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SHELF LIFE AND MICROBIOLOGICAL SAFETY STUDIES ON MINIMALLY PROCESSED, REFRIGERATED "SOUS-VIDE" PRODUCTS

by

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SHELF LIFE STUDIES ON MINIMALLY PROCESSED SOUS-VIDE PRODUCTS

To Benjamin for all his help and to our children, Ewurabena, Baaba,

and Aba Wendy-Anne

Foreword

This thesis is submitted in the form of original papers suitable for journal publications. The first two sections are a general introduction and literature review presenting the theory and background information on this topic. The next six sections represent the body of the thesis (each is a complete manuscript). The last section is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, section 7 "Manuscripts & Authorship" which are as follows :

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Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. J.P. Smith of the Department of Food Science & Agricultural Chemistry, Macdonald Campus of McGill University (as the principal supervisor) with Drs. K.L. Dodds (Microbial Hazards Bureau, Health Protection Branch, Ottawa) and H.S. Ramaswamy (Department of Food Science & Agricultural Chemistry, Macdonald Campus of McGill University) as co-supervisors.

Abstract

Pasteurization values (Pv) were established for reformulated sous-vide spaghetti/meat sauce and rice salmon products based on the thermal destruction kinetics of *Streptococcus faecium*. The pasteurization values were a function of both the pH and salt concentration of the heating medium. Microbiological and sensory changes occurring in these pasteurized products during storage at conventional (5°C), as well as abuse temperature conditions (15°C), were also evaluated; results of which indicate that lactic acid bacteria (LAB) and *Bacillus* spores were the predominant spoilage microorganisms in the minimally processed products after 35 days storage at 15°C. Furthermore, most packages were swollen due to carbon dioxide production by the LAB. The fact that spores survived the mild heat treatment is of concern since it implies that other more pathogenic spores eg. *Clostridium botulinum* would also survive and may pose a Public health problem in products stored at 15°C. As such, the influence of combination treatments (viz., mild heat processing treatment, pH, water activity, storage temperature, lactic acid bacteria) were evaluated through challenge studies with selected strains of *C. botulinum* type A, B, and E spores. The shelf life and safety of the products, (based on measurements of days until spoilage and/or toxin detection) following a 13D process at 75°C, could be extended to $\geq$42 days by a combination of pH $\leq$5.0 and storage at 5°C. Other combination treatments were also effective in controlling growth of, and toxin formation in the products by *C. botulinum*. For instance, botulinum toxin was not detected until day 35 in inoculated spaghetti and meat sauce products (pH 5.25 and a_w 0.992) with type A and B spores and stored at 15°C. Shelf life extension and inhibition of toxin production was also possible through reductions in a_w levels achieved by

addition of 1 - 3% salt (w/w) to the products. Water activity levels of $\leq$ 0.983 prevented botulinum toxin production in the minimally processed spaghetti and meat sauce product for $\geq$ 42 days during storage at 15°C.

Similar trends were observed in the sous-vide rice and salmon products. Furthermore, when sodium lactate was used as the humectant to lower the a_w of this product, toxin production was also delayed, but not to the same extent as achieved with NaCl. When two strains of bacteriocin-producing lactic acid bacteria were co-inoculated separately in the sous-vide rice and salmon products that had been challenged with *C. botulinum* type E spores, toxin formation was only slightly delayed in products with added NaCl (1%), while toxin formation was neither delayed nor prevented in similarly lactic acid bacteria-treated samples with no added NaCl. Furthermore, in most of the products in which botulinum toxin was detected, spoilage preceded toxigenesis, however, in some samples stored at 5°C toxigenesis preceded spoilage. Addition of α-2-macroglobulin at a level of 2.7 ppm did not delay or prevent toxin formation in the product. Microwave heating of products at half-power or full power (800 Watts) for 5 to 10 min proved effective in inactivating all of the pre-formed toxin in toxic samples.

Thus, the study has shown that barriers such as process temperature, pH, a_w, and storage temperature, all exert significant antimicrobial effects, and can be used in various combinations to enhance the shelf life and safety of sous-vide products.

Resumé

ETUDE SUR LA DURÉE DE VIE ET LA QUALITE SANITAIRE DE PRODUITS SOUS-VIDE, PASTEURISES ET REFRIGERES

Les valeurs pasteurisatrices (Pv) ont été établies pour reformuler deux plats cuisinés (spaghetti en sauce à la viande et riz au saumon) en utilisant des cinétiques de destruction thermique de *Streptococcus faecium*. Les Pv étaient fonctions à la fois du pH et de la concentration en sel du milieu de chauffage. Les modifications microbiologiques et organoleptiques de ces produits au cours du stockage et de la réfrigération ($5^{o}C$) ainsi que dans des conditions de température limites ($15^{o}C$) ont aussi été évaluées. Les résultats ont indiqué que les bactéries lactiques et les spores de Bacillus étaient les principaux microorganimes contaminants dans les produits ayant subit un traitement minimal après 35 jours de stockage à $30^{o}C$. De plus, la plupart des emballages étaient gonflés par le dioxyde de carbone produit par les batéries lactiques. Le fait que ces spores ont survécu au traitement thermique doux est trés important puisqu'il implique que d'autres spores plus pathogènes telles que Clostridium botulinum pourraient aussi survivre et poser de ce fait un problème de santé publique pour les produits stockés à 15oC. Ainsi, l'influence de traitements combinés (traitement thermique doux, pH, activité de l'eau, température de stockage, bactéries lactiques) a été étudié par ensemencements des produits avec des spores provenant de souches sélectionnées de *C. botulinum* de type A, B and E. La durée maximale de conservation et la qualité sanitaire (mesurées par le nombre de jours avant détérioration et/ou detection de toxine) peut être

étendue, après un traitement à 13D à 75°C, à 42 jours au moins par la combinaison d'un pH inférieur à 5 et d'un stockage à 5°C. D'autres combinaisons de traitements étaient efficaces pour limiter la croissance de *C. botulinum* et la formation de toxine dans les produits. Par exemple, la toxine botulinique n'était pas détectée jusqu'au 35ième jour dans tous les plats de spaghetti avec sauce à la viande (pH 5.3 et a_w 0.992) inoculés par des spores de type A et B et stockés à 15°C. L'extension de la duréee maximale de consommation et l'inhibition de la production de toxine étaient aussi possible par la réduction de l'a_w réalisée par addition de 1 - 3 % de sel (p/p) dans les produits. Une a_w inférieure à 0.983 empêchaient la production de toxine botulinique pendant 42 jours au moins à une température de stockage de 15°C dans un plat de spaghetti avec sauce à la viande ayant subi un traitement minimal.

Des résultats similaires ont été observés dans les plats cuisinés sous-vide de riz au saumon. De plus, quand le lactate de sodium était utilisé comme humectant pour diminuer l'a_w de ce produit, la production de toxines était aussi retardée mais de façon moins efficace qu'avec NaCl. Lorsque deux souches de bactéries lactiques productrices de bacteriocine étaient co-inoculées séparément dans des produits sous-vide ensemencés avec des spores de *C. botulinum* de type E, la formation de toxine était seulement légèrement retardée dans les produits additionnés de NaCl (1%). Cependant, la formation de toxine n'était ni retardée ni prévenue dans les échantillons traités de façon similaire avec les bactéries lactiques mais sans addition de NaCl. De plus, dans la plupart des produits dans lesquels la toxine botuilinique a été détectée, la dégradation a

précédé la production de toxine. Cependant, dans certains échantillons stockés à 5°C, la production de toxine précédait la dégradation. L'addition d'alpha-2-macroglobuline à la concentration de 2.7 ppm n'a pas retardé ni prévenu la formation de toxine dans les produits. Des traitements aux micro-ondes à demi-puissance ou pleine puissance (800W) pendant 5 à 10 min se sont avérés efficaces pour inactiver totalement la toxine préformée dans les échantillons toxiques.

Ainsi, cette étude a montré que des barrières telles que le traitement thermique, le pH, l'aw et la température de stockage exercent toutes des effets antimicrobiens significatifs, et peuvent être utilisés dans différentes combinaisons pour augmenter la durée de consommation et la qualité sanitaire des plats cuisinés sous-vide.

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I would like to express my sincere gratitude to my supervisor, Dr. J.P. Smith, for his tremendous assistance, patience, useful suggestions and guidance throughout the study.

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Finally, my thanks to the "unmentionable many" and above all to the Almighty for making all this possible.

Preface

Claim of Original Research

1. The use of *S. faecium* in establishing pasteurization conditions of sous-vide spaghetti and meat sauce and rice and salmon product.

2. Challenge studies with *C. botulinum* in sous-vide spaghetti and meat sauce and rice and salmon products.

3. The use of combination treatments, i.e., pH, a_w, and storage temperature, and their effects on shelf life and microbiological safety of sous-vide spaghetti and meat sauce and rice and salmon products, with respect to controlling toxigenesis by *C. botulinum.*

4. The use of sodium lactate as a humectant to lower water activity in sous-vide products, to result in prevention or retardation of toxigenesis by *C. botulinum* in sous-vide products.

5. The study of the capacity of α-2-macroglobulin, a naturally occurring protease inhibitor, to prevent or delay toxigenesis by *C. botulinum.*

A. *Part of this work has been published as follows :*

Smith, J.P., Toupin, C., Gagnon, G., Voger, R., Fiset, P.P. and Simpson, M.V. (1990). A Hazard Analysis Critical Control Point approach to ensure the microbiological quality of sous-vide processed meat/pasta products. *Food Microbiol.* 7 : 177.

B. *Part of this work has been accepted for publication, viz. :*

Simpson, M.V., Smith, J.P., Ramaswamy, H.S., Simpson, B.K. and Ghazala, S. (1993). Thermal resistance of *Streptococcus faecium* as influenced by pH and salt. *Food Res. Int.* (*In press*).

C. *Part of this work has been presented at Scientific Conference, viz.,*

Simpson, M.V., Smith, J.P., Ramaswamy, H.S., Simpson, B.K. and Ghazala, S. (1991). The effect of salt, pH and processing temperature on the thermal resistance of *Streptococcus faecium* in nutrient broth/sous vide products. *34th. Ann. CIFST Conf.* Montreal, Canada.

Simpson, M.V., Smith, J.P., Ramaswamy, H.S., Simpson, B.K. and Ghazala, S. (1991). Thermal kinetic studies on *Streptococcus faecium* in nutrient broth medium/sous vide products. *17th Int. Cong. of Refrigeration.* Montreal, Canada.

Ghazala, S., Ramaswamy, H.S., Smith, J.P., Simpson, B.K. and Simpson, M.V. (1991). Establishing processing schedules for sous-vide spaghetti/meat sauce and rice/salmon based on thermal destruction of *Streptococcus faecium. 17th. Int. Cong. of Refrigeration.* Montreal, Canada. Paper # 354.

D. *Part of this work has been submitted for publication as follows :*

Simpson, M.V., Smith, J.P., Ramaswamy, H.S., Dodds, K.L. and Simpson, B.K. (1993). Storage studies on a sous-vide spaghetti and meat sauce product. *Food Microbiol.*

Simpson, M.V., Smith, J.P., Dodds, K.L., Simpson, B.K., Ramaswamy, H.S. and Blanchfield, B. (1993). Challenge studies with *Clostridium botulinum* in a sous-vide spaghetti and meat sauce product. *J. Food Protect.*

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Permission to use part of this material has been obtained from the co-authors of the manuscripts listed above.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ATCC	➠	America type culture collection
a_w	➠	water activity
BHI	➠	brain heart infusion
CAP	➠	controlled atmosphere packaging
E_h	➠	oxidation / reduction potential
ERH	➠	equilibrium relative humidity
EVOH	➠	ethylene vinyl alcohol
FDA	➠	Food & Drug Administration
HACCP	➠	hazard analysis critical control points
LAB	➠	lactic acid bacteria
LT	➠	heat labile enterotoxin
MA(P)	➠	modified atmosphere (packaging)
MPRF	➠	minimally processed refrigerated foods
OPP	➠	oriented polypropylene
PP	➠	polypropylene
PVC	➠	polyvinyl chloride
PVDC	➠	polyvinylidene chloride
RH	➠	relative humidity
ST	➠	heat stable enterotoxin
TDT	➠	thermal destruction time
TSA	➠	trypticase soy agar
VP	➠	vacuum packaging

CHAPTER 1

INTRODUCTION

The need for food by humans on a continuing basis is probably second only to our need for air and water. Several physical, chemical, and biological methods of food preservation can be used to inhibit the deteriorative changes in foods thereby extending the shelf life and quality of foods. Physical methods of food preservation to delay microbial or enzyme activity include thermal processing, irradiation, controlled reduction in the products' water content (e.g., concentration, air dehydration, freeze drying) and the use of protective packages, e.g., modified atmosphere packaging. Chemical preservation involves the addition of ingredients such as sugars, salts, acids and/or their products, smoke or fumigants to foods. Examples of the more common preservation methods and their anti-microbial effects are as shown in Table 1, while biological preservation includes methods such as alcoholic or acidic fermentations, e.g., yogurt production.

In order to provide adequate diet for the world's ever increasing population, there is need to develop more effective, practical and less energy intensive methods of food preservation. Food losses through spoilage is a major problem, particularly in developing countries. It has been estimated that ~ 50% of all fresh food is spoiled due to mainly microbial/enzyme activity.

Technological developments in food processing and preservation have provided consumers with a greater selection of foods with a longer shelf life.

Table 1 : Comparison of Antimicrobial Efficiencies of Some Food Preservation Methods*

Preservation method	Primary anti-microbial property(ies)
Modified atmosphere	inhibits aerobes; facultative anaerobes & anaerobes can grow; combination with refrigeration can slow down growth; flushing with 100% CO_2 retards growth.
Canning & UHT treatments	destroys all vegetative cells as well as some spores (e.g., *C. botulinum*); partially effective toward thermophilic bacterial spores that grow at $\geq 40^oC$.
Pasteurization	destroys all vegetative pathogens & about 90% of non-pathogenic microorganisms. Ineffective toward spores & thermoduric vegetative cells.
Refrigeration (0 - 5^{o}C)	inhibits growth of mesophilic & thermophilic organisms; minimizes growth of several psychrotrophs.
Freezing ($\geq$ -20^{o}C)	inactivates certain vegetative cells; & growth is stopped in surviving cells.
Partial drying (a_w 0.8 - 0.9)	inhibits growth of many bacteria; but some microorganisms can grow.
Partial drying (a_w 0.6 - 0.8)	inhibits growth of bacteria, but yeasts & mold can grow.
Drying (a_w <0.6)	similar to freezing.
Ionizing radiation	destroys vegetative cells & spores; level of destruction depends on dose.

* Adapted from Ray (1992).

Consequently, consumers have been more selective, more conscious of quality, and more concerned about value for money. They are also more aware of, and sensitive to off-odors, strong flavors, discoloration, and other indications of spoilage in food.

Since food may be processed, and then distributed and sold at retail outlets often far removed from the point of processing, the processor must employ methods for both short- and long- term preservation of food. Such methods must be capable of either suppressing or killing both pathogenic and spoilage microorganisms without the addition of any toxic substances. In addition to prolonging shelf life and keeping quality, the preservative technique employed must also preserve the characteristics of the raw products as much as possible.

Of the various physical, chemical and thermal processing methods available for enhancing the prime quality shelf life and safety of foods, refrigeration is by far the most important method of preservation for the food industry. This technology has been practiced for several hundred years. The attractive features about refrigeration storage include the fact that it slows down (i) intrinsic biochemical processes potentiated by indigenous enzymes, (ii) microbial proliferation, and (iii) minimizes undesirable side reactions in foods (e.g., discoloration and flavor loss). As well, cold temperature storage protects heat-labile essential components in foods, e.g., vitamins.

However, it has been demonstrated that, even under refrigeration storage conditions (0 - 3^{o}C), food continues to spoil due the activities of aerobic, psychrotrophic strains of bacteria, predominantly *Pseudomonas* species.

However, it is possible to modify the storage conditions, and thus the environment to which spoilage bacteria are exposed at refrigerated temperatures. With regard to further spoilage control, it is widely accepted that microorganisms show greatest tolerance to a single adverse environmental factor when all other conditions are optimal (Anon., 1980). Conversely, two or more simultaneous suboptimal environmental conditions will be far more inhibitory than each component considered separately. The combined use of several preservation methods can be explained by the "hurdle concept" of Leistner and Rodel (1976) and Mossel (1983). This states that several hurdles or 'inhibition factors', even if any one of them individually cannot inhibit microorganisms, will nevertheless together reduce or inhibit microbial growth, if the hurdles are incorporated into a substrate in sufficient number and height. Moberg (1989) defined "hurdles" as the physical, chemical and/or biological factors which act as barriers to microbial growth and in addition to temperature (i.e., refrigeration, freezing, or heating) these include such factors as pH, chemical treatments, irradiation, modified atmospheres, vacuum packaging, naturally occurring inhibitors, moderate heat treatment (pasteurization), water activity (a_w), redox potential (E_h), and competitive microorganisms (Moberg, 1989; Scott, 1989; Wagner & Moberg, 1989; Genigeorgis, 1985). The advantages of using one or more hurdles in conjunction with each other to control spoilage in minimally processed refrigerated foods, and thereby enhance their stability and safety include : (i) the undesired side effects of an individual barrier may be avoided or reduced, (ii) it is less energy intensive and hence less expensive than conventional physical methods of preservation, such as freezing. Examples of combinations of these barriers in the preservation of processed foods is illustrated in Fig. 1.

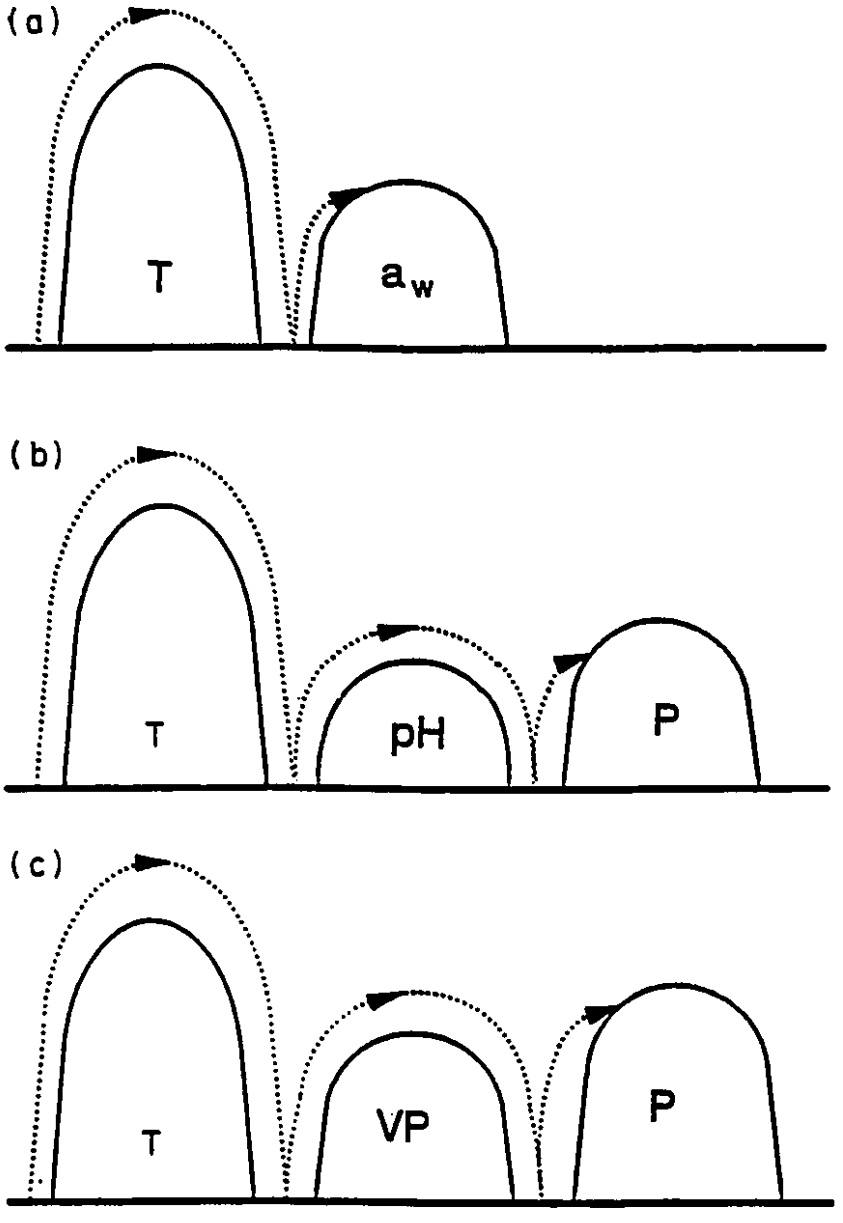


Fig. 1 : The hurdle approach to shelf life extension of processed foods (a) combination of low temperature T and a_w ; (b) combination of T, pH and preservative (P) ; (c) combination of T, vacuum packaging (PV) and P.

