0.05
2.3	50	0.19
1.3	30	0.14

* As proportion of the total mass of PCA

Preservative concentrations as shown in Table 7.2 were recalculated on the basis of the PCA moisture content in order to validate the developed model based on moisture content. The observed probabilities of growth from the validation experiment were compared with the predicted probability of growth calculated using the developed models.

7.3. Results

Table 7.3 shows the observed probabilities of growth for the preservative concentration combinations shown in Table 7.1, together with preservative concentrations recalculated on the basis of PCA average moisture content, PCA moisture content observed at zero time and eight weeks, average moisture content and PCA water activities at zero time and eight weeks.

The moisture content of the PCA samples decreased slightly due to evaporation during the eight weeks storage period. The a_w did not change and was almost constant over this storage period. The pH of all the PCA samples was adjusted to 5.5 and did not change after eight weeks storage time.

The OD of negative controls for all treatments did not increase during the eight weeks storage time. This indicated that no microbial contamination had occurred during the preparation of test broths. As expected, the OD of the positive control increased, indicating that growth occurred in the absence of the test preservatives. These findings support confidence in the probability of growth data presented in Table 7.3.

7.3.1. Development of the probability of growth models

Two types of model were developed to describe the inhibitory effects of different combinations of salt, potassium sorbate and nisin: one based on total PCA mass (Model A) and one based on PCA moisture content (Model B). Using this approach, the probability of growth of *C. sporogenes* can be managed by evaluating the effectiveness of different levels of the selected preservatives.

7.3.1.1. Model A: preservative concentrations based on total mass of PCA

The concentrations of preservatives were based on total weight of the PCA. The main effect of salt, nisin and potassium sorbate were the only significant terms (p -value < 0.05) and were retained in Model A (Equation (7.2)). The interaction and quadratic terms were not significant (p -value > 0.05) and were not included in the model. The significant terms with their corresponding p -values, goodness of fit criteria and the predictive power criteria are presented in Table 7.4.

Table 7.3. The observed probability of growth of *C. sporogenes* with the moisture content and a_w of the PCA samples at time zero and the end of the eight week storage period, and the average moisture content. Preservative concentrations are expressed on both a total mass basis and a moisture content basis.

Treatment	Salt (% w/w)*	SIM ¹ (%)	Nisin (ppm)*	NIM ² (ppm)	Potassium sorbate (% w/w)*	PIM ³ (%)	Observed probability of growth	Moisture content			Water activity		pH ₀	pH ₈
								M ₀ ⁴ %	M ₈ ⁵ %	M _a ⁶	a _{w0} ⁷	a _{w8} ⁸		
1	1	1.67	160	267.9	0.2	0.33	0	60.22	59.22	59.72	0.976	0.986	5.5	5.3
2	2	3.34	0	0	0.1	0.17	0.8	60.83	58.93	59.88	0.97	0.981	5.5	5.4
3	2	3.35	240	402.35	0.0	0	1	60.95	58.35	59.65	0.966	0.97	5.5	5.4
4	0	0	0	0	0.2	0.33	1	60.89	59.68	60.285	0.989	0.998	5.5	5.4
5	1	1.66	160	265.27	0.0	0	1	61.14	59.49	60.315	0.979	0.987	5.5	5.4
6	3	4.98	240	398.74	0.2	0.33	0	60.73	59.65	60.19	0.952	0.962	5.5	5.5
7	1	1.66	0	0	0.0	0	1	60.83	59.87	60.35	0.978	0.992	5.5	5.5
8	3	4.98	80	132.86	0.2	0.33	0	60.90	59.53	60.215	0.962	0.971	5.5	5.3
9	1	1.65	80	132.19	0.2	0.33	0	61.16	59.88	60.52	0.982	0.989	5.5	5.4
10	2	3.31	160	264.74	0.1	0.17	0	61.21	59.66	60.435	0.968	0.978	5.5	5.5
11	2	3.32	0	0	0.0	0	0.8	60.90	59.50	60.2	0.975	0.983	5.5	5.3
12	1	1.65	0	0	0.1	0.17	1	61.09	59.71	60.4	0.984	0.994	5.5	5.5
13	3	4.94	80	131.64	0.0	0	0	61.13	60.41	60.77	0.963	0.97	5.5	5.4

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Table 7.3. Continued.

Treatment	Salt (% w/w) *	SIM ¹ (%)	Nisin (ppm)*	NIM ² (ppm)	Potassium sorbate (% w/w)*	PIM ³ (%)	Observed probability of growth	Moisture content			Water activity			
								M ₀ ⁴ %	M _s ⁵ %	M _a ⁶	a _{w0} ⁷	a _{w8} ⁸	pH ₀	pH ₈
14	0	0	160	265.52	0.1	0.17	1	60.83	59.69	60.26	0.989	0.998	5.5	5.3
15	1	1.64	240	394.35	0.0	0	1	61.95	59.77	60.86	0.978	0.985	5.5	5.4
16	0	0	160	266.4	0.2	0.33	0	60.94	59.18	60.06	0.988	0.999	5.5	5.4
17	2	3.32	240	398.24	0.1	1.66	0	61.15	59.38	60.265	0.966	0.972	5.5	5.4
18	3	4.98	0	0	0.0	0	0.8	61.02	59.41	60.215	0.966	0.97	5.5	5.4
19	1	1.65	240	396.37	0.1	0.17	0	61.29	59.81	60.55	0.975	0.986	5.5	5.5
20	1	1.63	80	130	0.1	0.16	0.6	62.64	60.38	61.51	0.981	0.991	5.5	5.5
21	0	0	80	132.48	0.2	0.33	1	61.15	59.62	60.385	0.992	0.998	5.5	5.3
22	2	3.3	80	131.99	0.1	0.16	0.6	60.94	60.28	60.61	0.972	0.981	5.5	5.4
23	2	3.3	160	264.48	0.2	0.33	0	61.03	59.96	60.495	0.969	0.978	5.5	5.5
24	2	3.3	80	132.55	0.2	0.33	0.2	60.69	60.02	60.355	0.971	0.981	5.5	5.3
25	0	0	240	398.37	0.2	0.33	0.8	61.12	59.37	60.245	0.989	0.998	5.5	5.5
26	0	0	80	132.71	0.1	0.17	1	60.88	59.68	60.28	0.994	0.998	5.5	5.4
27	2	3.3	160	264	0.0	0	1	60.75	60.46	60.605	0.97	0.979	5.5	5.4

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Table 7.3. Continued.