Currently in North America, there is a high demand for convenient, fresh tasting, ready-to-eat, microwaveable refrigerated and shelf-stable foods due to factors like increases in dual income families, and single parent households, which have decreased the time and desire for elaborate meal preparation. The trend is towards minimally processed entrees, meats, vegetables, salads, pastas, sauces and other side dishes. Many of these foods are designated as the "new generation refrigerated" or "chilled" foods and they contain little or no preservatives, and may be packaged in modified atmospheres to extend their refrigerated shelf life. Sous-vide is in this category of new-generation refrigerated foods (Rhodehamel, 1992) and the technology involves vacuum packaging of the food products in thermo-retractable pouches with the intent to store the products at refrigerated temperatures. The term 'sous-vide' originated from France and was developed by Georges Pralus in the mid 70's who discovered that these products had improved yield with less shrinkage (Swientek, 1989).

Sous-vide usually refers to vacuum processed/packaged/pasteurized food entrees distributed and stored under refrigeration conditions, although some processors freeze their products for distribution reasons (Baird, 1990; Beauchemin, 1991). In sous-vide processing, foods are cooked in sealed vacuumized heat stable pouches, which is said to "seal-in" natural flavors, aromas and nutrients in the food products . This method of "sealing in" natural juices is not new. Early civilization used many ingenious ways of cooking foods in wrappings to retain natural flavors and maximize juiciness (Tannahill, 1973).

What is new about the sous-vide process is the highly controlled conditions of time and temperature specified for the production of the food.

This technique is widely used in Europe, Japan and North America. In 1988, about 138 industrial establishments in France alone used sous-vide technology to process 12,000 tons of fish and seafood (Beauchemin, 1991). In North America, unlike Europe, the demand for these products is consumer driven instead of being pushed through by the chain stores. Sale of these refrigerated foods, including sous-vide products, is predicted to reach $5 billion by 1995 in the North American market alone.

Today, however, one major concern of these foods is that they could pose a potential public health hazard if proper control measures are not in effect. This safety concern is justified in view of (i) the fact that sous-vide products undergo minimal heat processing, and are therefore not "commercially sterile", (ii) the ability of pathogenic resistant spore and non-spore formers, e.g., *Clostridium botulinum*, to withstand the pasteurization processing treatment, (iii) the anaerobic conditions in the product which may be conducive to the growth of pathogens, and (iv) the potential for temperature abuse. However, there is paucity of information on shelf life stability of these products.

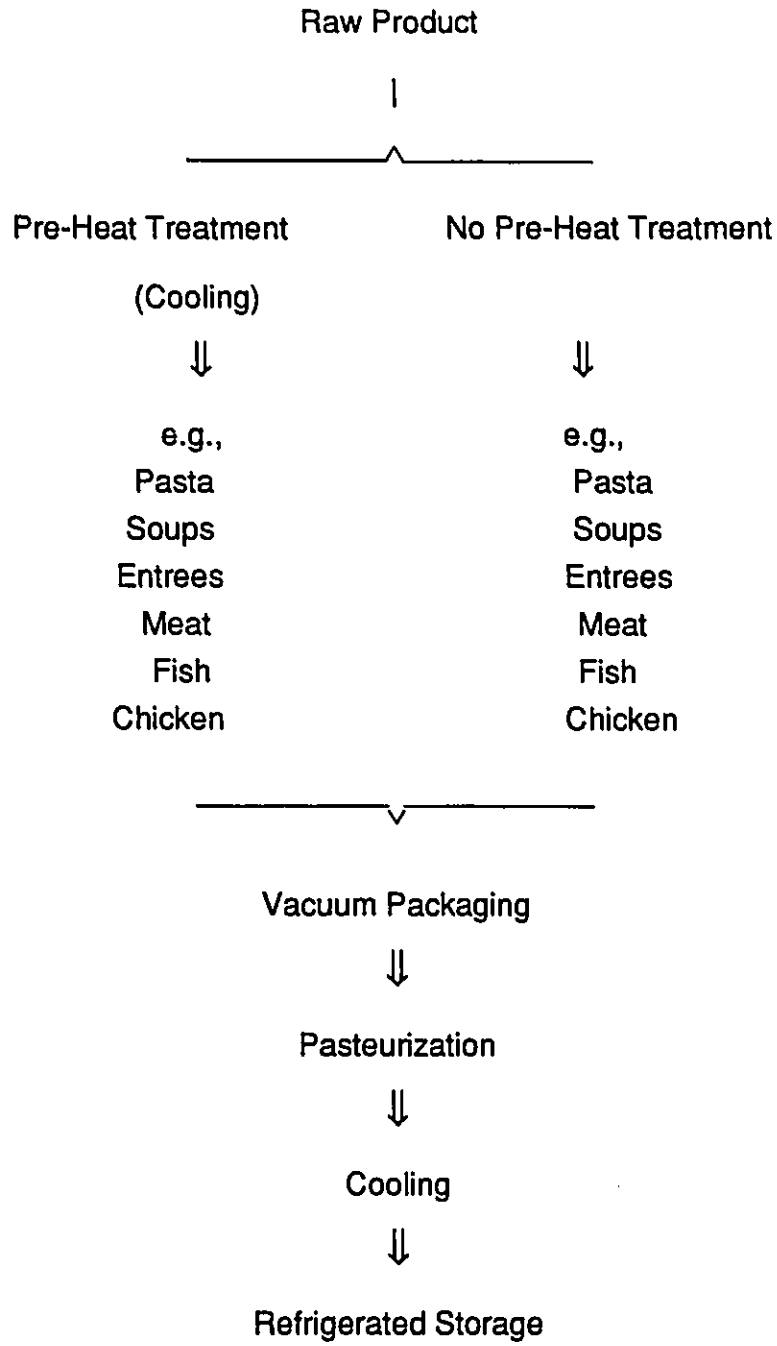
CHAPTER 2

LITERATURE REVIEW

2.1. "New Generation Refrigerated Foods"

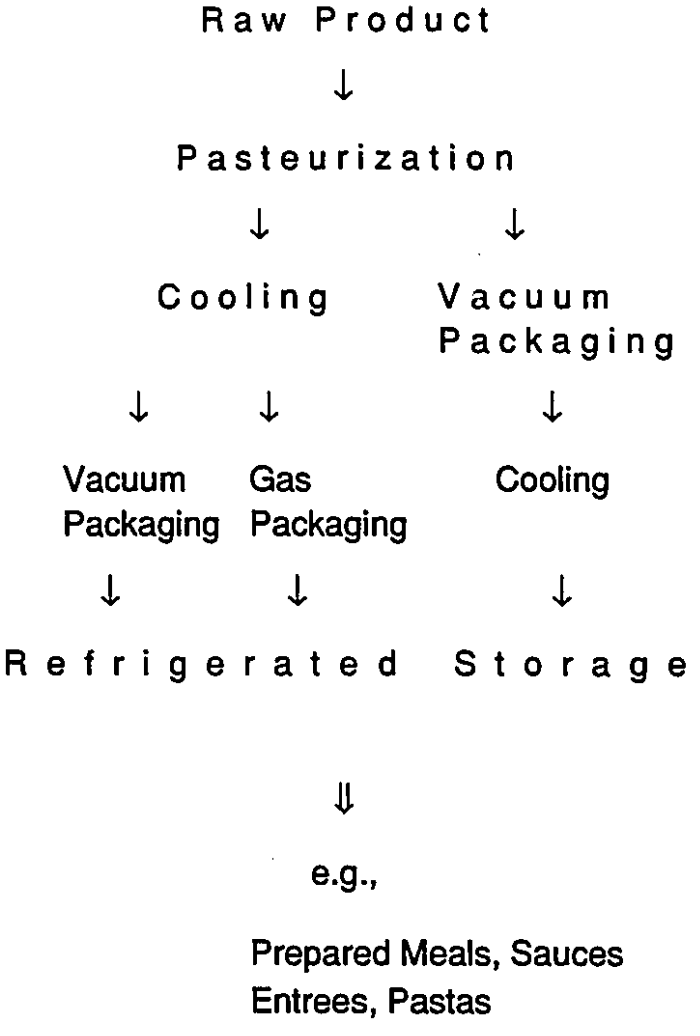
Traditional refrigerated foods include raw meats and fish, pasteurized milk, cured and cooked ready-to-eat meats, high-moisture cheeses and spreads, fermented cheeses, yogurt, pickles, perishable delicatessens and other similar products, while the term "new generation refrigerated" or "chilled" foods include minimally processed entrees such as pasta and sauces, dinners (complete meals), salads, meat etc., (Corlett, 1989; Swientek, 1988 & 1990; Eilers, 1990). Many of the traditional refrigerated foods have a backup preservation system, e.g., nitrite in cured and cooked meats, without which they will spoil and be a potential hazard to the consumer. Also, consumers, in general, are familiar with methods of handling these foods safely, i.e., refrigeration. In contrast, the "new generation" of refrigerated foods, e.g., sous-vide products, are minimally processed products with very little or no preservatives. Many of these products are subjected to a minimal heat treatment which does not inactivate thermoduric spoilage or pathogenic microbial cells and spores that can grow at normal refrigeration temperatures. Two methods of production of these minimally processed/refrigerated foods are shown schematically in Figs. 2 & 3. Vacuum packaging together with refrigeration can further increase shelf life by inhibiting the growth of certain spoilage or pathogenic organisms. Vacuum packaging inhibits the growth of aerobic pathogenic bacteria, but can facilitate growth of some other facultative pathogens (Eilers, 1990).

Fig. 2 : Preparation of New Generation Refrigerated Foods - Scenario A*



* Adapted from Gagnon (1990).

Fig. 3 : Preparation of New Generation Refrigerated Foods - Scenario B*



* Adapted from Gagnon (1990).

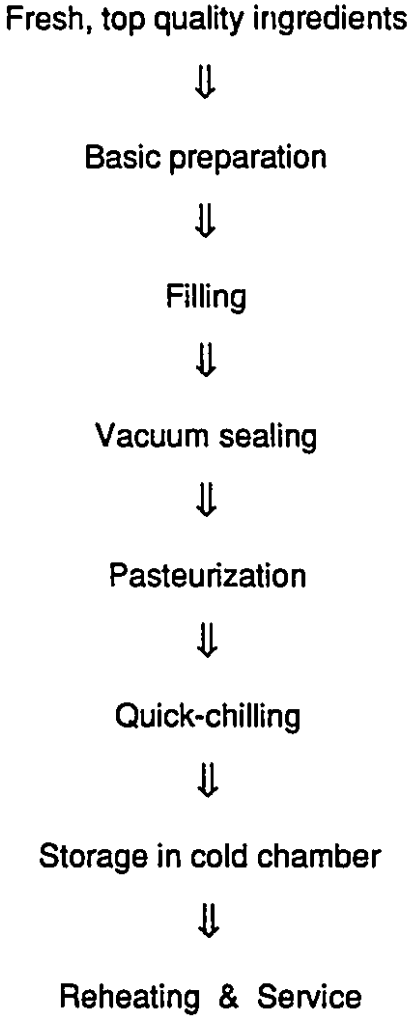
Carbon dioxide, a commonly used gas in modified atmosphere packaging (MAP) has a biostatic activity on certain microorganisms. However, temperature abuse and inadequate distribution systems can increase the risk of microbial hazards in these foods.

2.2. "Sous-Vide Products"

2.2.1. *Process for Making Sous-Vide Products*

The steps in sous-vide process (Fig. 4) involve the use of top quality ingredients, which are precooked or browned if necessary, packaged under vacuum into heat-stable air-impermeable containers, and finally cooked or pasteurized at a particular temperature for a specified time. The products are immediately cooled to $4^{0}C$ and stored or distributed under refrigeration (Raffael, 1984 & 1985; Light et al., 1988), then reheated before serving. Such products made by caterers and manufacturers have a good predicted shelf-life of 21 to 45 days (Schafheitle and Light, 1989). In addition to extending the shelf life, sous-vide processing :- (i) enhances the natural taste of food since all volatile compounds remain in the package, (ii) accentuates the tenderness of meats due to prevention of moisture loss during cooking, (iii) requires no cooking fats and hence is lower in calories, (iv) retains the maximum amount of vitamins and minerals, and (v) the food can be served after 10 - 15 min in boiling water or 4 - 5 min in a microwave. Also, vacuum conditions within the packages not only inhibit microbial growth, but also prevent oxidative and other chemical reactions.

Fig. 4 : Scheme for Vacuum Cooking Process



The problem of cross contamination is also eliminated since products are packaged before heat processing. However, the acceptance of sous-vide products in North America has been slow due to concerns about microbiological safety.

2.2.2. *Public Health Concerns about Microbiological Safety of Sous-Vide Products*

The main concern of sous-vide processing as a preservation method is the microbiological safety, i.e., potential for the growth of pathogenic, anaerobic, bacteria under temperature abuse conditions during transportation, distribution and retailing of the food products.

Sous-vide products generally contain no added preservatives, and no intrinsic inhibitory barriers, and they rely on strict temperature control as the only limiting factor to control the growth of pathogenic organisms. However, strict temperature control cannot be ensured since temperature abuse of the products can occur at any stage during handling, transportation and distribution (Table 2).

Table 2 : Temperature Range in Coolers and Display Cases in Selected Stores*

Temperature range (^{o}C)	Store type (in %)				
	Major stores	Independent stores	Ma & Pa stores	Convenience stores	Average
<1.7	9	3	6	8	6
1.8-4.4	31	19	26	38	28
4.5-7.2	30	36	16	38	30
7.3-10	23	28	26	0	19
10.1-12.8	3	9	10	8	9
≥12.9	4	5	16	8	8
Nº. audited	352	113	31	13	

* Adapted from Harris (1989).

2.2.3. *Foodborne Pathogens of Concern in Sous-Vide Products*

Many psychrotrophic organisms can grow in foods and cause spoilage under refrigerated conditions. These include several bacteria, yeasts and molds. The bacterial species include *Pseudomonas, Achromobacter, Flavobacterium, Acinetobacter, Altermonas putrefacience, Enterobacteriaceae*, and certain *Clostridium* species. Factors like the nature of the food, food environment and storage conditions greatly determine the predominant type or types of spoilage microorganisms.

Vacuum packaging will discourage the growth of aerobes such as *Pseudomonas, Achromobacter, Flavobacterium* species, yeasts and molds (Grau, 1980). Temperatures close to 0^{0}C used for storing certain foods will greatly reduce the growth of strains of *Enterobacteriaceae* (Newton & Gill, 1978), while a pH of 5.8 or below will reduce the growth of *A. putrefacieus* and *B. thermosphacta* (Grau, 1981). However, any shift in these parameters or temperature abuse will favour the growth of many other spoilage microorganisms (Gilliland et al., 1984; Jay, 1992).

In sous-vide technology, where products are packaged under anaerobic conditions and stored at refrigerated temperatures, the organisms of concern are anaerobes, mainly heat resistant psychrotrophic anaerobes or facultative anaerobic spore formers, such as spores of *Bacillus* and *Clostridium* which can germinate and undergo cell multiplication (Kornacki & Gabis, 1990; Shehata & Collins, 1971; Shehata et al., 1972; Bhadsvle et al., 1972). Growth of aerobic spoilage bacteria, yeasts and molds is greatly inhibited by packaging under

vacuum with low oxygen permeability films. As well, aerobic microorganisms, such as pseudomonads which are common in refrigerated muscle foods are also destroyed.

Food borne pathogens have been divided into three groups based on the severity of their hazards, viz., Group I - those that present severe hazards (e.g., *Brucella abortus*, *Clostridium botulinum*, *Salmonella cholerae-suis*; *Shigella* spp., & *Vibrios cholera*); Group II - those that present moderate hazards with potentially extensive spreads (e.g., Enterotoxigenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., & *Streptococcus pyogenes*); & Group III - those that pose moderate hazards with limited spread (e.g., *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, & *Yersinia enterocolitica*). Table 3 shows some growth requirements of some of the organisms of public health concern in sous-vide foods. The hazards caused by foodborne pathogens is classified into 3 main categories as : (i) intoxication (poisoning), (ii) infection, or (iii) toxico-infection (Bryan, 1990; Frazier & Westhoff, 1988; Banwart, 1989; Gravani, 1987). Intoxication occurs from the ingestion of food containing preformed toxin(s). Food infection on the other hand is caused when live cells of pathogenic bacteria or viruses are ingested through eating contaminated foods and the pathogens either invade the intestinal mucosa and multiply or pass to other organs. Toxico-infection occurs when a large number of live cells of pathogens are consumed with food and they produce and release enterotoxins into the gastrointestinal tract which then produce symptoms.

Table 3 : Some Pathogenic Microorganisms Capable of Growth at Low Temperatures

Pathogen	Food associated with outbreaks	Lowest growth temperature(^{o}C)	Other important characteristics
C. botulinum (NP-types B, E)	marine products	3.3	anaerobic spore formers
C. botulinum (P-types A, B, F)	marine products raw meats	10.0	anaerobic spore formers
L. monocytogenes	milk / cabbage	1.7	facultative anaerobe
Y. enterocolitica	animal origin	0	facultative anaerobe
E. coli	animal origin	2.5	facultative anaerobe
A. hydrophila	animal origin / water	3.0	facultative anaerobe
B. cereus	grains / vegetables meat products	5.0	facultative anaerobe
S. aureus	cooked meat products	6.5	facultative anaerobe
Vibrio parahaemolyticus	marine products	5.0	facultative anaerobe

NP-types => non-proteolytic types ; P-types => proteolytic types

The characteristics of some of the more important foodborne pathogens from the three groups referred to above include the following :

Y. enterocolitica (a Group III foodborne pathogen) is a facultative anaerobic, Gram-negative short rod shaped organism. It occurs widely in nature and in a wide variety of foods especially those of animal origin, and can grow at $0^{o}C$. Although *Y. enterecolitica* is known to cause food poisoning when consumed in high numbers (i.e., $>10^{7}/g$), not all the species are pathogenic, and poisoning by this organism is generally not fatal. *Y. enterecolitica* food poisoning is unique among other food poisoning bacteria in that symptoms differ for various age groups. When it afflicts children or adolescents, they suffer symptoms of gastroenteritis and pseudoappendicitis (in children) and abdominal disorders (adolescents). With adults, it is manifested as diarrhea and arthritis primarily. *Y. enterecolitica* may be eliminated by pasteurization. Under conditions of elevated CO_2 levels, the growth of *Y. enterecolitica* decreases. The organism is not killed by bacteriocins of starter cultures but low pH will destroy them.

B. cereus (a Group III foodborne pathogen) is a facultative anaerobic, Gram-positive, spore forming motile organism. The organism produces enterotoxins that are known to cause two distinct syndromes. One enterotoxin causes an illness with similar symptoms to that produced by the *S. aureus* enterotoxin (see below). A second enterotoxin results in disorders similar to those caused by *C. perfringens* enterotoxin. The organism is reported to grow between 10 and $12^{o}C$.

A. hydrophila (a Group III pathogen), is a facultative anaerobic, Gram-negative rod shaped bacterium. This organism occurs widely in nature and has

been isolated from samples of fresh sea food, poultry, red meat and milk. It has the ability to grow at 1 to 5°C. *A. hydrophila* is sensitive to pasteurization with different strains exhibiting varying degrees of sensitivity to bacteriocins of lactic acid bacteria (Bhunia et al., 1988). The organism is also sensitive to low pH.

S. aureus (a Group III foodborne pathogen), is a Gram-positive, facultatively anaerobic organism. It is relatively resistant to dehydration and is widely spread by workers, e.g., via infected wounds, and the human nose. The organism grows best in the presence of oxygen and produces enterotoxin under anaerobic conditions. The organism is significantly inhibited in 50% to 100% CO_2 at 10°C, with only slight inhibition at 25°C. This is to be expected because of the increased solubility of CO_2 at lower temperatures. Although the effect of this high solubility of CO_2 at low temperature on *S. aureus* can be microcidal, i.e., increase the cell death rate, and reduce the growth, this cannot be depended upon to eliminate the pathogen or its stable toxin. The spores of *S. aureus* although not killed by pasteurization, can be destroyed by some bacteriocins of LAB. However, it can grow in low a_w (0.84), low pH (4.0), and high salt concentrations (18%), (Hurst & Nasim, 1984; Bhunia et al., 1988). The toxins remain heat stable even after boiling for 25 min and after the death of the cells, but are inactivated at pH <4.5.

E. coli (a Group II foodborne pathogen), is a Gram-negative lactose fermenting rod shaped facultative anaerobe commonly found in the intestinal tract of man and animals. Its presence in foods or drinking water indicates faecal contamination. In pasteurized dairy products the presence of detectable *E. coli* is

indicative of either underprocessing or post-pasteurization contamination. Until recently, the presence of this organism in food was not considered hazardous. However, it is now recognized that certain strains of *E. coli* are pathogenic to both man and other homeothermic species, and food can serve as a vehicle for these organisms. The organism may be eliminated by pasteurization.

L. monocytogenes (a Group II foodborne pathogen), is a small coccoid to rod-shaped Gram-positive motile bacterium. It is a facultative anaerobe and grows better at reduced oxygen and increased CO_2 levels. Outbreaks of *L. monocytogenes* infection have been implicated with contaminated cabbage used in coleslaw production. *L. monocytogenes*, in contrast to common food poisoning organisms, generally causes a variety of gastrointestinal symptoms, pneumonia, urethritis and flu-like symptoms, which in pregnant women can cause abortions or stillbirths. *L. monocytogenes* has been shown to be capable of growth at temperatures as low as 1.7^{o}C, when it is more virulent, i.e., pathogenic. *Listeria* can be killed by mild heat treatment or pasteurization. It is also sensitive to low pH and is killed by some bacteriocins of lactic acid bacteria (Bhunia et al., 1988; Benkerron & Sandine, 1988; Schillinger & Lucke, 1989). The Regulatory requirement for *L. monocytogenes* is for "zero tolerance" in ready-to-eat foods.

Salmonella (some strains are Group II, while others are classified as Group I foodborne pathogens), is a Gram-negative facultatively anaerobic organism, whose minimum growth temperature is a function of various cultural conditions as well as the food itself. Heat injured *Salmonella* is capable of repair at 10^{o}C and once this occurs, the repaired cells will produce toxin and create a hazard in foods at 10^{o}C or above.

The symptoms, and the severity of the illness, depend on the number of organisms and the serotype as well as the resistance of the host. The most commonly reported symptoms are fever, nausea, vomiting, chills and headache. *Salmonella* is affected by vacuum conditions and high levels of CO_2 (60% CO_2 and 40% O_2) with the degree of inhibition increasing with decrease in temperature. The organism is killed by pasteurization, is sensitive to low pH, but insensitive to bacteriocins of lactic acid bacteria (LAB). Regulatory standards for this organism in foods is "zero tolerance".

C. botulinum (a Group I foodborne pathogen), is a Gram-positive, anaerobic, spore forming bacterium. *C. botulinum* is a very heterogeneous group of bacteria with 2 main groups recognized as causing human botulism, viz., Groups I and II, representing the proteolytic types (A, B, & E) and the non-proteolytic types (B, E, & F) respectively. The term "proteolytic" is used to denote the ability to digest casein, meat or coagulated egg and serum proteins, while "non-proteolytic" refers to the capacity to hydrolyze gelatin and other modified proteins. The proteolytic strains are mainly associated with botulism outbreaks in meats, poultry and vegetables, while the non-proteolytic strains have been associated with outbreaks mainly in fish/fish products. The classification by types into A, B, C, D, E, F and G, is based on the antigenicity of the toxin they produce. The types A, B, E and F are of significance to man. Table 4 shows the classification and growth requirements of the organism. Several studies have also shown that *C. botulinum* species can grow and produce toxin in beef, pork and fish products packaged in various gaseous conditions and stored under mild temperature abuse conditions.

Table 4 : Minimal Requirements for Growth and Heat Resistance of *C. botulinum* Types A, B, E, & F.

Properties	Group I	Group II
Toxin types	A, B, F	B, E, F
Inhibitory pH	4.6	5.0
Inhibitory NaCl concentration	10%	5%
Minimal a_w	0.94	0.97
Temperature range for growth	10 - 48°C	3.3 - 45°C
D_{100} of spores	25 min	<0.1 min
Maximum E_h (mV)	+250 (type A)	+250 (type E)
Susceptibility to preservatives (except nitrite)	minimal	minimal

Source : Hauschild (1989)

With fish, toxigenesis occurred within 3 days irrespective of storage temperature, with spoilage preceding toxigenesis (Stier et al., 1981).

C. botulinum types A, B and E spores pose the greatest threat to consumer safety in sous-vide products due to (i) the ability of this spore forming pathogen to withstand mild heat treatment, (ii) the complete or partial destruction of non-spore forming competing microorganisms and potential indicators of incipient spoilage, and (iii) the presence of anaerobic packaging conditions conducive to the growth and toxin production *by C. botulinum*. Oosterom et al., (1981) showed that spores of psychrotrophic strains of *C. botulinum* types B and E are generally less heat resistant than those of mesophilic strains. Spores of mesophilic *C. botulinum* (i.e., proteolytic types A and B) do not germinate at temperatures below $10^{o}C$, but spores of non-proteolytic psychrotrophic strains, e.g., *C. botulinum* type E, can germinate at temperatures as low as $3.3^{o}C$. Oosterom et al., (1981) tested the hypothesis that *C. botulinum* will not produce toxin in food that was heated prior to storage under proper refrigerated conditions. Although toxin was not produced in vacuum packaged potatoes previously heated to $95^{o}C$ for 40 min and stored at $4^{o}C$ for 45 days, toxin was detected after 9 days in similarly treated samples stored at $10^{o}C$. These findings provide further evidence of the need for refrigerated temperatures ($\leq 4^{o}C$) for minimally processed food to prevent the growth of *C. botulinum* spores.

Simunovic et al., (1985) emphasized in a review, that type E and non-proteolytic types B and F can grow and produce toxin at refrigeration temperatures as low as 3 to $4^{o}C$.

Vegetative cells of *C. botulinum* are killed at certain pasteurization temperatures, but the spores may survive. The spores can be inactivated by boiling in water for 6 h, low pH, or low water activity, by some bacteriocins of lactic acid bacteria, nitrites and salts.

The National Advisory Committee on Microbiological Criteria for Foods (Anon, 1991) recommended for refrigerated foods containing meat and poultry to be sufficiently processed to achieve a minimum of 4 log cycle reduction (4D) for *Listeria monocytogenes* (Anon, 1991; Brown, 1991), while Smith et al., (1990) recommended a thermal process to achieve a 12 - 13 log reduction of *S. faecium*.

2.2.4. *Incidence of C. botulinum in Food*

Various studies have confirmed the presence of *C. botulinum* in fish/fish products, meats and poultry, as well as in fruits and vegetables, and it appears the prevalence of the different types of the pathogen depends on geographic location (Dodds, 1993). For example, the type A spores are said to predominate in the soils of western United States, Brazil, Argentina and China; the type B spore is the predominant type found in eastern U.S., the U.K., and much of continental Europe; the type E spore is the major type in Northern Canada, Alaska, much of Scandinavia, the former Soviet Union and Japan; while the types C and D spores tend to be the major types in the relatively warmer climates. According to Dodds (1993), the level of contamination in fish is generally higher than in meat and poultry, and this distinction was attributed to the relatively

higher level of contamination in the aquatic environment. The organism has also been shown to be capable of growth and toxin production at 3.3^{0}C in beef stew medium (Palumbo, 1986). Although the incidence of *C. botulinum* spores is very low in meat and poultry products in Canada and the U.S., the organism has been found in meat products held at low temperature (Lechowich, 1988). The first confirmed isolation of psychrotrophic *C. botulinum* from meat was a non-proteolytic type B strain from raw smoked ham in Germany in 1981. Blood sausage was the source of a psychrotrophic type B strain implicated in botulism outbreak in Iceland in 1983. Greenberg et al., (1966) examined the incidence of mesophilic clostridium spores in raw chicken, beef and pork and observed most (including type C) to be putrefactive anaerobes. Most meat contamination occurred due to contamination from the animals rather than the environment (Klarmann, 1989; Narayan, 1967). Other investigators did not find the pathogen in various food products. For example, Taclindo et al., (1967) and Insalata et al., (1969) surveyed various foods including vegetables, and were unable to detect *C. botulinum* spores. Also Thatcher et al., (1967) were unable to show the presence of *C. botulinum* in 436 samples of vacuum packed sliced processed meat purchased in Canada. Lower incidence levels of *C. botulinum* spores in meat and meat products have been indicated in North America than in Europe. In Canada, of 208 samples of commercial bacon surveyed, only one was found positive (Hauschild & Hilsheimer, 1980 & 1983).

In North America, the presence of *C. botulinum* in fish has been extensively studied. The pathogen has been found in fish harvested from the Great Lakes as well as from the Atlantic and the Pacific coasts. Vacuum

packaging of seafoods has been controversial since the absence of oxygen and low temperature encourage the growth of type E spores. The main concern being that of type E toxin accumulation in the absence of obvious spoilage odors. Post et al., (1985) found that when the flesh of cod, whiting, and flounder fillets was inoculated with *C. botulinum* type E spores, and then packaged either in air, vacuum or gas mixtures, there was spoilage which resulted in rejection of the products either before or concurrent with toxin detection during storage at $12^{o}C$ and $26^{o}C$. Cann et al., (1965) studied the effect of inoculum load, storage temperatures and irradiation on toxin formation in a variety of vacuum packed seafoods and demonstrated that time until toxin production increased with decreasing storage temperature. They also demonstrated the relationship between the level of the spores and the storage temperature. Also, in the evaluation of the effect of competitive microorganism, Bremner and Statham (1983) indicated that the spoilage of vacuum-packed scallop, with or without lactobacilli, proceeded at similar rates. The potential risk from commercial seafood in California was assessed by Lerke (1973), who reported that *C. botulinum* type E was not detected in any of 35 samples preserved by acetic acid and refrigeration. Furthermore, Taclindo et al., (1967) examined 113 refrigerated samples and found only 1 sample of luncheon meat was positive with type B spores, while only one sample of unshucked oyster was positive with type E. Those foods examined which are considered vulnerable included processed meats as well as smoked fish products. Insalata et al., (1967) also examined the incidence of *C. botulinum* in convenience foods and found the levels of the pathogen to be low.

2.2.5. *Outbreaks of Botulism*

There seems to be a relationship between the occurrence of *C. botulinum* in the environment and the outbreaks of botulism (Hauschild, 1993). Examples of botulism outbreaks are summarized in Table 5. The outbreaks of botulism in the U.S. from 1971 to 1989 were caused mostly by type A followed by types B and E in that order (Hauschild, 1993). Most of the implicated foods in the U.S. were fruits and vegetables, although some of the outbreaks were linked to fish and meat products. Most of the botulism outbreaks in the U.S. (25 out of 39) were reported in Alaska alone.

Here in Canada, of the reported botulism outbreaks, 87% occurred in the northern native Canadian communities, and the prevalent type associated with the outbreaks was type E. Some of the foods implicated in the outbreaks were meat from marine mammals (raw, parboiled or fermented), caribou meat (raw or parboiled), fish (raw, parboiled, dried or smoked) and preserved vegetables or pork.

Seven of the outbreaks in North America were associated with foods served in restaurants between 1977 and 1987. It is estimated that, at least 177 patrons were affected with 3 fatalities. In some cases, the incriminating food was found to have been improperly processed, and as a result of this, Canadian restaurants were forbidden from serving in-house (i.e., non-commercial) canned or bottled foods (Todd, 1985). Some of the outbreaks were linked to temperature abuse of food ingredients or the finished products.

Table 5 : Examples of Outbreaks of Foodborne Human Botulism

Country	Period	# of Outbreaks	Total Cases	Fatalities[b]
U.S.[a]	1971 - 89	272	597	63 (11)
Canada	1971 - 89	79	202	28 (14)
France	1978 - 89	175	304	7 (2)
Norway	1961 - 90	19	42	3 (7)
China	1958 - 82	986	4377	548 (13)
Japan	1951 - 87	97	479	110 (23)

Country	***Outbreaks** (with type identified)*	***Type (%)***			
		A	***B***	***E***	***Other***
U.S.[a]	252	61	21	17	0.4
Canada	76	4	8	88	0
France	171	0	77	2	0.6
Norway	19	47	47	5	
China	733	93	5	1	0.4
Japan	97	2	2	96	0

Adapted from Hauschild (1993) ; ***a*** => including Alaska ; ***b*** => in bracket as percent of total cases

2.3. Biochemistry and Mechanism of General Food Spoilage

Mossel (1971 & 1982), described two main areas of concern in food microbiology as : (i) protection of the consumer against disease from food borne pathogens, and (ii) extension of shelf-life or retardation of spoilage from activity of other types of organisms. From the microbial aspect, this includes products of metabolism resulting from differences in biochemical characteristics between pathogens and spoilage bacteria.

2.3.1. *Food Spoilage*

Food spoilage can be defined as the loss of acceptance quality due to either microbial or non-microbial changes. Microbial spoilage can occur both from the metabolic activities of the live cells of bacteria, molds and yeasts, and in the absence of live cells, from the action of microbial enzymes, especially heat-stable ones in heat processed foods. In general, the incidence of food spoilage that is of microbial origin is much higher than that of non-microbial origin with bacteria being the prime culprit, followed by molds, then yeasts. Microbial spoilage can change the flavor, texture and the color of food products. Many of these changes are due to the end products formed after metabolism of the food components.

2.3.2. *Mechanism of Food Spoilage*

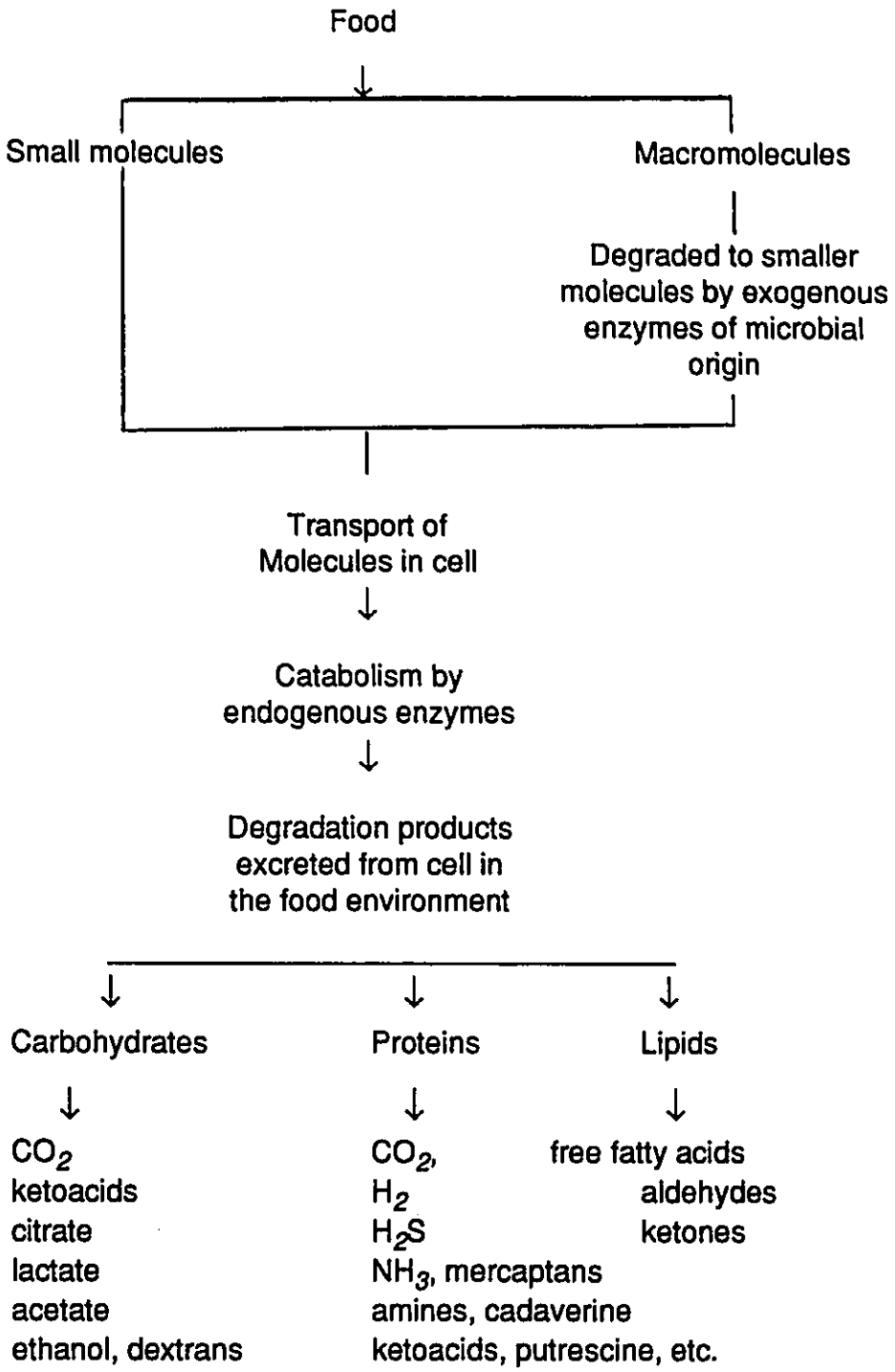
Microorganisms grow in food by metabolizing carbohydrates, proteins and lipids in the food to produce different metabolic end products. The effectiveness of metabolizing different food components and the nature of the end products are

dependent upon the chemical nature of the food components, the microbial types, and the metabolic pathways through which a component has been utilized, as shown in Fig. 5. The end products formed in metabolism depend on factors such as the chemical nature of the substrate, the concentration of the substrate, the type of organism, and the availability of oxygen. In general, microorganisms will preferentially utilize metabolizable carbohydrates as an energy source as opposed to using proteins and lipids. The metabolic end products formed by one microorganism can inhibit or stimulate growth of other microorganisms. Thus, metabolism of carbohydrates by lactic acid bacteria produces lactic acid that inhibits the growth of many Gram-negative spoilage bacteria capable of metabolizing proteins, and helps in preventing the spoilage of protein-rich foods, so called "protein-sparing" effect. The acid environment can also prevent or reduce the growth of many other bacteria, but not that of yeasts and molds, which can grow and cause spoilage of acid foods.