Treatment	Salt (% w/w)*	SIM ¹ (%)	Nisin (ppm)*	NIM ² (ppm)	Potassium sorbate (% w/w)*	PIM ³ (%)	Observed probability of growth	Moisture content			Water activity		pH ₀	pH ₈
								M ₀ ⁴ %	M ₈ ⁵ %	M _a ⁶	a _{w0} ⁷	a _{w8} ⁸		
28	3	4.96	160	264.38	0.2	0.33	0	60.81	60.23	60.52	0.96	0.967	5.5	5.3
29	2	3.3	240	396.76	0.2	0.33	0	60.6	60.38	60.49	0.968	0.972	5.5	5.4
30	3	5	240	399.63	0.0	0	0	60.7	59.41	60.055	0.957	0.966	5.5	5.4
31	2	3.3	80	132.44	0.0	0	1	60.88	59.93	60.405	0.976	0.98	5.5	5.4
32	2	3.3	0	0	0.2	0.33	0.2	60.78	60.38	60.58	0.978	0.985	5.5	5.4
33	0	0	80	132.89	0.0	0	1	60.97	59.43	60.2	0.992	0.998	5.5	5.5
34	3	4.95	240	395.88	0.1	0.16	0	60.72	60.53	60.625	0.955	0.967	5.5	5.5
35	1	1.65	160	263.79	0.1	0.16	1	60.89	60.42	60.655	0.981	0.988	5.5	5.3
36	1	1.65	80	132.12	0.0	0	1	60.8	60.3	60.55	0.984	0.99	5.5	5.4
37	3	4.93	160	263	0.1	0.16	0	61.01	60.64	60.825	0.96	0.969	5.5	5.5
38	0	0	240	397.68	0.1	0.17	1	60.87	59.83	60.35	0.9858	0.995	5.5	5.3
39	0	0	0	0	0.0	0	1	60.67	57.28	58.975	0.995	0.998	5.5	5.5
40	3	4.9	80	130.69	0.1	0.16	0	61.21	61.22	61.215	0.964	0.97	5.5	5.4
41	3	4.95	0	0	0.1	0.16	0	61.15	60.09	60.62	0.965	0.97	5.5	5.4

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Table 7.3. Continued.

Treatment	Salt (% w/w)*	SIM ¹ (%)	Nisin (ppm)*	NIM ² (ppm)	Potassium sorbate (% w/w)*	PIM ³ (%)	Observed probability of growth	Moisture content			Water activity		pH ₀	pH ₈
								M ₀ ⁴ %	M ₈ ⁵ %	M _a ⁶	a _{w0} ⁷	a _{w8} ⁸		
42	0	0	240	398.41	0.0	0	1	61.01	59.47	60.24	0.987	0.996	5.5	5.3
43	3	4.93	0	0	0.2	0.33	0	61.09	60.53	60.81	0.965	0.971	5.5	5.4
44	1	1.66	0	0	0.2	0.33	0.2	60.81	59.64	60.22	0.985	0.993	5.5	5.4
45	3	4.97	160	264.92	0.0	0	0	60.83	59.96	60.39	0.959	0.965	5.5	5.4
46	0	0	160	264.06	0.0	0	1	61.06	60.12	60.59	0.99	0.997	5.5	5.4
47	1	1.65	240	396.79	0.2	0.33	0	60.87	60.1	60.48	0.978	0.9850	5.5	5.5
48	0	0	0	0	0.1	0.17	1	60.91	57.24	59.07	0.996	0.998	5.5	5.5

* As a proportion of total mass of PCA

² Nisin in moisture content of PCA

⁴ Moisture content of PCA at time zero

⁶ Average moisture content of PCA

⁸ a_w of PCA at eight weeks

¹ Salt in moisture content of PCA

³ Potassium sorbate in moisture content of PCA

⁵ Moisture content of PCA at eight weeks

⁷ a_w of PCA at time zero

Table 7.4. Model A: coefficients and relevant statistics.

Parameter	Model A	
	Coefficient	<i>p</i> -value
Constant	7.8698	<0.0001
Salt	-2.6234	<0.0001
Potassium sorbate	-28.8735	<0.0001
Nisin	-0.0102	0.0004
Goodness of fit /predictive power		
-2lnL	267.981	
AIC	275.981	
HL	9.1	
	<i>p</i> -value=0.332	
c-value	0.948	
Correct prediction %	95	

$$\begin{aligned} \text{Logit (p)} &= \ln\left(\frac{p}{1-p}\right) \\ &= 7.8698 - (2.6234 \times \text{Salt}) - (28.8735 \times \text{PS}) \\ &\quad - (0.0102 \times \text{Nisin}) \end{aligned} \quad (7.2)$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), salt is the sodium chloride concentration (% w/w), PS is total potassium sorbate concentration (% w/w), and Nisin is nisin concentration (ppm).

In Model A the HL statistic takes a small value (9.1) and the corresponding *p*-value (0.332) was higher than 0.05. This indicates that the model fits the data adequately. The c-value was 0.948, close to 1, and the percentage of correct prediction was 95, indicating good predictive power of the model.

7.3.1.2. Model B: preservatives concentrations based on moisture content of the PCA

The concentrations of preservatives were expressed as proportions of PCA moisture content. The main terms of salt, nisin and potassium sorbate, and the significant

interaction and quadratic terms (p -value > 0.05) were retained in Model B (Equation (7.3)). The significant terms with their corresponding p -values, goodness of fit criteria and the predictive power of criteria are presented in Table 7.5.

$$\begin{aligned} \text{Logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\ &= 15.1727 - (5.3964 \times \text{Salt}) - (38.1177 \times \text{PS}) - (0.0104 \times \text{Nisin}) \\ &\quad + (0.3294 \times \text{Salt}^2) + (8.5681 \times \text{Salt} \times \text{PS}) \\ &\quad + (0.00268 \times \text{Salt} \times \text{Nisin}) \end{aligned} \quad (7.3)$$

where logit (p) is an abbreviation of $\ln\left(\frac{p}{1-p}\right)$, p is the probability of growth (in the range 0-1), Salt is the sodium chloride concentration (% w/w) in the PCA moisture, PS is the potassium sorbate concentration (% w/w) in the PCA moisture, and Nisin is the nisin concentration (ppm) in the PCA moisture.

In Model B the HL statistic takes a small value (5.0479) and the corresponding p -value (0.7524) was higher than 0.05. This indicates that the model fits the data adequately. The c -value was 0.953, close to 1, and the percentage of correct prediction was 95.1, indicating that the predictive power of the model was good.

The comparison between the observed and predicted probability of growth from the both models A and B showed close agreement. However, there was some discordance between observed and predicted probability of growth for both models that is inevitable.

The $-2\ln L$ and the AIC values for two models were similar. This indicates that both models could be used for predicting the probability of growth of *C. sporogenes* in the PCA.