Since microorganisms utilize food components as a source of energy, as well as for the synthesis of cellular, structural, and functional components, the biological molecules (from food) must be transported inside. Large food molecules such as starch, pectin, casein, myoglobin and triglycerides, cannot enter the microbial cells. However, many microorganisms, including important types of food spoilage microorganisms produce extracellular enzymes that hydrolyze the macromolecules into smaller components which are then transported into cell and utilized by the microorganism.

Thus, the action of microbial endo- and exoenzymes as well as the live

Fig. 5 : Scheme for the Formation of Degradation Products from Various Food Components by Microbial Activity *



* Adapted from Ray (1992).

cells effect the changes that are associated with food spoilage. In addition, exoenzymes and endoenzymes released after death of microorganisms bring about changes in the food quality in the absence of live cells. This is especially important for heat stable enzymes in many heat-processed foods with extended shelf-life, e.g., sous-vide products.

2.4. Methods for Controlling Foodborne Pathogens and Food Spoilage

The potential health hazard of minimally processed refrigerated foods (MPRF) or new generation foods is recognized by the Refrigerated Foods and Microbiological Criteria Committee of the National Food Processors Association (Anon, 1988a). According to Moberg (1989), refrigeration alone is not sufficient to provide a means for insuring safety of these foods from pathogenic bacteria since temperature abuse is possible. Further, destruction of competitive vegetative microorganisms by the mild heat treatment may allow spore forming bacteria to grow (Moberg, 1989).

Raw and processed foods contain different types of microorganisms and the relative numbers of the organisms vary greatly depending on the nature of the food and the handling method. Food can provide the nutrients necessary for the growth (i.e., metabolism and multiplication) of most microorganisms (Frazier & Westhoff, 1988; Banwart, 1989; Jay, 1992).

The potential for growth of the individual species or strain among the mixed microorganisms in the sous-vide packages is influenced by the intrinsic, extrinsic and the processing factors which are all interdependent, especially for the processed foods (Scott, 1989; Anon, 1980). The intrinsic factors include the chemical, physical and biochemical characteristics such as nutrient composition, presence of natural antimicrobials, pH, a_w, oxidation-reduction potential (E_h), and the structure of the cell, e.g., membrane or cuticle, porosity, etc.

The extrinsic factors include environmental factors such as storage temperature, storage gaseous atmosphere, and the packaging method. The processing factors include the survival and growth of microorganisms during processing. The interdependency and combinations of these factors all influence microbial growth and determine which types and proportion of bacteria will predominate in the food product over a period of time. Therefore, in order to assure the microbiological safety of the finished sous-vide products, the parameters to be controlled are quality of raw materials, effective processing during pasteurization, pH, a_w and relative humidity (RH), oxidation reduction potential (E_h), packaging material and temperature control.

Sous-vide products are more susceptible to spoilage because they contain little or no added anti-microbial preservatives to control microbial growth during storage. The main controlling factors in these products are heat, oxygen removal and temperature control. Therefore, the most important factors determining the shelf life and the resistance to temperature abuse are the initial microbial load, the effectiveness or lethality of the heat treatment (or F value), and the means to

prevent the growth of microorganisms through temperature control, packaging integrity and the use of additional barriers.

2.4.1. *Prevention of Growth and Toxin Production by C. botulinum*

C. botulinum toxins are produced under conditions that permit vegetative cell growth (Baird-Parker, 1971). In most foods, *C. botulinum* is inhibited by a combination of factors rather than a single factor (Hauschild, 1989). The main factors limiting growth and toxin production are temperature, pH, water activity, redox potential, added food preservatives, and competing microorganisms. The effects of these factors are interrelated. Combinations of inhibitory factors are more restrictive than each factor alone (Ohye & Christian, 1967).

The initial level of bacteria load in raw food material is important in determining the finished product's quality. Generally speaking, the greater the number of organisms present, the shorter is the lag phase of growth, and the more rapid bacterial multiplication in the growth phase.

In food spoilage due to microbial action, a relatively low initial population will require a relatively longer time to multiply and reach the spoilage level ($>10^{6-7}$/g) under proper storage conditions. Careful screening of raw materials and hygienic processing conditions are essential. Identification of sources of microbial contamination, possible growth and points of re-contamination from raw materials at all phases of processing and subsequent handling would provide useful information needed for formulating and/or rationalizing effective control procedures to inhibit the deleterious effects of microorganisms. The effectiveness

of these controls is made by corrective measures known as the Hazard Analysis Critical Control Point (HACCP) procedure (Adams, 1991; Alli, 1989 & 1990; Anon, 1988b; Beauchemin, 1991; Bryan, 1990; Corlett, 1989; Smith, 1991; Smith et al., 1990; Snyder, 1990). Thus, it is often possible to lengthen the lag phase of bacterial growth to permit sufficient completion of a processing operation before active growth occurs in the product, to lower the bacterial load by manipulating temperature alone. For instance, the lag phase of bacterial growth in many perishable foods may be lengthened by ~100% by maintaining the temperature of such food at $10^{o}C$ instead of $18^{o}C$ during certain processing operations.

2.4.1.1. *Water Activity (a_w)*

The water activity of a food product is a measure of free water available in a food system, and has been defined as the ratio of the vapor pressure of the food to that of pure water at a specified temperature and also expressed by Raoult's law.

$$\text{i.e., } a_w = P/P_o = n_2 / n_1 + n_2$$

where P and P_o are the vapor pressures of a solution and pure water respectively and n_1 is the moles of solute and n_2 is the moles of pure water.

The a_w of a food significantly influences its storage life and even its wholesomeness. The texture, browning and other color reactions and quality of the food may be altered by its a_w level. In addition, microbial growth rates and in some cases, production of metabolites may be particularly sensitive to alterations

in a_w. Minimum levels of a_w permitting growth of some psychrotrophs are shown in Table 6. The a_w of a food can be reduced either by adding humectants which may be in the form of specific inorganic salts (e.g., NaCl), or organic compounds (e.g., carbohydrates, proteins), or by removing part of the free water from a food in drying.

Different types of solutes influence growth and toxin production differently. Generally, NaCl, KCl, glucose and sucrose show similar patterns, while glycerol permits growth at lower a_w levels (Ray, 1992). Pelroy et al., (1985) demonstrated that NaCl and KCl had equivalent inhibitory effects on toxin production by *C. botulinum* type E strains in smoked white fish. Salt (NaCl) remains one of the most important factors in the control of food borne *C. botulinum*. For *C. botulinum*, an organism of public health concern in sous-vide food products (the main focus of this study), it is generally accepted that a_w values $\geq$ 0.94 of food products enable the growth and toxin production by the proteolytic *C. botulinum* types A & B strains, while a_w levels $\geq$ 0.97 are conducive for growth and toxin production by type E strains (Baird-Parker & Freame, 1967; Denny et al., 1969; Emodi & Lechowich, 1969; Marshall et al., 1971). Emodi and Lechowich (1969) found that 38.5% sucrose or 22.5% glucose inhibited growth of 4 strains of type E spores.

The perceived advantages of a_w in inhibiting growth and toxin production by *C. botulinum* in foods have been investigated by various researchers.

Table 6 : Minimum a_w for Growth of Some Major Food Spoilage Bacteria *

Microorganism	Characteristics	Minimum for growth	
		a_w	pH
Aeromonas spp.	facultative anaerobe	0.95	5.5
Enterobacter spp.	facultative anaerobe	0.95	5.0
Escherichia coli	facultative anaerobe	0.94	4.5
Lactobacillus spp.	facultative anaerobe	0.93	3.6
Enterococcus spp.	facultative anaerobe	0.94	4.6
Clostridium spp.	obligatory anaerobe		
Proteolytics		0.94	4.6
Non-proteolytics		0.97	5.0
Bacillus spp.	facultative anaerobe	0.90	4.2
Pseudomonas spp.	obligatory aerobe	0.95	5.0

* When all other conditions are optimal.

For example, the addition of salt (3 - 5%), was reported to inhibit botulinum toxin formation in smoked fish chubs that had been challenged with the organism (Christiansen et al., 1968). Furthermore, controlled a_w levels delayed botulinal toxin production in vacuum packaged potatoes (Dodds, 1989), and also extended the shelf life and safety of heat-treated canned pears (Jacobsen & Jensen, 1975). The general effects of reduced a_w on microbial growth is shown in Fig. 6.

2.4.1.2. *pH or Acidity of Food*

The hydrogen ion concentration influences the overall acidity, and hence the quality of foods. In foods, the acids of interest are either naturally present organic acids, or formed during fermentation (e.g., lactic acid in fermented foods), or may be added in the formulation (e.g., sorbic acid). The pH of a food is important because it can influence the survival and growth of microorganisms during processing, storage, and distribution. Several studies have shown that the internal pH of microorganisms is greatly affected by the presence of acids in the menstrum. For example, Kashket and Wong (1969) showed that organic acid acidified the interior of the cell more effectively than strong inorganic acid. It has also been suggested that the undissociated form of certain weak acids diffuse freely through the cell membrane and ionize in the cell to yield protons that acidify the alkaline cell interior (Hunter & Segel, 1973; Ingram et al., 1956). Freese et al., (1973), suggested that the underlying mechanism for the activity of weak acids used as preservatives is due to the rate of proton leakage into the cell relative to the proton-rejecting capacity of the cell and this determines the inhibition capacity of an environment.

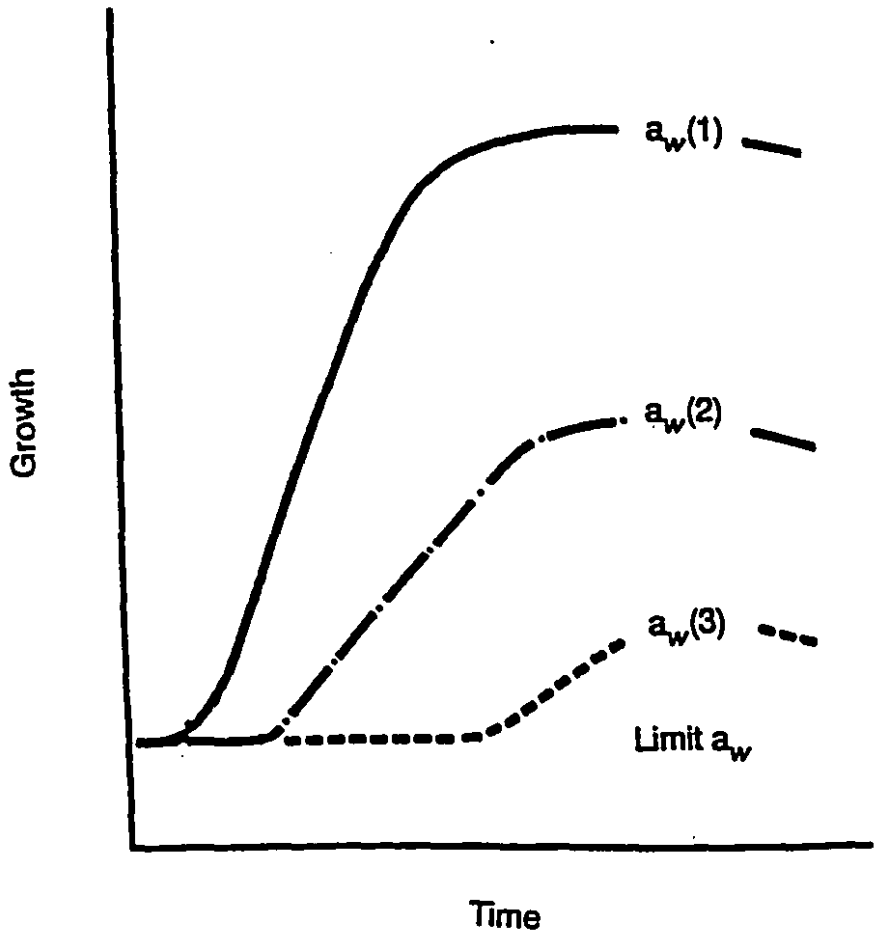


Fig. 6 : General Effects of Reduced a_w on Microbial Growth

$$a_w(1) > a_w(2) > a_w(3)$$

The acidification of the cell interior leads to inhibition of nutrient transport as well as other cell activities.

Different microorganisms have different tolerances to acidification. Thus, many spoilage bacteria, such as Pseudomonas species will not grow at pH $\leq$ 5.0; some lactic acid bacteria will grow at pH below 4.0; *Leuconostoc* species will not grow below pH of 4.0; yeasts and molds can grow at pH 2.0, etc. The optimum pH range for the growth of some *Clostridium* species has been reported as 5.4 - 5.7. For *C. botulinum* spores, the optimum pH for growth has been reported as $\geq$ 7. In a mixed flora, acidity may select the predominant initial population.

Alderton and Snell (1963) suggested that spores act as weak cation exchangers so that low pH permits the replacement of other bound ions in the spore wall by hydrogen ions, thus lowering the stability of the spore. Therefore, low pH can be used in food preservation to either inhibit microbial growth directly, or reduce microbial heat resistance in foods that will be subsequently heat processed. Lechowich (1988) described the potential hazard from refrigerated foods under several categories, based mainly on pH and a_w - two intrinsic factors that affect selection of types of organisms present in foods (Mossel, 1971). These two properties are highly important in judging the potential for a food to become a public health hazard. Low acid foods (i.e., foods of pH greater than 4.6), as may be the case with sous-vide products, are considered to be hazardous if improperly processed and stored, and many types of refrigerated food products are in this category. These products are intended to provide a shelf life of two weeks or more but their safety is questionable.

The upper limits of growth of, and toxin production by *C. botulinum* is within the pH range of 8 - 9 (Hobbs, 1976). The minimum pH requirement for growth and toxin production by proteolytic *C. botulinum* strains is in the range of 4.6 - 4.8, and for the non-proteolytics the minimum pH is about 5 (Hauschild, 1989; Ito & Chen, 1978; Post et al., 1985; Segner et al., 1966). Other investigators have reported growth and toxin production by the pathogen at pH 4 (Raetjes & Smelt, 1979; Smelt et al., 1982; Young-Perkins & Merson, 1989). Wong et al., (1988) observed that leaf protein at pH values below 4.6 permitted germination, outgrowth and toxin production to a greater extent than soy protein. Therefore, pH alone is not a suitable index for predicting toxicity end points in media. According to Blocher and Busta (1983), factors such as strain, medium composition, growth temperature, etc., influence the acid tolerance.

Corlett (1989), reported that a pH level of $\leq$4.6 has long been known to inhibit the growth and toxin production by *C. botulinum* and also retards growth of most other pathogens. The antibotulinal effects of pH was also demonstrated by Kang et al., (1969).

Furthermore, various acidulants used in foods have different antimicrobial activities. Smelt et al., (1982) found that citric acid was less inhibitory than lactic acid or acetic acid.

2.4.1.3. *Oxidation-Reduction Potential (E_h)*

Microorganisms display varying degrees of sensitivity to the oxidation-reduction potential (E_h) of their growth medium (Hewitt, 1950). The E_h of a

substrate is defined as the ability to lose or gain electrons and is expressed in millivolts. Aerobic microorganisms require positive E_h values (oxidized) for growth, while anaerobes require negative values (reduced). For example, *C. botulinum* requires reduced conditions for growth initiation and an optimum E_h of ~ -200 mV has been reported for the pathogen (Hauschild, 1989), although maximum E_h values for initiation and growth is said to range from +30 to 250 mV (Ando & Iida, 1970; Huss et al., 1979; Morris, 1976).

In general, microaerophilics (e.g., Lactobacilli and Streptococci) grow better under slightly reduced conditions. Vacuum packaging (e.g., sous-vide), therefore, selects microorganism types depending on their requirements for oxygen. It is usually believed that O_2 levels below 4% create anaerobic conditions for the organisms, and at this level other factors such as temperature control, pH and a_w also influence bacterial growth. Some anaerobes can grow in atmospheres of 20% O_2, probably because the Eh of the microenvironment may be sufficient to permit growth. According to Ando and Iida (1970), germination of *C. botulinum* spores can occur at much higher E_h values. Factors such as NaCl and acidity lower the maximum E_h level for growth of *C. botulinum* (Smoot & Pierson, 1979). The E_h of most foods exposed to air is usually low enough to permit growth of *C. botulinum* (Sperber, 1982), and the organism has been found to grow in foods exposed to air. For example, Christiansen and Foster (1965) reported the same rate of toxin production by *C. botulinum* type A in sliced bologna, whether or not it was vacuum packaged; while Ajmal (1968) reported that *C. botulinum* type E inoculated in meat and fish produced toxin under both aerobic and anaerobic conditions.

In a study with strains of *C. perfringens*, E_h values $\leq$ -45 mV had no influence on growth of the organism, while more positive E_h values produced an increase in the lag phase in the growth of the organism (Barnes & Ingram, 1956).

2.4.1.4. *Packaging - Modified Atmospheres*

In "Modified Atmosphere Packaging" (MAP), perishable food products such as fish, meat, poultry and some forms of produce, all packaged in an atmosphere which has been modified so that its composition differs from that of air.

MAP represents one of the most significant food technologies of the 'New Generation' of food products with extended shelf-life. Also, the quality of foods prepared by this approach is considered to be superior to that of thermally processed foods such as canned, or aseptically, or retort pouch/tray packaged foods. The technology of Modified Atmosphere Packaging is not exactly new and has been practiced for over a century. As far back as 1882, it was known that elevated levels of CO_2 made it possible for meats to be stored for up to 5 weeks (Holland, 1980), as shown in Table 7. During the 1930's, the storage lockers of ships transporting meat carcasses were enriched with CO_2 as a means of increasing storage (Genigeorges, 1985; Seideman & Durland, 1984). By 1940, approximately 25% of all New Zealand beef and 60% of Australian beef was shipped under CO_2 atmospheres.

Table 7 : Examples of Commercial Applications of MAP

Country	Year	Atmosphere used	Product
New Zealand	1938	CO_2	beef
Australia	1938	CO_2	beef
United States	1972	* CO_2 / O_2 / N_2	beef / pork / lamb poultry / salmon
United States	1972	O_2 / N_2	beef
United States	1976	CO_2	meat / poultry / fish

* => 35 - 75% CO_2 ; 21 - 28% O_2, and N_2 as the remainder.

Food packaging preserves the sensory and microbiological quality of cooked and chilled foods, and this has been shown by several investigators to be of great value (Snyder & Matthews, 1984; Daniels et al., 1985; Young et al., 1988; Young et al., 1989). Sous-vide products are packaged in either preformed rigid trays or flexible thermoformed pouches, multi layered by co-extrusion or with adhesives to maintain barriers after thermal processing. Factors such as the permeation to gases, water vapor and organic acids, plastics migration into food, the heat sealing integrity, transparency to light and mechanical resistance, dictate the microbiological safety of the product, particularly after prolonged storage. For sous-vide products, flexible materials generally incorporate polyethylene (PE), ethylene vinyl alcohol (EVOH), or polyvinylidine chloride (PVDC), and polypropylene (PP), while for semi rigid trays PP, EVOH and PP combined may be used. The integrity of the heat seal is just as critical in ensuring the efficiency of the barrier properties.

Some food processors use compensated vacuum with bacteriostatic gases to extend the shelf life of food products. However, in Japan, oxygen scavengers are used to control the level of oxygen in the products. Use of these scavengers in the sous-vide products may not be applicable because of the heat treatment following packaging.

A food can either be stored in the presence of air or under modified atmosphere, (MA). In sous-vide processing, the products are packaged under vacuum, and this removal of air from the packages constitutes a modification of the atmosphere (Genigeorgis, 1985), as well microorganisms present respire

(i.e., utilize the residual oxygen) and carry on enzymatic activities to result in elevated levels of carbon dioxide.

In vacuum packaging, the elevated levels of CO_2 (10% - 20%) are produced within vacuum packages by microorganisms on the food as they utilize residual oxygen (Silliker & Wolfe, 1980) or by respiring produce. This condition of low O_2 (<1%) and elevated CO_2 levels help extend the shelf-life by inhibiting the growth of undesirable microorganisms just as would be achieved with added CO_2. However the shelf life of vacuum packaged products depends on interrelated factors such as the microbiological load, pH at the time of packaging, packaging film permeability, package integrity and storage temperature. Examples of gas mixtures used for packaging some food products are shown in Table 8.

Although mixtures of various gases (e.g., CO_2, N_2, O_2, and sometimes CO) are used in MAP, the most commonly used and perhaps the most effective is CO_2, used either exclusively or in combination with other gases.

Table 8 : Examples of Gas Mixtures Used for Packaging Selected Food Products

Product	Temperature (°C)	O_2%	CO_2%	N_2%
Fresh meat	0 - 2	70	20	10
Cured meat	1 - 3	0	50	50
Poultry	0 - 2	60 - 80	20 - 40	-
Fatty fish	0 - 2	0	60	40
Cheese	1 - 3	0	60	40
Dry snacks	RT	0	20 - 30	70 - 80

RT => room temperature (20 - 25°C)

CO_2 is important because of its distinct biostatic activity toward molds, yeasts and strictly aerobic spoilage organisms which grow at refrigerated temperatures (Enfors & Molin, 1978). It also has an inhibitory effect on the respiration of the products (Kader, 1980). CO_2 can extend the lag phase and generation time of microorganisms (especially, Gram-negative psychrophilic organisms), thereby decreasing their growth rate and delaying spoilage of products caused by these organisms. However, CO_2 does not retard the growth of all microbes. For example, the growth of lactic acid bacteria is actually improved in the presence of CO_2 and under low O_2 content.

To be an effective biostat the CO_2 must dissolve in the aqueous phase of the product leading to the formation and ionization of carbonic acid and subsequently a change in the intracellular pH.

i.e., : $CO_2 + H_2O \quad <===> \quad H_2CO_3 \quad <===> \quad H^+ + HCO_3^-$

The exact mechanism by which CO_2 inhibits microorganisms is not known but two explanations have been offered. King and Nagel (1975), found that CO_2 blocked the metabolism of *Pseudomonas aeruginosa* and appeared to effect a mass action on enzymatic decarboxylation reactions. Sears and Eisenberg (1961), found that CO_2 affected the permeability of the cell membranes resulting in an increased fluidity, and this theory has been endorsed by Enfors and Molin (1978) and Daniels et al., (1985).

Nitrogen is an inert gas and is used as a filler gas to reduce the concentration of other gases in MA packaged products, and to prevent the

product from shrinking and the package from collapsing as the CO_2 dissolves into the product. However, N_2 increases off flavor development in food products, at the same time, it does not curtail growth of aerobic bacterial flora in the food material when applied at a level of 100% of the atmosphere (Huffman, 1974; Karmas, 1975; Holland, 1980).

In a MA environment, oxygen acts to inhibit growth of anaerobic pathogens, improve the red color of meat by combining with the ferrous ion of the heme pigment of myoglobin (MB) to form bright red oxymyoglobin. The effects of MA on various microorganisms are shown in Table 9.

Since the 1970's and the 1980's there has been renewed interest in the use of MA (Wolfe, 1980) to extend the marketable shelf-life of fresh muscle foods at reasonable cost, and the market demand for the use of this technology to retain freshness of food products for retail distribution has correspondingly increased.

Vacuum packaging of foods (as in sous-vide products), is of great concern since this might facilitate *C. botulinum* growth, although other pathogens may be inhibited. Pace and Krumbiegel (1973) reported the rate of toxin production to be higher in vacuum packaged fish than in similar samples packaged without vacuum. However, Johannsen (1965) stated that the formation of toxin in vacuum packaged products is lower than in those not packaged in vacuum. He proposed that the microaerophilic environment in the vacuum packages encourages the growth of lactobacilli, which antagonize growth and toxin formation by *C. botulinum.*

Table 9 : Summary of Effect of MA on Selected Microorganisms

Microorganism	Treatment	Effect of Treatment
Y. enterocolitica	vacuum packaging	enhanced growth of organism
C. perfringens	MA (elevated CO_2)	no effect on growth of organism
S. aureus	[1] MA (O_2/CO_2/N_2)	growth of organism inhibited at 10°C; normal growth at 20°C.
Salmonella	MA (elevated CO_2)	retarded growth of organism at 10°C; normal growth at 20°C.
Salmonella	[2] MA (CO_2/O_2)	growth of organism inhibited at 10°C.
C. botulinum	[3] MA (O_2/CO_2/N_2)	growth & release of toxin in 24 h
C. botulinum	[4] MA (O_2/CO_2/CO/N_2)	growth & release of toxin in 24 h
Enterobacter	MA (elevated CO_2)	no effect on growth of organism

[1] => 25% O_2 + 60% CO_2 + 15% N_2 ; [2] => 60% CO_2 + 40% O_2

[3] => 20% O_2 + 60% CO_2 + N_2 balance at 27°C ; [4] => 20% O_2 + 60% CO_2 + 0.5% CO + N_2 balance at 27°C.

Also, CO_2 present in some modified atmosphere packages may inhibit or stimulate *C. botulinum* depending on its concentration (Foegeding & Busta, 1983).

2.4.1.5. *Competing Microorganisms*

Although preservatives such as nitrites, sorbic acid, parabens (alkyl esters of parabenzoic acid), polyphosphates, bacteriocins, phenolic antioxidants such as butylated hydroxytoluene (BHT), ascorbates, SO_2, etc., have been used in inhibiting growth and toxin production by *C. botulinum* in various food products, addition of certain microorganisms directly to the food is becoming a popular method of preservation since this is regarded as a "natural preservative". Some microorganisms when present in foods may inhibit growth of *C. botulinum* by either changing the environment unfavorably, or by the production of specific inhibitory substances, including organic acids (e.g., lactic acid, acetic acid, propionic acid), alcohols (e.g., ethanol), H_2O_2, bacteriocins (e.g., nisin), etc., (Daeschel, 1989).

Lactic acid bacteria such as *Lactobacillus, Pediococcus* and *Streptococcus* (*Lactococcus* spp.) have been shown to produce acid and inhibit growth and toxin production in meat products (Tanaka et al., 1980 & 1985). To ensure lactic acid production, additional fermentable sugars may be required. Another source of inhibition may be due to bacteriocins produced by the lactic acid bacteria. Nisin is an example of a bacteriocin, and is a polypeptide antimicrobial agent produced by *Streptococcus lactis*, which has been shown to

be effective against *C. botulinum* (Hurst, 1981; Rayman et al., 1981; Scott & Taylor, 1981a & 1981b). Thus, cells of food-grade starter culture microorganisms are added to foods to produce safe, shelf stable, and desirable fermented foods.

2.4.1.6. *Temperature Classification of Organisms*

The most important factor that affect microorganisms growth is temperature. Four major physiological bacterial groups are distinguished based on their temperature ranges of growth, namely thermophiles, mesophiles, psychrophiles and psychrotrophs (Silliker and Wolfe, 1980). The various temperature requirements for growth are as shown in Table 10. Mesophiles, many of which are of animal or human origin, prefer moderate temperatures with an optimum generally between 30 and 45^{o}C, and with a minimum growth range of about 5 to 10^{o}C. Mesophiles include all pathogens and many food spoilage types, e.g., *C. botulinum, B. cereus*. Thermophiles have an optimum temperature range for growth between 55 and 65^{o}C, with a maximum of about 90^{o}C and a minimum of about 35^{o}C, examples are *C. thermosaccharolyticum, B. stearothermophilus* and *B. coagulans*. According to Olson and Nottingham (1980), psychrotrophs and psychrophiles have the same minimum temperature range (-5 to +5^{o}C), but psychrotrophs (e.g., *Pseudomonas* species) have optimum growth at 25 to 30^{o}C, while psychrophiles (e.g., *Vibrio fischeri*) have optimum growth temperature range of 12 to 15^{o}C.

Table 10 : Temperatures for Growth of Microorganisms (in ^{o}C)

Group	Growth Temperature		
	Minimum	Optimum	Maximum
Thermophiles	40 - 45	55 - 75	60 - 90
Mesophiles	5 - 15	30 - 45	35 - 47
Psychrophiles	-5 - +5	12 - 15	15 - 20
Psychrotrophs	-5 - +5	25 - 30	30 - 35

Source : Anon (1980)

The term thermoduric has been used to differentiate microbial cells that survive pasteurization or low heat treatments. Over the years, different definitions of psychrotrophs have been proposed depending on their ability to grow at 7°C, 5°C or even 3°C (Cousin, 1982). However, this group includes all the psychrophiles and some mesophiles as well as some thermodurics. Spores, especially bacterial spores, are all thermoduric; however they are much more heat resistant than the thermoduric vegetative cells and some can survive much higher temperature treatments. Among the microorganisms, the thermophilics and the psychrotrophic groups are probably the more important groups in food spoilage. Thermophilics are particularly important in foods that are processed or held at temperatures above 40°C and below 60°C for considerable periods of time, while psychrotrophs are important in foods that are kept at refrigeration temperatures for long periods of time and can undergo temperature abuse. Psychrotrophs include many kinds of bacteria such as Gram-negative and Gram-positive bacteria, aerobes, anaerobes, facultative organisms, spore formers and non-spore formers, motile and non-motile bacteria, rods, cocci and vibrios (Suhren, 1989; Olson & Nottingham, 1980).

2.4.1.6.1. *Temperature Control*

Temperature control in refrigerated foods is one of the major hurdles of minimizing microbial growth. Although, growth of *C. botulinum* may not occur at temperatures of 3.3°C and below, refrigeration is not enough to totally inhibit all microbial growth. A combination or synergistic effect of other factors at sub-inhibitory levels can be devised to further control growth of microorganisms

during temperature abuse (Leistner, 1992).

2.5. *Thermal Kinetics*

When temperatures are elevated slightly above the maximum for growth and multiplication of bacteria, death of the vegetative bacterial cells occurs. However, thermal destruction depends on the initial type of microorganism, quantity and nature of the cells. Different microorganisms are affected differently by heat. A bacterium is said to be dead if it has lost its ability to reproduce. It has been generally accepted that the number of viable cells reduces exponentially with time of exposure to a lethal temperature. Hence, a plot of the logarithm of numbers of surviving microorganisms versus time of exposure gives a straight line (Fig. 7, survivor curve) and this is referred to as the logarithmic order of death. The slope of the line defines the D value or the decimal reduction time, which is the time required to reduce the microbial load by a factor of 10 or 1 log cycle. Higher temperatures decrease the D values. D values may be used to compare the relative resistance of spores or vegetative cells to heat. The most resistant strains of *C. botulinum* types A and B spores have a D_{100C} value of 50 min and D_{121C} of 0.1 - 0.2 min, while the most heat resistant thermophilic *B. stearothermophilus* spores have D_{100C} of 3000 min and D_{121C} of 4 - 5 min, (Anon, 1980). Table 11, lists D values of other microorganisms.

According to Rahn (1929 & 1945), the loss of reproductive power of a bacterial cell when subjected to moist heat is due to the denaturation of one gene essential to reproduction.

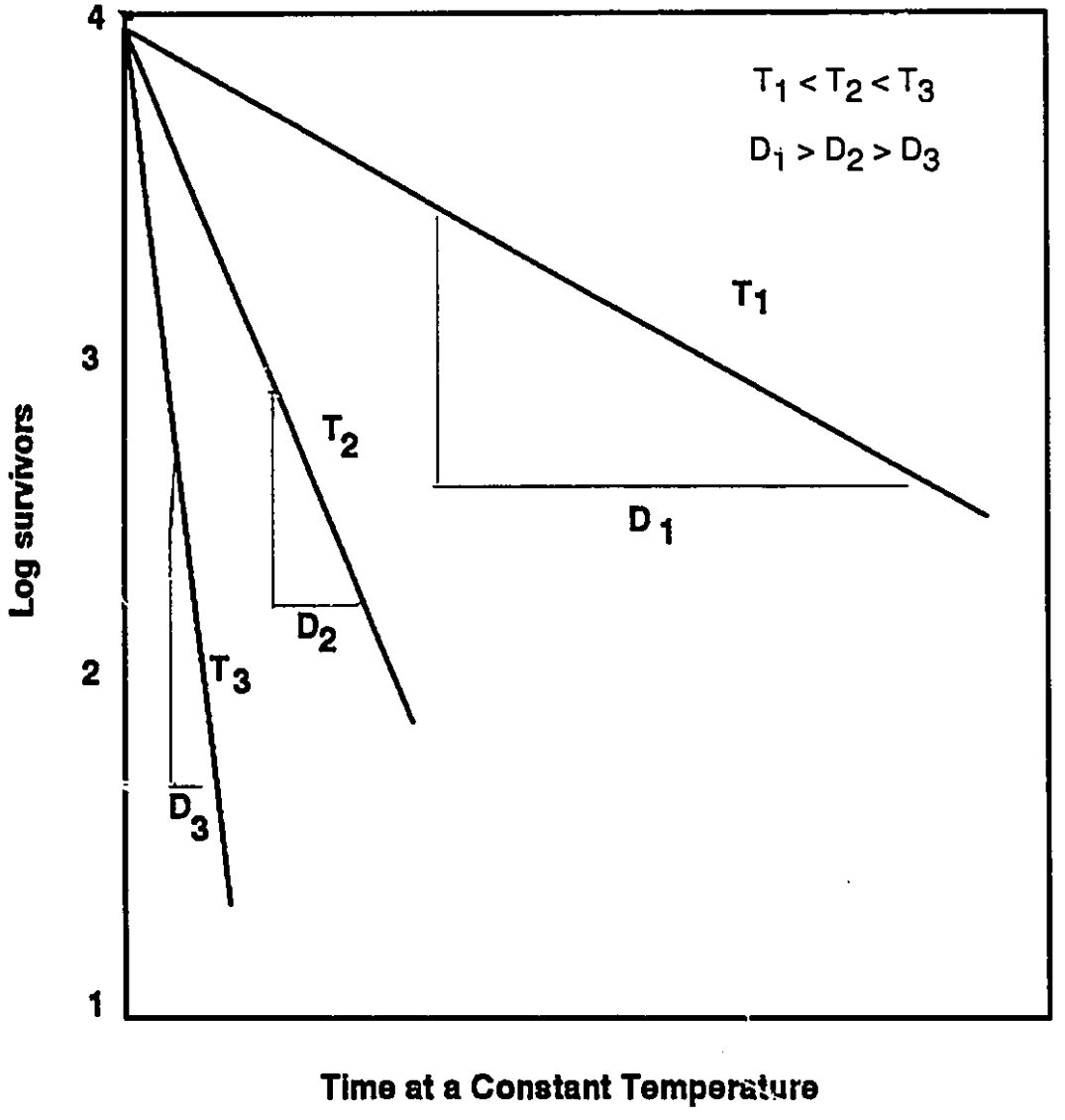


Fig. 7 : A typical Semi-logarithmic plot of a survivor curve

Table 11 : Resistance (D values) of Some Bacteria to Heat

Species	D value (min) at					
	55°C	65°C	66°C	82°C	100°C	120°C
Mesophiles						
S. lactis	2	-	-	-	-	-
E. coli	5	0.1	-	-	-	-
P. aeruginosa		2	-	-	-	-
Salmonella seftenberg	-	1 - 30	-	-	-	-
Salmonella sp (other than *S. seftenberg*)	-	-	0.02 - 0.03	-	-	-
S. aureus	-	-	0.2 - 2.0	-	-	-
C. botulinum - types A & B spores		-	-	-	50	0.1 - 0.2
C. botulinum - type E spores		-	-	0.1 - 3.0	-	-

Adapted from Anon, (1980).

Although, complete destruction of a microbial population is not theoretically

Although, complete destruction of a microbial population is not theoretically feasible with the logarithmic order, a thermal destruction time (TDT) is generally defined to indicate a minimal heating treatment following which no growth occurs. Obiviously, TDT will depend on the initial load of microbial population and the exprimental limit for growth detection. Therefore, it represents a certain multiple of D value which is independent of initial microbial population. Mathematically, D value can be obtained from the following relationship:

$$D = t / (\log a - \log b)$$

where, t = time in min of heating at a constant lethal temperature; a = initial number of viable cells in the population; b = number of viable cells in the population after time, t.

The D value is a function of temperature and mathematically related as shown :

$$D_1 / D_2 = 10^{(T_2 - T_1)/z}$$

The z value is obtained from a a plot of the thermal resistance curve (log D values versus temperature) as the negative reciprocal of the slope. In other words, z represents a temperature range between which the D value (or TDT) changes by a factor of ten.

2.5.1. *Pasteurization*

Pasteurization is a mild heat treatment designed to destroy all the

pathogenic microorganisms and significantly reduce number of vegetative spoilage bacteria. Obviously, target organism is of low thermal resistance. In sous-vide processing, the mild heat treatment or pasteurization applied to the products kills part, but not all, of the microorganism present in the food and consequently the products are further stored under refrigeration conditions to minimize microbial proliferation. The shelf life of sous-vide product is a function of the severity of the applied pasteurization process i.e., magnitude of the total pasteurizing value. Calculation of the total pasteurizing value (Pv) requires information on D and z values and the required number of decimal reductions in the target microbial population.

The time necessary to result in a certain designated number of decimal reductions (n) of a microbial population at a reference temperature (T_o) is called the Minimum Pasteurizing Value (Pm^z_{To}) and is defined by the equation:

$$Pm^z_{To} = n\, D_{To}$$

The total pasteurization value or the Pv value which represents the accumulated lethality effects of the time-temperature treatment. This can be obtained through integration of kinetic parameters of target organism with heat penetration data of the particular food product. The optimization of the cooking process provides for the destruction of certain toxins and bacteria, while preserving organoleptic qualities and minimizing cooking losses.

that at the reference temperature using the following relationship:

$$P_T = P_{To}\, 10^{(T_o - T)/z}$$

The total pasteurizing value of a process can be obtained by summation:

$$Pv^z{}_{To} = \int_o^{tf} 10^{(T_o - T)/z} \, . \, dt$$

between time 0 and t_f, where, "t_f" is the total process time including the cooling.

This equation therefore permits the calculation of the lethality based on the target organism and can be used to ascertain the quality of the treatment. In France, for example, it is legislated that sous-vide products, be processed after packaging at Pv of 100 min at 70°C be exempted from submission of products to the authorities for shelf life studies and could be labelled with a maximum of 21 days shelf life (Lund & Notermans, 1993).

2.5.2. *Heat Penetration*

The use of thermocouples for heat penetration measurements was reported by several authors (Stumbo, 1973). The heating profiles of a product are measured by thermocouples inserted or located at the geometric center or the slowest heating point of the product. The packages are filled and vacuum packaged. The time-temperature data are then gathered through lead wires from the thermocouples attached to a temperature indicator or temperature recording potentiometer. Temperature readings are taken at frequent intervals. Heat penetration parameters such as j_{ch}, f_h, j_{cc}, f_c can be evaluated (Stumbo, 1973)

from the heat pentration data for use in various process calculation procedures.

2.6. Process Evaluation Methods

Process calculation methods are broadly divided into two classes: (i) General methods, and (ii) Formula methods.

The General method for process evaluation, described by Bigelow et al., (1920), integrates the lethal effects of heat treatment by a graphical or numerical integration procedure based on the time-temperature data obtained from test samples processed under actual commercial processing conditions. The time-temperature data gathered are tabulated. From TDT data (using $TDT = 10^{(To - T)/z}$), TDT at the various temperatures are obtained. The reciprocal of TDT defines the lethal rate at the corresponding temperature. From a plot of lethal rate versus time, a lethal rate curve is drawn that integrates the lethal effects of all temperatures during heating and cooling. The area under the curve is equivalent to the sterilization value. A value of unity (or an area of 1) is the minimal requirement with respect to the test organism. For sterilization values greater or lesser than 1, the curve is shifted either to the right or left in order to achieve the desired value.