Table 7.5. Model B: coefficient and relative statistics.

Parameter	Model B	
	Based on total mass of PCA	
	Coefficient	<i>p</i> -value
Constant	15.1727	<0.0001
Salt	-5.3964	<0.0001
Potassium sorbate	-38.1177	<0.0001
Nisin	-0.0104	0.005
Salt ²	0.3294	0.0083
Salt× potassium sorbate	8.5681	<0.0001
Salt× nisin	0.00268	0.0118
Goodness of fit /predictive power		
-2lnL	266.085	
AIC	280.085	
HL	5.0479	
	<i>p</i> -value=0.7524	
c-value	0.953	
Correct prediction %	95.1	

7.3.2. Model validation

Models A and B were validated with new experimental data obtained using the preservative concentration combinations shown in Table 7.6 and Table 7.7. The predicted probabilities of growth are compared with the observed probabilities of growth in Table 7.6 (Model A) and Table 7.7 (Model B).

There is close agreement between the predicted and observed probability of growth (experimental data) for both models. Thus both can be considered to be safe models and can be used for the prediction of the probability of growth in PCA samples.

Table 7.6. Validation of Model A: comparison between observed and predicted probabilities of growth.

Salt (%) [*]	Nisin (ppm) [*]	Potassium sorbate (%) [*]	Probability of growth	
			Observed	Predicted by Model A
0.5	0.05	100	1	0.983
1.5	0.15	150	0	0.127
2.5	0.09	40	0	0.155
1.7	0.16	200	0	0.037
1.2	0.07	40	0.8	0.908
2.1	0.02	60	0.6	0.763
0.7	0.18	220	0.2	0.196
1.4	0.05	100	0.8	0.849
2.3	0.19	50	0	0.0153
1.3	0.14	30	0.4	0.527

^{*} As a proportion of the total mass of PCA

Table 7.7. Validation of Model B: comparison between observed and predicted probabilities of growth.

SIM (%) ¹	NIM (ppm) ²	PIM (%) ³	Probability of growth	
			Observed	Predicted by Model B
0.83	165.67	0.083	1	0.999
2.49	248.5	0.25	0	0.206
4.14	66.27	0.15	0	0.133
2.82	331.35	0.27	0	0.105
1.99	66.27	0.12	0.8	0.946
3.48	99.4	0.033	0.6	0.5
1.16	364.48	0.3	0.2	0.147
2.32	165.67	0.083	0.8	0.901
3.81	82.84	0.31	0	0.089
2.15	49.7	0.23	0.4	0.582

¹ Salt in moisture content

² Nisin in moisture content

³ Potassium sorbate in moisture content

Model A is preferable to Model B because there is no need to recalculate preservative concentrations on the basis of PCA moisture content for any further calculation as the preservative concentrations are based on total weight of the PCA. In contrast, the preservative concentrations for Model B need to be recalculated on the basis of the moisture content of the PCA.

7.3.3. Exploration of the relative preservative effects of salt, nisin and sorbic acid using Model A

Based on the results of this study, the use of Model A for predicting the probability of growth of *C. sporogenes* is favoured. It is possible to predict the probability of growth using this model for any given combination of preservative concentrations within the ranges tested. Figures 7.1-7.3, which display comparisons between measured and predicted probabilities for various combinations of salt, nisin and sorbic acid concentrations, show that except for some slight, inevitable discordance, there is generally close agreement between observed and predicted values.

Model A shows how combinations of different concentrations of the preservatives tested affect the probability of growth. Concentrations required to give a 'conservative' probability of growth of 0.1, a probability of 0.5, and a 'liberal' probability of growth of 0.9, were calculated using Model A. Lines of constant probability were then compared graphically with experimentally observed probabilities (Figures 7.1-7.3) to illustrate both the degree of accuracy of Model A, and interactions between the three preservatives investigated.

7.3.3.1. The inhibitory effects of potassium sorbate and nisin as influenced by salt concentration

The inhibitory effect of potassium sorbate and nisin as influenced by different levels of salt is shown in Figure 7.1. Salt had a significant inhibitory effect on the probability of growth of *C. sporogenes*. Figures (7.1 a-d) show that as salt concentration increased from 0 to 3%, lower concentrations of potassium sorbate and nisin were required to achieve a given probability of growth.

The no growth area (red triangles) increased as salt concentration was increased from 0 to 3%. Salt at the highest concentration (3%) was unable to prevent growth in the absence of nisin and potassium sorbate; nisin concentrations of ≥ 80 ppm and potassium sorbate concentrations of $\geq 0.1\%$ were required to halt growth experimentally at 3% salt (Figure 7.1d).

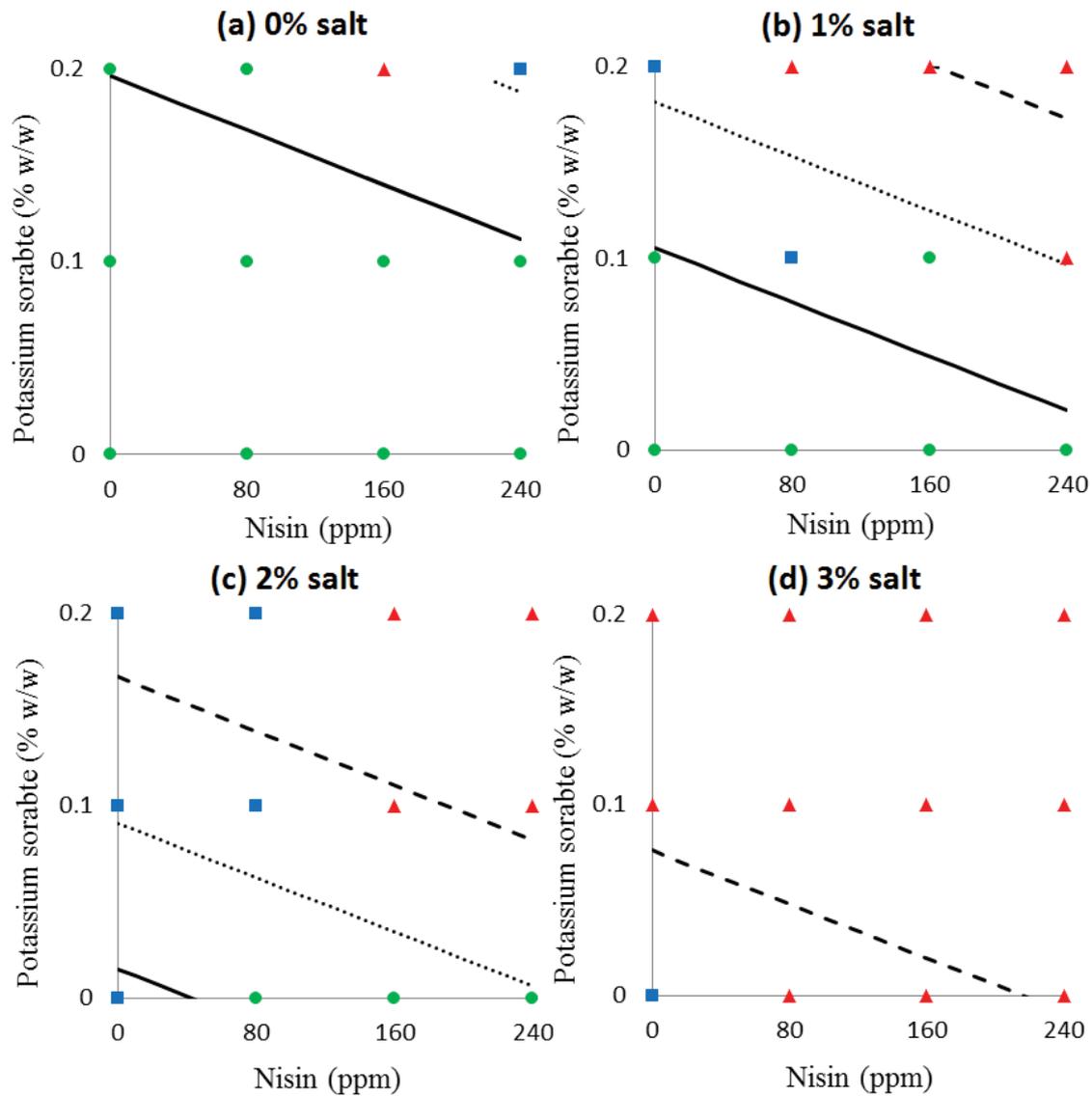


Figure 7.1. Comparison, highlighting the effect of salt concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by model A. Red triangle: observed $p = 0$; blue square: $0.1 \leq$ observed $p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

7.3.3.2. The inhibitory effects of salt and nisin as influenced by sorbic acid (potassium sorbate)

The inhibitory effects of salt and nisin as influenced by potassium sorbate concentration are shown in Figure 7.2. Increasing the potassium sorbate concentration from 0 to 0.2% resulted in the predicted probability of growth lines shifting to lower concentrations of salt and nisin (Figure 7.2 a-c), and the no growth area (red triangles) gradually increasing in size.

7.3.3.3. The inhibitory effects of potassium sorbate and salt as influenced by the nisin concentration

The inhibitory effects of salt and potassium sorbate as influenced by different levels of nisin are shown in Figure 7.3. By increasing nisin concentrations from 0 to 240 ppm the predicted probability of growth lines shifted to lower salt and potassium sorbate concentrations (Figure (7.3 a-d)). However, the probabilities of growth lines shifted only slightly at nisin concentration higher than 160 ppm.

The result of this study demonstrated that it is feasible to produce a high moisture and low salt PCA if sufficient concentrations of potassium sorbate and nisin are added to the product. A combination of salt, potassium sorbate and nisin enables the production of a microbiologically safe high moisture PCA. Any change in the concentration of one of the selected preservatives has to be compensated for by changes in the concentrations of the other two preservatives.

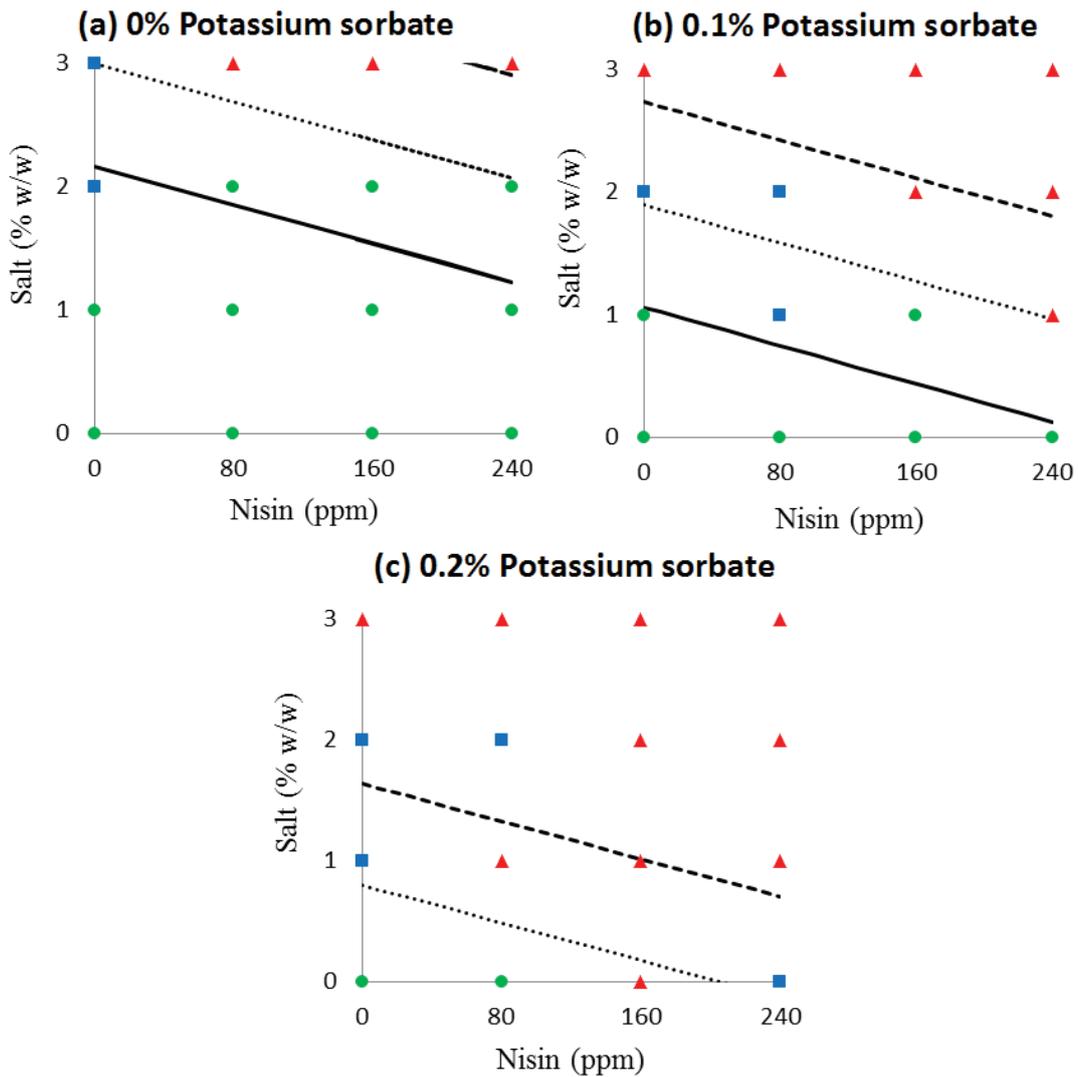


Figure 7.2. Comparison, highlighting the effect of potassium sorbate concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by model A. Red triangle: observed $p = 0$; blue square: $0.1 \leq$ observed $p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