The new improved General method based on contributions by Ball (1928) and Schultz and Olson (1940), employs a thermal destruction curve as in the case of the General method, to obtain the lethal rate (L) at any particular temperature. The summation of product of lethal rate and heating time interval Δt gives the cumulative (integrated) lethality of the process during the entire heating

and cooling:

$$F_o = \sum L \, \Delta t$$

The formula method makes use of parameters obtained from the heat penetration data together with several mathematical procedures to integrate the lethal effects. Balls formula method uses the equation :

$$B = f_h \log (j_{ch} I_h / g_c)$$

where B is the process time, f_h the heating rate index, j_{ch} the lag factor, I_h the initial temperature difference ($T_r - T_i$), and "g_c" the temperature difference at the end of the cook (i.e., $T_r - T$ at time => B), T_r and Ti are the retort and initial temperatures, respectively. Based on the TDT curve concept , the TDT or F value at the retort temperature (U) is obtained as a product of the desired process lethality (F_o) and F_i, the number of min at T_r equivalent to 1 min at 121.1°C. $F_i = 10^{(121.1 - Tr)/z}$. The cooling lag factor is approximated by an average j_{cc} value of 1.41. A given value of f_h/U has an associated value of g_c for a single value of z, and $T_r - T_w$ and a relationship between f_h/U and g_c is provided in Tables of physical constants.

The Stumbo's formula method is essentially similar to Ball's, but it is more versatile in accounting for the thermal effects of cooling when the cooling lag factor (j_{cc}) differs from 1.41 as assumed by Ball. Both Ball's and Stumbo's methods are widely used in the industry. These methods, although originally developed for establishing commercial sterilization processes, can easily be applied to pasteurization systmes by changing the reference temperature from

121.1°C to a lower value such as 70°C.

2.7. Multiple Barrier Concept in Food Preservation

With regard to control of *C. botulinum*, the most effective method is by heat inactivation. However, in sous-vide processing, the low temperatures used in the process will not achieve this objective.

It is not always practical or desirable to inactivate *C. botulinum* spores to prevent the production of botulinum toxin, since such harsh heating treatment may reduce the sensory quality of foods and increase processing cost. Furthermore, the presence of spores does not imply that an outbreak of botulism. For this to happen, the food must provide the proper environment for germination and growth of, and toxin production by *C. botulinum*. Factors affecting the growth and toxin production by *C botulinum* include a carbon source (such as glucose, fructose, etc.), pH, water activity, presence of CO_2, etc. (Kim & Foegeding, 1992). By appropriately manipulating some or all of these factors, an unfavorable environment could be created to control growth and toxin production by the pathogen.

Assuring the safety of minimally processed, refrigerated foods (MPRF), such as sous-vide products, involves the concept of hurdles, which is the combining of different inhibitory factors that act either individually or synergistically to improve the microbiological safety of the foods (Moberg, 1989; Scott 1989; Liska, 1986). Suitable additional barriers such as water activity, pH, added preservatives such as biological preservatives, oxygen scavengers and

oxygen permeability of packaging films. Examples of the application of additional barriers to control growth of *C. botulinum* in certain processed food products include appropriate combinations of heat treatment, smoking and salt or brine to prevent growth of *C. botulinum* type E in smoked fish, while combinations of a_w and pH have been used to prevent growth of *C. botulinum* type E in caviar at ambient temperatures.

Biopreservatives such as cells of food grade starter culture microorganisms, particularly lactic acid bacteria, are added to food to either make it safe, shelf stable or produce a desirable fermented product. In refrigerated foods, these cells which are mesophilic may indicate temperature abuse, while in semi-preserved refrigerated foods, they act to control spoilage and pathogenic bacteria. At abusive temperatures for example, the mesophilic lactic acid bacteria will grow and produce acid in sufficient amounts to lower the pH so as to prevent growth of the pathogen. Lee et al., (1974) found that addition of viable cells together with irradiation provided much better safety against growth of *C. botulinum* and *S. aureus* in semi-preserved meat products. This inhibitory effect of the added biopreservatives in combination with other barriers has also been demonstrated by other researchers (Christiansen, 1975; Bartholomew & Blumer, 1977; Tanaka et al., 1985).

The beneficial effects of the multiple barrier concept as a preservation technique for controlling botulism were demonstrated in studies with "tsuyu" - a Japanese noodle soup (Imai et al., 1990). These authors showed that the interactive effects of pH (6.5) and NaCl (4%), inhibited growth and toxin

production in the product after inoculation with *C. botulinum* and incubation at 20°C. When pH and NaCl concentration were lowered to 5.0 and 1% respectively, antibotulinal effects were again achieved in the products during storage at 30°C (Imai et al., 1990)

2.8. Rationale and Objectives of Study

In traditional refrigerated foods, spoilage can serve as a safety factor to signal temperature abuse, thus preventing the consumption of food products which might not only be spoiled, but be of public health concern. Many of the traditional refrigerated foods have backup preservation systems, e.g., nitrite in cured and cooked meats, without which they will spoil and be a potential hazard to the consumer. Also, consumers in general, are familiar with methods of handling these foods safely, i.e., refrigeration. In contrast, sous-vide products are minimally processed products with very little or no preservatives. Furthermore, the heat treatment does not inactivate thermoduric spoilage or pathogenic microbial cells and spores that can grow at normal refrigeration temperatures, rather the sous-vide process has potential to destroy cells of vegetative spoilage microorganisms which might otherwise warn consumers.

Therefore, the question to be addressed here is whether pathogens, especially *C. botulinum* will proliferate in these "New Generation" refrigerated foods (e.g., sous-vide), under conditions of temperature abuse.

The proteolytic strains of *C. botulinum* require retort temperatures for destruction. In sous-vide processing, heating at retort temperatures to destroy

proteolytic spores is not desirable since this will mean sterilizing the product to result in possible destruction of heat labile essential nutrients.

Since (i) there are no guarantees that the product would be heated sufficiently to destroy heat labile toxins present in the product, and (ii) there is high potential for consumers to abuse, mishandle and over-extend the products shelf stability limits, refrigeration alone is not sufficient as a means to inhibit the growth and activity of pathogenic microorganisms in sous-vide foods, in order to eliminate the hazard of botulinum poisoning in these products.

Also, because of the difficulty in ensuring the stipulated temperature control (3 - 5°C), it has been recommended that additional inhibitory factors (such as reduced a_w, pH, added preservatives, etc.), be incorporated into minimally processed refrigerated foods so that their safety in relation to *C. botulinum* is not dependent solely on refrigeration (Conner et al., 1989; Gagnon, 1990). Since proper refrigeration of sous-vide products at 0 to 3°C to limit the growth of *C. botulinum* cannot be assured throughout the food distribution chain, there is justifiable concern that the wide temperature fluctuations in supermarket and household refrigerators could enable spores of *C. botulinum* which survive the minimal heat processing treatment used for sous-vide processing, to grow and produce their lethal toxins.

Thus far, little or no meaningful studies aimed at elucidating the microbial safety of food products formulated with this technology have been carried out, and there is real concern regarding the (im)proper application of the technology and its implications to public safety in the hands of a manufacturer improperly

trained in the technology. There is also the need for further research in order to establish the heat treatments needed to achieve specified lethality for spores of the non-proteolytic strains of *C. botulinum* in order to obviate the likelihood of applying excessive heat treatments which could cause other undesirable changes in sous-vide type food products.

Thus, the objectives of the study were :

(a) to determine the "Decimal Reduction Time" of a reference microorganism (*S. faecium*) at selected pasteurization temperatures used in sous-vide processing ;

(b) to determine "Pasteurization Values" of sous-vide processed meat and fish products to ensure total reduction of non-spore forming pathogens ;

(c) to monitor the physical, chemical, microbiological and sensory changes that occur during storage of sous-vide meat and fish products ;

(d) to investigate the public health safety of sous-vide products through "challenge studies" with *C. botulinum* type A, B and E spores at various storage temperatures ; &

(e) to incorporate additional barriers with sous-vide processing in order to verify their ability to improve safety and extend shelf life of sous-vide products.

CHAPTER 3

THERMAL RESISTANCE OF *STREPTOCOCCUS FAECIUM* AS INFLUENCED BY pH AND SALT

3.1. Summary

Decimal reduction times (D-value) of *Streptococcus faecium* in brain heart infusion (BHI) broth were evaluated at selected temperatures (60 - 75^{o}C), pH (5 - 8) and NaCl concentrations (0.5 - 10%). The D values were influenced by all three factors. Acidic conditions (pH 5 & 6) gave lower D values than their neutral/basic counterparts. Higher NaCl concentrations generally resulted in higher D values. The z-values within the temperature studied (60 & 75^{o}C) varied slightly according to pH and NaCl content of test medium, ranging from 8.3 to 10.3C^{o}.

3.2. Introduction

The heat treatment applied to foods should only be as severe as is necessary to free the food from undesirable microorganisms since the nutritive and sensory properties of the food are aiso adversely affected. Increasing consumer demand for convenience foods with extended shelf-life and closer to fresh characteristics have resulted in a new type of vacuum-packed (*sous-vide*) precooked foods that are marketed under refrigerated conditions (Raffael, 1985; Light et al., 1988; Schafheitle & Light, 1989). In practice, a *sous-vide* product is prepared from high quality ingredients which are precooked (if necessary), vacuum packaged in high-barrier plastic containers, pasteurized/cooked, cooled

to and stored/marketed at < 4^{o}C.

The pasteurizing conditions are designed to destroy the non-spore forming pathogenic bacteria which may be present in raw ingredients used in sous-vide products. Indicator organisms have been generally *E. coli, Salmonella, Aeromonas, Listeria* and *Yersinia* species (Wyatt & Guy, 1980; Palumbo, 1986). *Streptococcus faecium* is a thermoduric enterococcus microorganism which has also been implicated as a potential spoilage type (Ingram & Barnes, 1955; Brown et al., 1960; Drake et al., 1960; Houben, 1980 & 1982). This organisms is most likely to survive the mild pasteurization heat treatment given to some foods and withstand the presence of salt and nitrite at normal usage levels (Gardner & Patton, 1975; Houben, 1980; Bell & Delacy, 1984).

The objective of this study was to determine thermal resistance of *S. faecium* over a range of temperatures (60 - 75^{o}C) in brain heart infusion (BHI) broth as influenced by pH (5, 6, 7 & 8) and sodium chloride concentration (0.5 - 10%). Conditions selected cover a broad range of pasteurization temperatures, pH conditions and NaCl concentrations encountered in typical *sous-vide* products, such that gathered data may be useful in establishing pasteurization procedures.

3.3. Materials & Methods

3.3.1. *Media Preparation and Inoculation*

S. faecium (ATCC 19432) was obtained from the Dept of Microbiology &

Immunology, McGill University, and stock cultures were maintained on brain heart infusion (BHI) agar slants (DIFCO). *S. faecium*, a facultatively anaerobic, Lancefield group D organism was chosen as the target organism for the study because of its reported high thermal resistance (Magnus et al., 1988; Banwart, 1989) and occurrence as a contaminant in the raw ingredients used in various food processes. For heat resistance study, these were subcultured in brain heart infusion (BHI) broth, incubated at 37^{o}C for 24 h and stored refrigerated at 4^{o}C. BHI broth was used because thermal resistance reported for this organism was higher in BHI broth than in conventional phosphate buffer (Greenberg & Silliker, 1961; Bell & Delacy, 1984; Gardner & Patton, 1975; Brown et al., 1960). Also, the broth resembles a food system more closely than a buffer system.

Glass vial (10 mL capacity) with screw caps containing 0.9 mL of the BHI broth were inoculated with 0.1 mL of stock culture of *S. faecium* and incubated at 37^{o}C for 24 h to obtain maximum cell concentration. Initial cell concentration was evaluated by serial dilution in BHI broth and surface plating on KF *Streptococcus* agar (abbreviated hereafter as KF-agar) and m-*Enterococcus* agar (abbreviated hereafter as mE-agar) followed by incubation under aerobic condition at 35^{o}C for 48 h. KF-agar and mE-agar were used to maximize the number of surviving microorganism (Pagel & Hardy, 1980). Both media were selective differential media for the organism and employ sodium azide as the chief selective agent and triphenyltetrazolium chloride for differential purposes.

3.3.2. *Determination of Thermal Resistance*

Prepared glass vials were placed in a temperature controlled water bath (Haake, Berlin, Germany) and heated for various times at selected temperatures (60, 65, 70 & 75^{o}C). Volume of the menstrum was kept small (1 mL) in order to promote rapid heating conditions (to minimize the come-up lag) and to allow a more accurate determination of the heat resistance. Vials were withdrawn at selected time intervals, cooled on ice and *S. faecium* enumerated as outlined before. The bath temperature as well as the internal broth temperature in three vials were recorded during the thermal treatment. Temperature stability in the bath during the runs was $\pm$ 0.5^{o}C from the set point. The temperature lag in the broth was below 30 s due to small sample volumes employed. No corrections were made for this lag. However, in order to avoid possible errors due to the come-up lag, only data from thermal treatments beyond 30 s were used in the computation of decimal reduction times (D values).

3.3.3. *Calculation of D and z-values*

The D value (time required to destroy a given microbial population by 90%) of *S. faecium* at each temperature was determined from a plot of logarithm of microbial survivors vs time. To normalize data handling, the initial microbial concentration was adjusted in each run by multiplying by a factor to give an initial count of 10^{8} CFU/mL. The same factor was used to normalize the concentration of surviving population following heating for various times in a given run. Such normalization should not have any influence on the evaluated D values since D

values are independent of initial concentration (Stumbo, 1973). D values were obtained by regression of logarithm of survivors vs time (t > 30 s) as negative reciprocal slopes. Thermal resistance curves were established by plotting logarithm of D values vs temperature and the z-value was obtained as the negative reciprocal slope of the thermal resistance curve by regression.

The effect of pH on the thermal resistance of *S. faecium* was evaluated in the BHI broth adjusted to pH 5 - 6 with 1 N HCl and pH 8 with 1 N NaOH. The effect of NaCl concentration (2.5 - 10%) was evaluated at pH 5 & pH 7 by adding appropriate amounts of NaCl to the broth whose initial NaCl concentration was 0.5%. The actual NaCl concentration in each broth was adjusted using a Horiba compact NaCl meter Model C-121 (Horiba Instruments Inc., Irvine, calif. U.S.A.) previously calibrated with 1 and 5% standard NaCl solutions. The NaCl concentrations were accurate to within $\pm$ 0.1% of the required level. These adjustments of the broth were made prior to inoculation.

3.3.4. *Random Verification of Thermal Kinetics*

For verification of the thermal kinetics, a small volume (1 mL) *of S. faecium* containing 10^7 - 10^8 CFU/mL in BHI broth (pH 5 or pH 7) was placed in a glass vial and heated in a water bath to a predetermined time (5 times the decimal reduction time at the selected temperature) followed by rapid cooling in ice water. An extra 30 s was added to the heating time to compensate for the temperature lag. The survivor counts were determined as described before, and the number of decimal reductions were determined using : $n = \log (N_0/N)$, where

N_o and N were the microbial numbers before and after the heat treatment.

3.4. Results and Discussion

3.4.1. *Survivor Curves and D values*

Survivor curves of *S. faecium* at pH 7 in BHI broth (control - 0.5% NaCl) at various temperatures characteristically demonstrated first order destruction rates (Fig. 8). The r^2 values obtained from the regression of logarithm of survivors vs heating time (>30 s) were generally high (the minimum r^2 observed was 0.96 for this condition, Table 12). The D values obtained from the survivor curves ranged from 24.1 min at 60°C to 0.6 min at 75°C. These observations were similar to those reported by Rosset et al., (1986) and Magnus et al., (1988) for a Lancefield group D organism. The two agar media employed for plating (KF-agar and mE-agar) showed some small statistically nonsignificant ($p > 0.05$) variations with reference to the D values (Table 12). Logarithmic order of destruction ($r^2 > 0.90$) was also observed at pH 5, but the relative magnitudes of D values were considerably lower (Table 12) ranging from 15.7 - 18.1 min at 60°C to 0.48 - 0.54 min at 75°C. These two pH levels (pH 5 and 7) were employed as controls because they roughly corresponded to the pH levels of two laboratory formulated *sous-vide* products (spaghetti and meat sauce, pH 5 and rice and salmon sauce, pH 7). Table 12 also gives the D values at various temperatures as influenced by a NaCl concentration of 2.5% (perhaps the maximum tolerable in food products). At the 2.5% level, the influence of NaCl on D values was insignificant (Table 12).

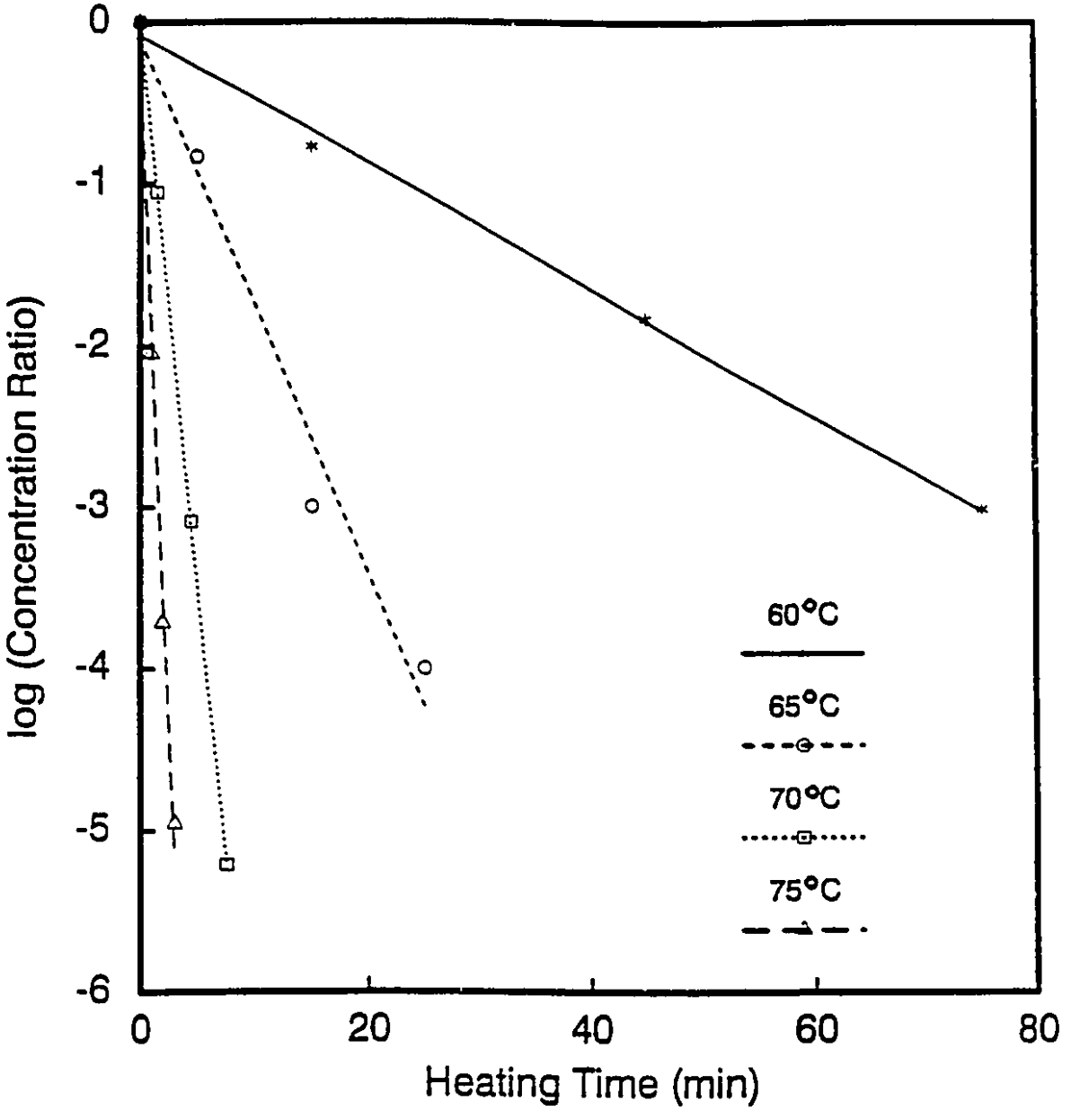


Fig. 8 : Survivor curves of *Streptococcus faecium* at selected temperatures

Table 12 : D values of *S. faecium* as Influenced by Temperature, pH and Sodium chloride Concentration

Temp °C	Decimal Reduction Time					
	KF-agar[1]			mE-agar[2]		
	Mean min	s.d. min	Min r^2	Mean min	s.d. min(n=6)	Min r^2
pH 7, 0.5% NaCl						
60	24.1	0.50	0.99	22.1	0.68	0.97
65	5.59	0.32	0.97	5.59	0.21	0.95
70	1.45	0.08	0.99	1.81	0.37	0.99
75	0.60	0.08	0.96	0.76	0.08	0.96
pH 5, 0.5% NaCl						
60	18.1	0.05	0.99	15.7	0.50	0.97
65	5.48	0.20	0.99	5.14	0.60	0.96
70	1.19	0.15	0.98	1.19	0.16	0.92
75	0.54	0.02	0.92	0.48	0.02	0.90
pH 7, 2.5% NaCl						
60	26.1	1.50	0.97	27.6	0.11	0.95
65	5.33	1.03	0.96	6.32	0.67	0.98
70	0.89	0.04	0.94	0.95	0.08	0.97
75	0.57	0.04	0.87	0.52	0.04	0.87
pH 5, 2.5% NaCl						
60	18.1	0.80	0.86	16.5	1.40	0.87
65	4.02	0.33	0.89	3.48	0.16	0.88
70	1.08	0.02	0.97	1.08	0.01	0.97
75	0.39	0.09	0.91	0.39	0.11	0.90

[1] enumerated on KF *Streptococcus* agar ; [2] enumerated on m *Enterococcus* agar

3.4.2. *Thermal Resistance Curve and z Values*

Figure 9 is a semi-logarithmic plot of decimal reduction time vs temperature of *S. faecium* at pH 7 and pH 5 (BHI broth control) with KF-agar medium. The individual z values obtained by regression were 9.5 and 9.7C^{o} with the KF-agar and mE-agar, respectively, at pH 7. The corresponding values at pH 5 were 9.3 and 10.3C^{o}, respectively. At 2.5% NaCl concentration, the z values were 8.6 and 8.3C^{o} at pH 7 and 9.1 and 9.3C^{o} at pH 5, respectively with the KF and mE media. Thus 2.5% NaCl concentration appeared to slightly increase the sensitivity of the microorganism to changes in temperature. Again the results show that the two media were somewhat comparable in performance.