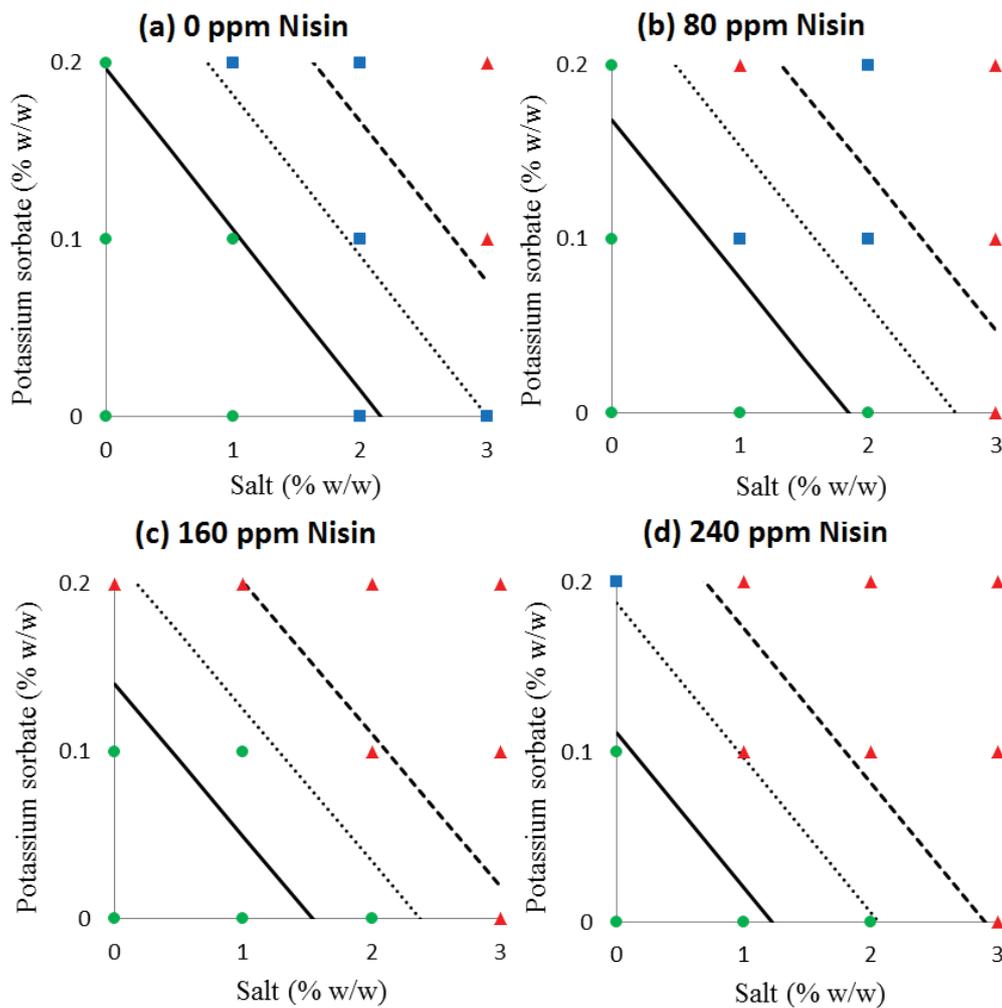


Figure 7.3. Comparison, highlighting the effect of nisin concentration, between the observed probability of growth (coloured symbols) and probability of growth predicted by model A. Red triangle: observed $p = 0$; blue square: $0.1 \leq \text{observed } p \leq 0.9$; green circle: observed $p = 1$. Dashed line: predicted $p = 0.1$; dotted line: predicted $p = 0.5$; solid line: predicted $p = 0.9$.

7.4. Discussion

The microbial stability of foods is based upon a combination of several factors (hurdles) which, when acting together, inhibit the growth of micro-organisms. In this study, salt, potassium sorbate and nisin used individually, even at high concentration, were not able to prevent the growth of *C. sporogenes* in the PCA. However, salt, potassium sorbate

and nisin used in combination were found to have significant effects in controlling the growth of *C. sporogenes*. Logistic regression provided a useful statistically sound description of the probability of growth of *C. sporogenes* in the PCA as functions of salt, nisin and potassium sorbate concentrations. The models developed can be used to calculate the probability of growth for a given combination of preservative concentrations, or to calculate the combinations required to give a set probability of growth.

The results of the present study show that salt at 3% (or relatively high concentration) was not able alone to prevent the growth of *C. sporogenes* in the high moisture PCA samples (60%) at pH 5.5. Concentrations of salt (1-3%) in combination with potassium sorbate and nisin, were able to prevent the growth of *C. sporogenes*. This result aligns with the Tanaka et al. (1986) study. They demonstrated a microbiologically safe processed cheese spread could be prepared with high moisture levels (up to 60%) with combinations of 2% sodium chloride, 2.5% disodium phosphate and low pH (< 5.7) (Tanaka et al., 1986).

Potassium sorbate as an agent to inhibit clostridia has been used in previous studies (Emard & Preese, 1951; Huhtanen & Feinberg, 1980; Ivey & Robach, 1976; Sofos, 1989; Sofos & Busta, 1981; Sofos et al., 1979). The present study demonstrated that potassium sorbate had a significant inhibitory effect on the probability of growth of *C. sporogenes* in the PCA. However, potassium sorbate at 0.2% (a high concentration) was alone unable to prevent the growth of *C. sporogenes*; it needed to be used in combination with salt and nisin. Several studies indicated that the antimicrobial activity of potassium sorbate is enhanced when used in combination with other preservatives. A combination of potassium sorbate and salt has been recommended to produce safe products with pH > 4.5 such as pasteurized cheese spread (Tanaka et al., 1986; Yigit & Korukluoglu, 2007). Avery and Buncic (1997) demonstrated that a combination of potassium sorbate and nisin reduced and inhibited the growth of *L. monocytogenes* on carbon dioxide packed beef and vacuum packed beef.