3.4.3. *Effect of pH on D Values*

Additional experiments were carried out at 60^{o}C to further test the effect of pH and NaCl concentration on the decimal reduction times. For this analysis, results from KF-agar and mE-agar plates were pooled since the two media were not significantly different in their ability to support the growth of *S. faecium* for enumeration. The effect of pH on the thermal resistance of *S. faecium* at 60^{o}C is shown in Table 13. Between pH 5 - 6, the D values varied between 14 and 17 min. On the other hand, D values at the higher pH values (pH 7 - 8) were considerably higher (23 - 25 min). These observations are in general agreement with previous findings made by Pflug and Holcomb (1983) who reported that microorganisms generally exhibit an increase in thermal resistance at neutral or slightly alkaline pH conditions.

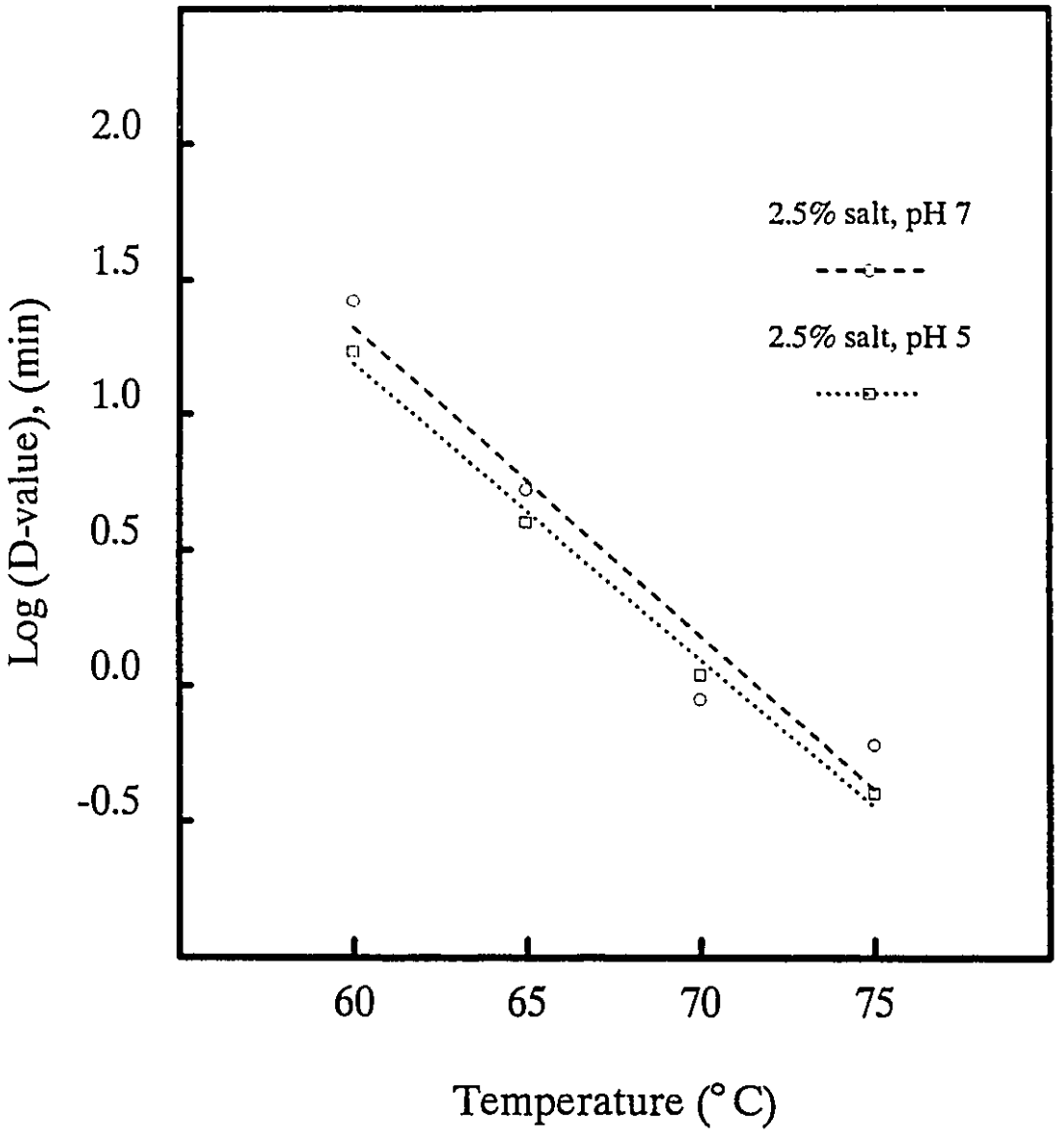


Fig. 9 : D value curves of *Streptococcus faecium* at pH 5 and 7

Table 13 : Mean D values of *S. faecium* and their Standard Deviations at 60°C as Influenced by pH (n = 12)

pH	D-values (min)		
	Mean	s.d.	Min r^2
5	16.9	0.8	0.99
6	13.9	1.9	0.97
7	23.1	0.7	0.96
8	25.1	2.1	0.99

s.d. => standard deviation

Although the mechanism of protection is unknown, it is surmised that solutes can reduce the water activity in the environment immediately surrounding the cells (similar to the effect of drying) thereby increasing the heat resistance of the organism and allow it to survive at normally inhibitory temperatures (Greenberg & Silliker, 1961).

3.4.4. *Effect of Sodium chloride Concentration*

Table 14 summarizes the effect of sodium chloride (salt) on the D values of *S. faecium* at both pH 5 and pH 7 at a temperature of 60^{0}C. The addition of salt, especially at concentrations of 7.5% and above, affected the thermal resistance at both pH 5 and pH 7. For example, at pH 7 the D values which ranged from 20 - 28 min up to a salt concentration of 5%, increased to 26 - 36 min at 7.5 - 10% salt concentration. Similar trends were observed in broth adjusted to pH 5.0 (16 - 17 min at $\leq$ 5% and 20 - 28 min at $\geq$ 7.5% salt). These observations are consistent with published results (Pflug & Holcomb, 1983; Bell & Delacy, 1984). The combination of pH, salt and processing temperature had pronounced effects on the thermal stability of *S. faecium* in the BHI broth. The D values ranged from a high of 35.3 min at 60^{0}C (mE-agar, pH 7, 10% salt) to a low of 0.39 min at 75^{0}C (mE-agar, 2.5% salt, pH 5).

Table 14 : Mean D-values and their Standard Deviations of *S. faecium* at 60°C (pH 7 & 5) as Influenced by Salt Concentration (n = 12)

Salt Concentration (%)	Decimal Reduction Time					
	pH 7.0			pH 5.0		
	Mean (min)	s.d. (min)	Min r^2	Mean (min)	s.d. (min)	Min r^2
0.5	23.1	0.6	0.97	16.9	0.8	0.97
2.5	26.8	0.9	0.95	16.7	1.7	0.86
5.0	20.0	0.7	0.98	16.7	3.0	0.98
7.5	26.7	3.4	0.94	20.2	3.1	0.91
10.0	33.9	3.4	0.91	27.5	0.2	0.99

s.d. => standard deviation

3.4.5. *Random Verification*

The number of decimal reductions following heating equivalent to a 5D process at 65 and 75^{o}C at pH 5 and 7 are shown in Table 15. Based on the average values of kinetic data, 5D values of *S. faecium* were calculated as 27.4 and 2.6 min, respectively at 65 and 75^{o}C at pH 5. The corresponding values at pH 7 were 27.9 and 3.4 min, respectively. The resulting decimal reductions varied from 5.5 to 6.0 at 65^{o}C and 5.4 to 6.1 at 75^{o}C, which was considered to be in reasonable agreement with the 5D process (with some deviation on the conservative side). The contribution of the additional 30 s provided to compensate for the temperature lag would have some influence, especially at the higher temperatures. Further, some discrepancy may have been due to the fact that D values in this random verification were calculated from the initial count and count reduction following a single heating time (unlike kinetic studies where initial values were not included).

3.5. Conclusion

The study has shown that the thermal resistance of *S. faecium* was dependent on pH, salt and processing temperature. Data obtained in this study could be used for predicting the time/temperature required to process various *sous-vide* products to ensure destruction of less heat resistant non-spore forming bacteria of public health concern.

Table 15 : Verification of Destruction Kinetics for a 5D Process at 65°C and 75°C (n=4)

Temp. (°C)	Heating Time (min)	pH	No. Decimal Reductions KF-agar[1]	mE-agar[2]
65	27.9	5	5.5±0.8	5.6±0.3
65	28.4	7	5.7±0.6	6.0±0.1
75	3.1	5	6.1±0.4	5.5±0.3
75	4.1	7	5.4±0.1	5.8±0.2

[1] KF *Streptococcus* agar plating ; [2] m*Enterococcus* agar plating

CHAPTER 4

ESTABLISHMENT OF PASTEURIZATION VALUES FOR SOUS-VIDE SPAGHETTI & MEAT SAUCE AND RICE & SALMON BASED ON KINETICS OF *S. FAECIUM*

4.1. Summary

Heat penetration data were obtained for two formulated sous-vide products (i.e., spaghetti and meat sauce; & rice and salmon) to establish 5D and 13D pasteurization processes based on the thermal kinetics of *Streptococcus faecium*. A numerical integration technique (General Method) was used to obtain pasteurization times from the heat penetration data. Stumbo's formula method was also used for obtaining pasteurization times based on gathered heat penetration data. Process times in the temperature range, 60 - 75°C, calculated from the two methods were generally comparable for both products.

4.2. Introduction

Sous-vide processing involves the packaging of partially cooked meal components in hermetically sealed barrier bags from which air has been removed. The products are then cooked or pasteurized to a desired pasteurized value (Pv_o). The products are then rapidly chilled and stored either at 0 to 3°C, or distributed under refrigeration (Beauchemin, 1990). General pasteurization principles related to container size, initial bacteria load, cooling, and bacterial survivors apply equally well to sous-vide foods (Hackney et al., 1991).

While some companies have the technology, quality assurance programs and trained personnel to ensure adequate processing and compliance with regulatory specifications at all stages of processing, handling and distribution, other companies do not. In the U.S., the FDA has limited the production of sous-vide products to approved food processing operations and currently does not allow production at retail establishments, such as grocery stores.

Recently, there have been concerns by regulatory agencies, both nationally and internationally, about the public health safety of such minimally processed sous-vide products. This concern is justified in view of the ability of many food-borne psychrotrophic pathogens to grow at temperatures between 3 and 12^{o}C, particularly *C. botulinum* (Eklund et al., 1988).

The pasteurization times for sous-vide products should be kept to a minimum in order to maintain maximum quality, at the same time it should be an effective means of rendering the product free from non-spore forming vegetative organisms. The time-temperature treatment is designed such that the coldest point of the product (center) receives the designated pasteurization value (Pv) to ensure a certain number of decimal reductions in the target organism. In France, *S. faecalis* is the selected target microorganism due to its high thermal resistance (Gagnon, 1990). As a consequence, pasteurization values represent the minimum time (min) at a specific temperature required for the product's coldest point to bring about the 13 - 14 decimal reductions of the target microorganism.

The objective of the study was to evaluate and compare process times obtained using the General and Stumbo's formula methods based on kinetics of *S. faecium* and heat penetration parameters of two sous-vide products.

4.3. Materials and Methods

4.3.1. *Preparation of Sous-Vide Products*

Two formulated sous-vide products were prepared as follows : a spaghetti and meat sauce product, and a rice and salmon product, weighing 375 g each. All ingredients were obtained from a local supermarket. The spaghetti and meat sauce was prepared by weighing 200 g of pre-cooked spaghetti with 175 g meat sauce into thermoformed oriented polypropylene, OPP, (Modern Plastic Inc, Montréal, Canada) rectangular test trays (8cm x 12cm x 3 cm). The containers were vacuum packaged using a vacuum packaging system (Trigon Model # RM331, MMIS Incorporated, Ontario, Canada) and heat sealed with a top film of OPP. The rice and salmon product was prepared by weighing 200 g of pre-cooked rice with equal weights (87.5 g) of canned pink salmon and canned white gravy. The products were then packaged as described previously for spaghetti and meat sauce. Several packages (~60) of the two products were prepared and kept refrigerated at $2^{o}C$ until needed.

4.3.2. *Evaluation of Heat Penetration Parameters of Sous-Vide Products*

For the heat penetration studies, several test trays were prepared by installing stainless steel needle type copper/constantan thermocouples (Ecklund-Harrison Technologies, Inc., Fort Myers, FL), such that the sensitive tip was located at the geometric center of the test tray. The trays were then filled with the sous-vide product and vacuum packaged as described previously. The test trays

were then heated in a temperature controlled water bath maintained at selected temperatures between 60 and 75^{0}C for 60 min. Following heating, samples were cooled immediately in a cold water bath (~ 5 - 10^{0}C) to below 25^{0}C. The internal temperatures of three packages during the entire heating and cooling processes were measured at 30 s intervals using a Molytek data logger (Model 3702, Molytek Inc., Pittsburgh, PA).

The time-temperature data gathered were used to evaluate heat penetration parameters such as heating rate index (f_h), cooling rate index (f_c), heating rate lag factor (j_{ch}), and cooling rate lag factor (j_{cc}). The come-up time used was zero since the water bath was maintained at the required temperature prior to its use.

4.3.3. *Estimation of Thermal Kinetics Data (D and z Values) of S. faecium*

The thermal resistance of *S. faecium* in brain heart infusion broth was determined as described in Chapter 3. The pH of the broth was adjusted to 5 and 7, to represent the pH of the spaghetti and meat sauce and rice and salmon products respectively. The D value of the organism at each temperature (60 - 75^{0}C) was obtained from a linear regression of the survival curve (Stumbo, 1973). The temperature sensitivity (z value) of the organism was determined as the negative reciprocal slope of thermal death time (TDT) curve plotted as logarithm of D value versus temperature. Pasteurization times were established for a 5D and 13D process.

4.3.4. *Determination of Pasteurization Values / Times of the Processed Sous-Vide Products*

Process times were calculated based on evaluated heat penetration parameters (section 4.3.2.), and processing conditions using Stumbo's method (Stumbo, 1973). Stumbo's formula method was chosen since it has been reported to produce results similar to those of the General method (Smith & Tung, 1982 ; Ghazala et al., 1991). The process times were also evaluated by numerical integration of the partial pasteurization values (Improved General method) using the time-temperature data gathered for the products under test conditions. For the General method, the Pasteurization rate (similar to Lethal rate) corresponding to each temperature was obtained as $10^{(T-70)/z}$ employing the z of *S. faecium* of 9.54C^{o} (pH 5) and a reference TDT of 1 min at 70^{o}C (Patashnik, 1953). The pasteurization rate was then multiplied by the time interval to obtain the partial pasteurization values which, when summed up or integrated numerically, gave the total pasteurization value. This was then adjusted by a trial and error technique to determine Pateurization times to achieve 5D and 13D Pasteurization processes. Percentage deviation of t_{Stumbo} from $t_{General}$ was calculated as : Error (%) = $(t_{Stumbo} - t_{General})/t_{General} \times 100\%$. Positive error associated with Stumbo's formula method indicated possible overprocessing result while negative error implied the contrary.

4.4. Results and Discussion

4.4.1. *Heat Penetration Parameters for Sous-Vide Products*

The spaghetti and meat sauce and the rice and salmon sous-vide products showed a characteristically linear conduction heating behavior during the heat processing. The evaluated heat penetration parameters are as shown in Table 16. The observed variability in the f_h and f_c values (~10%) was considered normal due to variations in product consistency.

4.4.2. Pasteurization Values/Times of the Processed Sous-Vide Products

The following kinetic data were obtained for *S. faecium* in the brain heart infusion broth : D values at 60, 65 70 and 75^{o}C, were 18.1, 5.48, 1.19 and 0.54 min at pH 5 and 24.1, 5.59, 1.45 and 0.60 min at pH 7, respectively (Table 12, Chapter 3). The pasteurization value (Pv), calculated for 13D of *S. faecium* at 60, 65, 70 and 75^{o}C based on the above kinetic data are summarized in Table 17. The Pv equivalents to 13D reduction were 71 min at 65^{o}C, and 7 min at 75^{o}C for the spaghetti and meat sauce product. The corresponding values for the rice and salmon products were 72.7 min at 65^{o}C and 7.8 min at 75^{o}C, respectively. The Pv equivalent to 5D reduction for both spaghetti and sauce, and rice and salmon using the General method are as shown in Table 18.