Nisin had a significant inhibitory effect; however, a high concentration of nisin (240 ppm) was unable to prevent the growth of *C. sporogenes* in the absence of salt and

potassium sorbate in this study. The use of nisin as an antibotulinal agent in pasteurized process cheese spreads was studied by Somers and Tylor (1987). They found that 12.5 ppm of nisin completely inhibited outgrowth and toxin production by *C. botulinum* in cheese spreads containing 54% moisture. As the moisture content increased or as the salt concentration decreased, more nisin was required to inhibit growth. In the present study, higher concentrations of nisin were required with decreasing salt or potassium sorbate concentrations in PCA to prevent the growth of *C. sporogenes*. The addition of nisin has been shown to enhance flexibility in processed cheese spread formulation, allowing the manufacture of a safe product with reduced salt or higher moisture levels (Somers & Taylor, 1987). The nisin levels required for the inhibition of *C. sporogenes* differ in different media due to the binding of nisin by some of media components such as protein and phospholipid (Scott & Taylor, 1981a). High protein and phospholipid concentrations decrease the ability of nisin to inhibit the growth of *C. botulinum* (Rayman et al., 1981; Rogers & Montville, 1994).

The stability and activity of hurdles have been changed by phenomena such as binding to food components (proteins and fats), pH and temperature (Alzamora et al., 1998). The fat component of processed cheese might also reduce the inhibitory effect of preservatives such as nisin, potassium sorbate and polyphosphate (Glass & Johnson, 2004b, 2004c). This aligns with previous studies demonstrating that the levels of nisin required to prevent *C. botulinum* spore outgrowth and toxin production in high moisture processed cheese spreads were higher than the levels required in trypticase-peptone-glucose yeast (TPGY) broth (Scott & Taylor, 1981a; Taylor, Somers, & Krueger, 1985). There is no information available to explain the difference between the inhibitory levels of nisin in PCA versus nutrient broth. However, the binding of nisin by the cheese matrix or matrix-related limitations on contact between nisin and the spores may have been responsible. The presence of cations such as Mg^{2+} and Ca^{2+} in foods may reduce the efficacy of nisin against Gram-positive bacteria. These cations interact with the negatively charged phospholipid groups in the cytoplasmic membrane of cells and prevent inhibitory interactions between nisin and these groups (Abee et al., 1994). In this study the pH of the PCA samples was adjusted to 5.5 and the pH of the PCA samples did not change during storage (Table 3.7). Therefore it can be concluded that pH, in this study, does not have a significant impact on the mineral solubility and thus

nisin activity via mineral solubilisation. The effects of possible antagonistic interactions during preservation and care must be selected in choosing preservatives in order to achieve the most effective combination of treatments.

Several other factors have been previously identified to control *C. botulinum* growth in processed cheese: moisture, salt type, level and type of emulsifier, pH, fat and lactate levels (Glass & Johnson, 2004c; Tanaka et al., 1986; Ter Steeg et al., 1995). The pH of processed cheese is a major factor in processed cheese stability and the stability will rapidly decrease at higher pH values (Ter Steeg et al., 1995). Phosphate based emulsifiers have a greater inhibitory effect on *C. botulinum* compared with other emulsifiers such as sodium citrate in processed cheese spread (Glass & Doyle, 2005; Roberts & Zottola, 1993; Tanaka et al., 1986). Therefore, it can be concluded that models developed in the present study may not be applicable to processed cheese products that contain sodium citrate as an emulsifier, a different pH to that used in the present study, reduced-fat and fat-free processed cheese or other non-standard processed cheese products. Additional studies are needed in order to determine the effect of altering processed cheese compositions and pH on the survival of *C. botulinum* in processed cheese.

7.5. Conclusion

The influence of salt, potassium sorbate and nisin on the probability of growth of *C. sporogenes* in PCA can be estimated quickly by using the developed models instead of time consuming shelf life studies or challenge tests. An important feature of the model developed in this study is that they enable the level of probability to be set to give the level of stringency required. It should be considered that the models were developed with a large inoculum size in this study. This large inoculum size is greater than the expected contamination in processed cheese products. Therefore, the probabilities of growth would be less under realistic inoculum conditions and the models will trend to be fail-safe.

The models developed in this part of the study are considered valid for evaluating the safety of PCA formulations similar to those in this study. The models have limitations

in the case of strongly deviating formulations, since minor changes in processed cheese formulation or its production might significantly alter its ability to support toxin production. If new ingredients or changes in processed cheese composition are introduced, a new safety study should be conducted (Roberts et al., 1981).

Chapter Eight. General discussion and conclusion

8.1. Introduction

There is considerable interest in increasing food quality by applying mild preservatives and reducing salt levels while maintaining safety and shelf stability. A combination of preservatives approach is an extremely valuable technique for creating new products which satisfy consumer and manufacturer demands. This study examined the feasibility of producing a safe and ambient shelf-stable high moisture and low acid food product that satisfies both the consumer and the manufacturer. Hurdle technology has been used in order to achieve high moisture, low acid ambient shelf-stable food products. *C. botulinum* produces an extremely powerful neurotoxin. Therefore, its growth in food products presents a severe hazard and it is important to ensure that all foods are safe with respect to this micro-organism (Russell & Gould, 2003). *C. botulinum* is the only food-borne pathogen that must be completely absent in food products. There are difficulties in working with this highly potent food poisoning micro-organism in laboratory trials. Therefore, *C. sporogenes* can be used to represent *C. botulinum* in the laboratory as *C. sporogenes* is very closely related to *C. botulinum* in growth characteristics but is not dangerous (Brown et al., 2012; Goldoni et al., 1980). *C. sporogenes* was used as an analogue of *C. botulinum* in this project. The results show that producing a safe and ambient shelf-stable high moisture processed cheese is achievable by choosing the appropriate concentrations of the selected preservatives. Probability of growth models are the most efficient way of quantifying hurdle effects in terms of preservative concentrations.

8.2. Exploration of the relative preservative effects of salt, sorbic acid, nisin and lysozyme on the growth of *C. sporogenes* in nutrient broth

The inhibitory effect of salt, sorbic acid (in the form of potassium sorbate), nisin and lysozyme on *C. sporogenes* growth in nutrient broth at the optimum pH of *C. sporogenes* (pH 7) was investigated as described in Chapter 4. This showed that salt and potassium sorbate were the most effective preservatives in preventing the growth of *C.*

sporogenes. The use of nisin and lysozyme did not have significant inhibitory effects on *C. sporogenes*. However, nisin acted synergistically in combination with the other preservatives. Previous studies have shown that the presence of salt can enhance the preservative effect of nisin against *L. monocytogenes* and *C. botulinum* (Harris et al., 1991; Mazzotta et al., 1997; Parente et al., 1998; Thomas & Wimpenny, 1996). Therefore, nisin was selected for further study along with salt and potassium sorbate. Lysozyme, a common preservative in processed cheese, was not selected for further study because it might amplify the heat resistance of spores and may cause allergic reactions (Alderton et al., 1974).

8.3. Development of probability of growth models

As reported in Chapter 5, the inhibitory effects of salt, potassium sorbate and nisin were modelled to determine the probability of growth of *C. sporogenes* in nutrient broth at pH 5.5 and 7. All three selected preservatives had significant inhibitory effects on the growth of *C. sporogenes*. In addition, a combination of salt, nisin and potassium sorbate allowed lower concentrations of each preservative to be used to inhibit growth compared with the concentrations required for individual preservatives. If one of these selected preservatives were omitted, the other two preservatives would need to be used at higher concentrations to achieve a given probability of growth. This study found that high moisture, ambient shelf-stable, low salt products can be produced by using higher nisin and potassium sorbate concentrations to compensate for reduced salt levels. The magnitude of the predicted probability of growth depends on the combination of salt, potassium sorbate and nisin concentrations. The influence of changes in these concentrations on the probability of growth of *C. sporogenes* can be quickly estimated by using the developed models. This provides a guide for testing in a food system. However, there will be always variation that needs to be considered. In practice, the application of a model developed under laboratory conditions to a less controlled environment of an industrial process usually involves uncertainty. The developed model should be tested using challenge trials in the specific product of interest before applying to a particular manufactured product.

It is essential that the reliability of a developed nutrient broth model is assessed in food products (Cole et al., 1987; Perrot, Trelea, Baudrit, Trystram, & Bourguine, 2011; Stewart et al., 2002; Valero et al., 2006; Vermeulen et al., 2008; Vermeulen, Devlieghere, et al., 2007). Therefore, the work described in Chapter 6 focused on testing the accuracy of the model developed in nutrient broth (Chapter 5) in the PCA. It is important that a model give realistic predictions without being fail-dangerous or excessively fail-safe. This study illustrated that the broth model (Chapter 5) could not precisely predict the growth probability of *C. sporogenes* in the PCA. This confirmed that it was necessary to use the PCA to develop an accurate probability of growth model for this product.

Therefore, the inhibitory effects of salt, nisin and potassium sorbate in the PCA were modelled as described in Chapter 7. Combination of 2% salt, 160 ppm nisin and 0.2 % potassium sorbate is suitable combination to produce a safe high moisture ambient shelf-stable PCA. The results showed that all three selected preservatives had significant inhibitory effects on the growth of *C. sporogenes*. Precise prediction can be obtained when the complexity of the applied mathematical models matches the complexity of the PCA with respect to the number of parameters influencing growth of *C. sporogenes*. The model validation illustrated that the developed PCA model can accurately predict the growth probability of *C. sporogenes* in PCA.

Spore-forming bacteria, such as *C. botulinum* are a concern in processed cheese because they survive heat processing and heat may activate the spores. The microbiological safety of processed cheese has traditionally relied on heat to destroy vegetative pathogens and the specific formulation of processed cheese (such as pH, salt) to prevent the growth of surviving heat resistant spore-formers or to prevent the growth of any re-contaminating bacteria. Several factors such as moisture, salt type, level and type of emulsifier, pH and lactate levels, influence the growth of *C. botulinum* in processed cheese (Glass & Johnson, 2004c). If processed cheese products vary from these established formulations, the safety of these products must be verified through *C. botulinum* challenge tests (Tanaka et al., 1986; Ter Steeg et al., 1995). The pH of processed cheese is a major predictor of processed cheese stability, which will rapidly decrease at higher pH values (Ter Steeg et al., 1995). Phosphate based emulsifiers have

a greater inhibitory effect on *C. botulinum* compared to other emulsifiers such as sodium citrate used in processed cheese spreads (Glass & Doyle, 2005; Roberts & Zottola, 1993; Tanaka et al., 1986). Wanger and Busta (1985) measured the effects of sodium acid pyrophosphate, salt and potassium sorbate on *C. botulinum* spores. Combinations of sodium acid pyrophosphate and potassium sorbate in culture media were effective in delaying the growth of *C. botulinum*. However, one has to be aware of model limitations in the case of strongly varying formulations, since changes in a processed cheese formulation or its production methods might significantly affect the safety of cheese. If new ingredients or changes in processed cheese composition are introduced, a new safety study should be conducted (Roberts et al., 1981). Thus the model for the PCA developed in the present study might not be applicable to processed cheese products that contain different emulsifiers or have a pH different from that in the present study. This provides a guide-line for industries. The model developed at this stage of the study is considered valid for evaluating the safety of standard PCA formulations produced with polyphosphate as an emulsifier. Additional studies are needed to determine the effect of altering processed cheese composition and pH on the survival of *C. botulinum* in this product. Normally, a developed model applies to a specific food product or a specific model system, and the applicability of models developed in this way to other products is uncertain. It is essential that a probability of growth model for a particular application in the food industry be developed in the actual food product.

The developed probability of growth models presented in this study describe the experimental data satisfactorily and quantify the preservative concentrations needed to result in a chosen probability of growth of *C. sporogenes*. It must be noted that the developed models are empirical in nature and do not contribute to understanding the mechanism involved in the growth inhibition of *C. sporogenes*. However, this type of model offers the possibility to include the no-growth responses, which are particularly important when efforts are made to prevent an event such as the growth of *C. sporogenes*, providing a significant degree of safety.

8.4. General recommendations for future study

The probability of growth modelling research for *C. botulinum* is expected to expand in the future as this organism is a most critical food safety threat. The following are recommendations for further research:

- Validate a model by working with *C. botulinum* itself. Because of difficulties in working with this highly potent food poisoning organism in laboratory trials, this was not achieved in the current study.

- The result of this study shows that a low salt concentration, high moisture, high pH product that is shelf-stable at ambient temperatures is achievable using combinations of potassium sorbate and nisin and salt. Russell et al. (1982) observed that lower salt levels act synergistically with other preservatives including nitrite, potassium sorbate and benzoate. In recent years, there has been a trend towards reducing salt concentration in the diet for health reasons. Similar approaches to this study could be used for other high moisture, low acid food products such as meat and vegetables to produce ambient shelf-stable products (Russell et al., 1992).

- Two different pHs, 5.5 and 7, were used in this study. The results show that the inhibitory effects of the selected preservatives were pH dependent. The probability of growth decreased when the pH was reduced from 7 to 5.5. The results of this study agree with the findings on the effect of pH, sodium chloride, sucrose, potassium sorbate and benzoate on the growth of food spoilage yeasts (Praphailong & Fleet, 1997). An additional level of utility of the model could be achieved in the future by expanding the model to include pH as a variable.

- Several other factors have been previously identified to control *C. botulinum* growth in processed cheese. These include low moisture, salt type, level and type of emulsifier, pH and lactate levels (Glass & Johnson, 2004c). The safety of processed cheese must be verified through *C. botulinum* challenge tests if processed cheese formulations vary (Tanaka et al., 1986; Ter Steeg et al., 1995). Therefore, a series of experiments with varying PCA composition will determine the influence of the PCA components on *C.*

botulinum in PCA. An additional level of utility in the PCA model could be achieved in the future by expanding the model to include components of PCA that have significant inhibitory effects.

- The developed models are able to describe changes in the probability of growth. To obtain precise probability of growth, more replicates must be performed at all conditions. For instance, 100 replicates for each condition should be performed if a reliable prediction of 1% chance of growth probability is needed (Vermeulen, Devlieghere, et al., 2007). However, this is very labour intensive and expensive.

- The developed models permit the interactive effects between selected preservatives to be quantified. Finally, the models give the ability to change preservative levels but still predict the probability of growth. This approach is somewhat limited as studies normally deal with a specific processed cheese or a specific model system and the applicability of the models developed this way to other products is uncertain. However, more general and robust models can be developed if more data is generated and significant factors are incorporated into the model (Dodds, 1993).

- In this study, eight weeks of storage time was used in experiments as a result of time constraints. A longer time might be useful to give greater confidence in the results. For instance, compositions which are stable for 12 weeks at ambient temperature will generally be found to be stable for 6 to 12 months (Eckner et al., 1994; Karahadian et al., 1985; Tanaka, 1982; Ter Steeg et al., 1995). A prediction of 12 weeks can be proposed as a practical criterion for botulinum stability at ambient temperature (Ter Steeg & Cuppers, 1995).

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Appendix

Appendix 1

NZMP™ Rennet Casein 771

PRODUCT BULLETIN

PB.010
Version 6.0209

NZMP™ Rennet Casein 771 from Fonterra is produced by controlled precipitation of casein from pure, pasteurised skim milk by the action of microbial rennet enzymes. Using a continuous and hygienic process, the curd is extensively washed, then dried and ground to a consistent particle size.

PRODUCT CHARACTERISTICS

- Very good nutritive value
- Excellent flavour stability
- Low fat and cholesterol levels
- Insoluble in water

SUGGESTED USES

- Imitation cheeses
- Processed cheeses

PACKAGING

Multi-wall paper which incorporates a moisture barrier and contains the product within an inner polyethylene bag. No staples or metal fasteners are used.

Net Weight	25.0 kg
Gross Weight	25.4 kg

STORAGE AND HANDLING

Caseins are hygroscopic and can absorb odours. Therefore adequate protection is essential. It is recommended that product is stored at temperatures below 25°C, relative humidity below 65% and in an odour free environment. Stocks should be used in rotation preferably within 24 months of manufacture.

TYPICAL COMPOSITIONAL ANALYSIS

The analysis results listed in this product bulletin are typical as measured on an "as is" basis. Refer to the selling specification for minimum & maximum limits by parameter.

Protein (N x 6.38) (g/100g) as is	82.4
Moisture (g/100g)	10.8
Fat (g/100g)	0.5
Total Carbohydrate (g/100g)	0.1
Ash (g/100g)	8.2
Inhibitory substances (IU/ml)	<0.005

TYPICAL NUTRITIONAL ANALYSIS

Energy (kJ/100g)	1420
Calories (kcal/100g)	340
Energy from fat (kJ/100g)	19
Calories from fat (kcal/100g)	4
Total Sugars (lactose) (g/100g)	0.1
Fibre (g/100g)	0
Cholesterol (mg/100g)	20
Saturated fat (g/100g)	0.3
Trans fat (g/100g)	0.02
Vitamin A (µg/100g)	<6
Vitamin A (IU/100g)	<20
Vitamin C (mg/100g)	<0.4
Iron (mg/100g)	0.36
Sodium (mg/100g)	7.9
Calcium (mg/100g)	2935



Dairy for life

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NZMP™ Rennet Casein 771

PRODUCT BULLETIN

PB.010
Version 6.0209

TYPICAL PHYSICAL PROPERTIES

Colour	Cream
Flavour	Clean
Particle size	30 mesh
Bulk density (packed, g/mL)	0.63
Scorched particles (50g)	Disc A

TYPICAL MICROBIOLOGICAL ANALYSIS

Aerobic Plate Count (cfu/g)	<5000
Coliforms (cfu/g)	<1
E. coli (/g)	Not Detected
Yeast & mould (cfu/g)	<10
Staph. coag. pos. (/g)	Not Detected
Salmonella (750g)	Absent
Listeria (25g)	Absent

QUALITY ASSURANCE

Strict quality control procedures are enforced during manufacture. The manufacturing environment is also subject to regular monitoring and control.

Final product is sampled and tested for chemical, sensory and microbial parameters using internationally recognised procedures.

During storage and shipment, precautions are taken to ensure that the product quality is maintained. Each package is identified, enabling traceback.

COMPLIANCE

- Halal

SUGGESTED LABELLING

Casein
Allergens: Contains Milk and Dairy products.

Country regulations for product labelling vary. Fonterra advises customers that they need to check local regulations to determine the correct labelling of this ingredient.



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Appendix 2

NZMP™ Skimmilk Powder

Regular High Heat

SELLING SPEC

PB.744
Version 1.0110

CHEMICAL	UNITS	MIN.	MAX.
Protein (N x 6.38) As is	% m/m	32.4	36.7
Protein SNF Basis	% m/m	34.0	
Moisture	% m/m		4.0
Fat	% m/m		1.25
Titrateable Acidity	% m/v		0.15
WPNI	mg/g		1.5

MICROBIOLOGICAL	UNITS	MIN.	MAX.
Aerobic Plate Count	cfu/g		10000
Coliforms	/g		Not Detected
E.coli	/g		Not Detected
Yeast & Moulds	cfu/g		50
Staph. coag. pos.	/g		Not Detected
Salmonella	/750g		Absent

PHYSICAL	UNITS	MIN.	MAX.
Colour	Typical/Atypical	Typical	
Foreign matter	/25g		Not Detected
Insolubility Index	ml		1.0
Scorched particles	/25g		B

SENSORY	UNITS	MIN.	MAX.
Flavour	Typical/Atypical	Typical	



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NZMP™ Skimmilk Powder

Regular High Heat

SELLING SPEC

PB.744
Version 1.0110

OTHER

Meets Codex standards for Lead

Meets Codex standards for Heavy metals

Meets Codex standards and FDA/EPA limits for Pesticides

Meets FDA requirements for Phosphatase

Meets FDA requirements Inhibitory substances



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