Table 16 : Heat Penetration Parameters for the Processed Sous-Vide Products

Product	Parameter	Mean Value	S.D.* n=6
Spaghetti and meat sauce			
	Heating rate index (f_h), min	27.44	3.52
	Cooling rate index (f_c), min	29.88	2.90
	Heating rate lag factor (j_{ch})	0.98	0.04
	Cooling rate lag factor (j_{cc})	1.09	0.04
Rice and salmon			
	Heating rate index (f_h), min	29.32	2.33
	Cooling rate index (f_c), min	32.72	3.29
	Heating rate lag factor (j_{ch})	1.02	0.04
	Cooling rate lag factor (j_{cc})	1.07	0.06

* => standard deviation

Table 17 : Comparison of Process Times Evaluated by both the General and Stumbo's Formula Methods for 13D Process

Product	Temperature (Pv)		Process time (min)		Error (%)
	(^{o}C)	(min)	Stumbo's method	General method	
Spaghetti & meat sauce					
	65	71.0	104.2	105.7	-1.42
	70	15.5	49.8	50.1	-0.59
	75	7.0	39.1	39.3	-0.51
Rice and salmon					
	65	72.7	109.6	110.1	-0.45
	70	18.9	56.0	56.2	-0.36
	75	7.8	42.5	42.6	-0.24

Table 18 : Comparison of Process Times Evaluated by the General Method for 5D Process

Product	Temperature (^{o}C)	(Pv) (min)	Process time (min)	S.D
Spaghetti & meat sauce				
	60	90.5	127.0	2.21
	65	27.4	71.0	1.60
	70	5.95	42.0	0.02
	75	2.70	36.3	0.10
Rice and salmon				
	60	120.25	161.0	1.43
	65	27.97	57.7	2.14
	70	7.25	44.3	0.02
	75	3.01	38.3	0.02

S.D. => standard deviation

The process times obtained by both Stumbo's formula and the General methods are shown in Table 17. Pasteurization at 60^{o}C to achieve a 13D process was not practical due to the long process times (> 5 h) involved, and hence not included in Table 17. The process times obtained by Stumbo's method were comparable to that of the General method indicating that the traditional process calculation method is applicable to the pasteurization of the sous-vide products. This observation is in agreement with that of Ghazala et al., (1991), who evaluated different formula methods including Stumbo's for process time calculations involving packaged foods in thin profile forms.

4.5. Conclusion

Since accurate process time calculations for sous-vide products can be performed using the formula method which is less time consuming than the General method, the formula method can therefore be used to establish processing schedules for sous-vide products.

CHAPTER 5

STORAGE STUDIES ON A SOUS-VIDE SPAGHETTI AND MEAT SAUCE PRODUCT

5.1. Summary

Shelf life studies were done on a minimally processed sous-vide spaghetti and meat sauce product subjected to a process for *Streptococcus faecium* sufficient to achieve 5D and 13D heat processing at 65°C or 75°C, then stored at refrigeration (5°C) or under mild temperature abuse storage conditions (15°C). Products stored at 5°C had a shelf life of $\geq$35 days irrespective of the processing treatment. However, for products stored at 15°C, packages were visibly swollen due to additional carbon dioxide production after 14 to 21 days depending on the severity of the heat processing treatment and the product had a distinct fruity odor when opened. Initial spoilage microorganisms consisted predominantly of *Bacillus* species. However, after 7 - 12 days at 15°C, the spoilage pattern changed with lactic acid bacteria accounting for almost all of the spoilage microorganisms. As lactic acid bacteria increased in numbers, there was a concomitant increase in carbon dioxide and lactic acid production and a drop in pH of the product. This study has shown that physical, chemical and microbiological changes can be useful indicators to demonstrate temperature abuse of a minimally processed pasta product.

5.2. Introduction

The increase in consumer demands for packaged minimally processed refrigerated convenience foods with closer to fresh characteristics has led to a tremendous growth in the use of *sous-vide* processing technology to extend the shelf life and keeping quality of fresh foods (Light & Walker, 1990a,b; Smith et al., 1990). In *sous-vide* processing, products are prepared from top quality raw ingredients and precooked (if necessary), packaged under vacuum in heat-stable air-impermeable trays, sealed and cooked/pasteurized at pre-established time/temperature combinations, and cooled immediately to 4^{o}C. The products are then stored and distributed under refrigeration to inhibit the growth of heat resistant spoilage and pathogenic microorganisms (Raffael, 1984 & 1985; Light et al., 1988). Produced under good manufacturing practices, *sous-vide* processed products have a shelf life of 20 to 30 days (Schafheitle & Light, 1989; Young et al., 1988). In addition to sealing in natural juices of the food, the high barrier film used in packaging *sous-vide* products prevents oxidative rancidity as well as any post-process contamination of the product. However, in spite of these advantages, commercial application of *sous-vide* technology in North America is limited due to public health safety concerns (Shamsuzzaman et al., 1992). These concerns are justified in view of : (i) the fact that *sous-vide* products undergo minimal heat processing and are therefore not "commercially sterile", (ii) the ability of pathogenic spore and heat resistant non-spore formers to withstand the pasteurization processing treatment, (iii) the anaerobic conditions in the product which may be conducive to the growth of pathogens, and (iv) the potential for temperature abuse. However, there is a paucity of information on the physical,

chemical and microbiological changes occurring in *minimally processed sous-vide* products throughout their shelf life storage period. This information would be useful in rationalizing proper handling procedures and determining appropriate indicators of microbiological safety to address public health and safety concerns about these products.

Therefore, the objectives of this present study were to monitor the physical, chemical, microbiological, and sensory changes occurring in a minimally processed spaghetti and meat sauce product stored at mild temperature abuse storage conditions.

5.3. Materials and Methods

5.3.1. *Preparation of Sous-Vide Product*

The product used in the study was a spaghetti and meat sauce product. All ingredients used in the product formulation were obtained from a local supermarket and packaged by weighing 200 g of pre-cooked spaghetti with 175 g of meat sauce into a thermoformed oriented polypropylene (OPP) tray (8 cm x 12 cm x 3 cm) as described in section 4.3.1. This was then vacuum packaged (950 mbar) with a top film of Wallo peel (polyester/polyamide/EVOH, O_2 transmission rate, 12 cc/m^2 atm/day @ 23°C) using a vacuum skin/thermoforming packaging system (Trigon Model # RM331 MMIS Incorporated, Toronto, Ontario, Canada).

5.3.2. *Proximate Analysis*

The formulated spaghetti and meat sauce product and a commercially prepared fettuccini (brand name) product were analyzed for their proximate composition, water activity, pH and salt content.

Proximate composition of products, i.e., moisture, ash, crude protein and crude fat were determined using standard AOAC methods (A.O.A.C. 1980). Carbohydrate was determined by difference. For the crude protein determination, a conversion factor of 5.7 (cereal products factor) was used to calculate percent protein from total nitrogen.

Water activity (a_w) was measured using a Decagon Model CX-II water activity meter calibrated with a saturated NaCl solution (a_w ~0.75). All measurements were made at 25^{0}C using a temperature controlled water bath, (Decagon Devices Inc., Pullman, WA).

Measurement of pH was done by inserting a previously calibrated pH probe (Corning pH meter, model 240, Canlab, Montréal, Québec, Canada) directly into each product. Sodium chloride concentration was determined by the method of Hackney and Dicharry (1988). One gram of sample was added to 20 mL of distilled water, mixed and titrated with silver nitrate (0.171 N) using dichlorofluorescein as indicator. The NaCl concentration was also measured using a Horiba compact salt meter (Horiba Instrument Inc., Irvine, CA. U.S.A.), previously calibrated with a 1% and 5% standard NaCl solution for comparison with the titration method. Since the results of the two measurements showed a

significant correlation, subsequent measurements of salt were carried out using the salt meter method only. All measurements for proximate analysis, water activity, pH and salt content were done on triplicate samples.

5.3.3. *Thermal Processing of Samples*

The prepared samples were heated at 65°C and 75°C for a specific time equivalent to either a 5D or a 13D reduction in the number of *S. faecium* according to procedures described by Simpson et al., (1993a). The thermally processed products were then immediately cooled under running tap water for 30 min and stored in a low temperature incubator (Fisher Scientific, Model 307, Montréal, Canada) maintained at either 5°C or 15°C. Samples stored at 5°C were analyzed after 0, 18 and 35 days, and those stored at 15°C were examined after 0, 7, 14, 21, 28 and 35 days for physical, chemical and microbiological changes.

5.3.4. *Physical and Chemical Analyses*

At day 0 and after each storage interval, samples were analyzed for changes in pH, total acidity (expressed as lactic acid), and headspace gas composition. Measurement of pH was done according to procedures described previously (section 5.3.2.). Total acidity was measured by titrating 10 g of the sample in 100 g water with 0.1 M NaOH using 1% phenolphthalein as indicator, and was expressed as the amount of lactic acid (in grams per 100 g sample) using the following equation :

g of lactic acid per 100 g sample = 0.009 x mL of 0.1 M NaOH

where 1 mL N/10 NaOH is equivalent to 0.009 g of lactic acid (Salfield, 1975).

On each sampling day, samples which were visibly swollen were analyzed for headspace gas composition by withdrawing gas samples using a 0.5 mL gas-tight pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, LA.) through silicone seals attached to the outside of each package. Headspace gas was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector and using Porapack, with molecular sieve 5A (80 - 100 mesh) columns in series. Helium was used as carrier gas at a flow rate of 20 mL min^{-1}. The column oven was set at 80^{o}C. The injector and detector were set at 100^{o}C. Peaks were recorded and analyzed with a Hewlett-Packard integrator (model 3390A, Hewlett-Packard Co., Avondale, PA).

5.3.5. *Microbiological Analysis*

On each sampling day, packaged trays were aseptically opened and 50 g of the sample were weighed with a sterile spoon into stomacher bags and blended with 450 mL of 0.1% peptone water in a stomacher (Model 400, A. J. Seward, London, UK) for 2 min. All subsequent dilutions were made from this 10^{-1} dilution.

Total aerobic counts were determined by plating appropriate dilutions on Plate Count Agar (Difco) using the pour plate technique. The plates were incubated at 35^{o}C for 48 h (HPB, 1989). Lactic acid bacteria were enumerated

on plates of *Lactobacillus* MRS agar (Difco MRS broth, plus 1.5% agar) incubated at 35°C for 48 h (de Man et al., 1960). Total anaerobic counts were determined by plating appropriate dilutions on trypticase soy agar (TSA; BBL Inc.), and incubating plates in anaerobic jars at 35°C for 48 h. Plates were incubated aerobically and anaerobically as described above (HPB, Health Protection Branch, 1989). All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. Although biochemical tests were not used for identification, Gram stains and catalase tests were performed on some randomly selected colonies to aid classification.

5.3.6. *Sensory Evaluation*

Samples were monitored visually by untrained panel of three, upon opening for changes in odor and color. A fresh sample was used as control for both color and odor. Packages were assigned a score of between 0 (fresh smell, excellent color) to +5 (foul smell, highly discolored) in a random sequence.

5.3.7. *Statistical Analysis*

A one way analysis of variance (ANOVA) for comparison of differences between means was done using Statistical Analysis System (SAS, 1991, release 6.04).

5.4. Results and Discussion

The chemical composition of the formulated spaghetti and meat sauce and the commercially produced pasta product are summarized in Table 19. Both products were similar with respect to pH, protein, and ash, they differed significantly in terms of moisture, carbohydrate, and salt contents, as well as water activity levels ($P < 0.05$).

The lower salt concentration and higher water activity of the formulated product was perhaps due to the fact that no salt was added to the product during preparation. The moisture content of formulated product was maintained relatively low compared to the commercially produced product, to prevent fluctuations in the heat penetration characteristics of the products.

Changes in the microbiological counts for the formulated spaghetti and meat sauce product given various heat processing treatments after 35 days storage at $5^{o}C$ and $15^{o}C$ are shown in Figs. 10 - 13. The points on each curve represent the means of three packages of each treatment sampled in duplicate. Generally, there was a gradual increase in total aerobic, anaerobic and lactic acid bacteria plate counts in all products throughout storage. Both processing conditions and storage temperature influenced the total plate counts. Heat processing at 65 and $75^{o}C$ equivalent to a 5D process resulted in a reduction in the initial microbial load to ~ 3 log CFU/g.

Table 19 : Proximate and Chemical Composition of Spaghetti and Meat Sauce

Sample	Formulated spaghetti & meat sauce	Fettuccini (commercial product)
% Moisture	69.98 ± 0.39	83.48 ± 0.07
% Ash	1.01 ± 0.01	1.24 ± 0.05
% Crude fat	0.19 ± 0.19	0.67 ± 0.01
% Crude protein	4.63 ± 0.05	3.20 ± 0.01
% Carbohydrate*	24.19 ± 0.12	11.41 ± 0.15
% Salt	0.45 ± 0.10	0.93 ± 0.10
pH	5.10 ± 0.10	5.30 ± 0.10
a_w	0.992 ± 0.00	0.980 ± 0.00

* Carbohydrate was calculated by difference

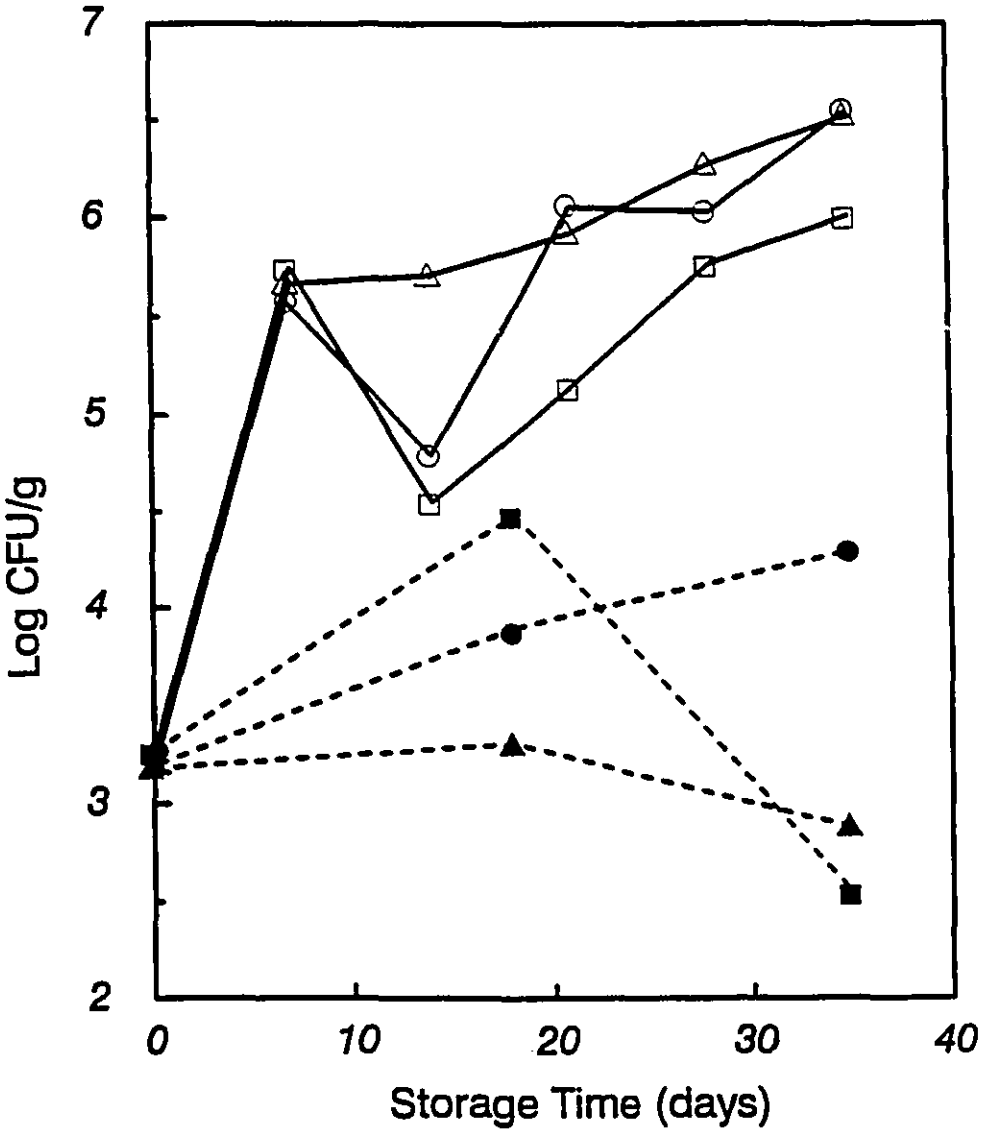


Fig. 10 : Total aerobic, anaerobic and LAB plate counts in sous-vide spaghetti and meat sauce product given a 5D process at 65°C and stored at 5°C and 15°C

▲ Aerobic (5°C) ; ■ Anaerobic (5°C) ; ● LAB (5°C)

Δ Aerobic (15°C) ; ❑ Anaerobic (15°C) ; ○ LAB (15°C)

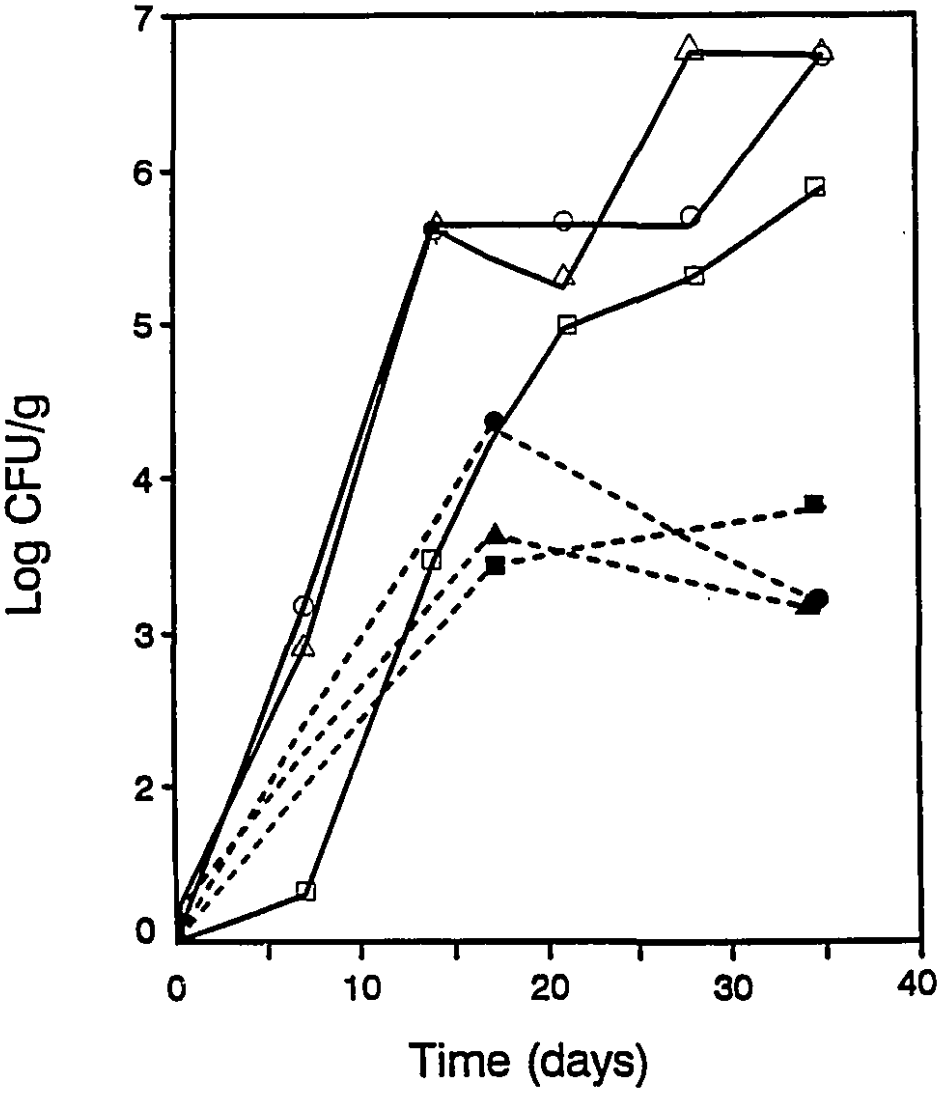


Fig. 11 : Total aerobic, anaerobic and LAB plate counts in sous-vide spaghetti and meat sauce product given a 13D process at 65°C and stored at 5°C and 15°C

▲ Aerobic (5°C) ; ■ Anaerobic (5°C) ; ● LAB (5°C)

Δ Aerobic (15°C) ; ❑ Anaerobic (15°C) ; ○ LAB (15°C)

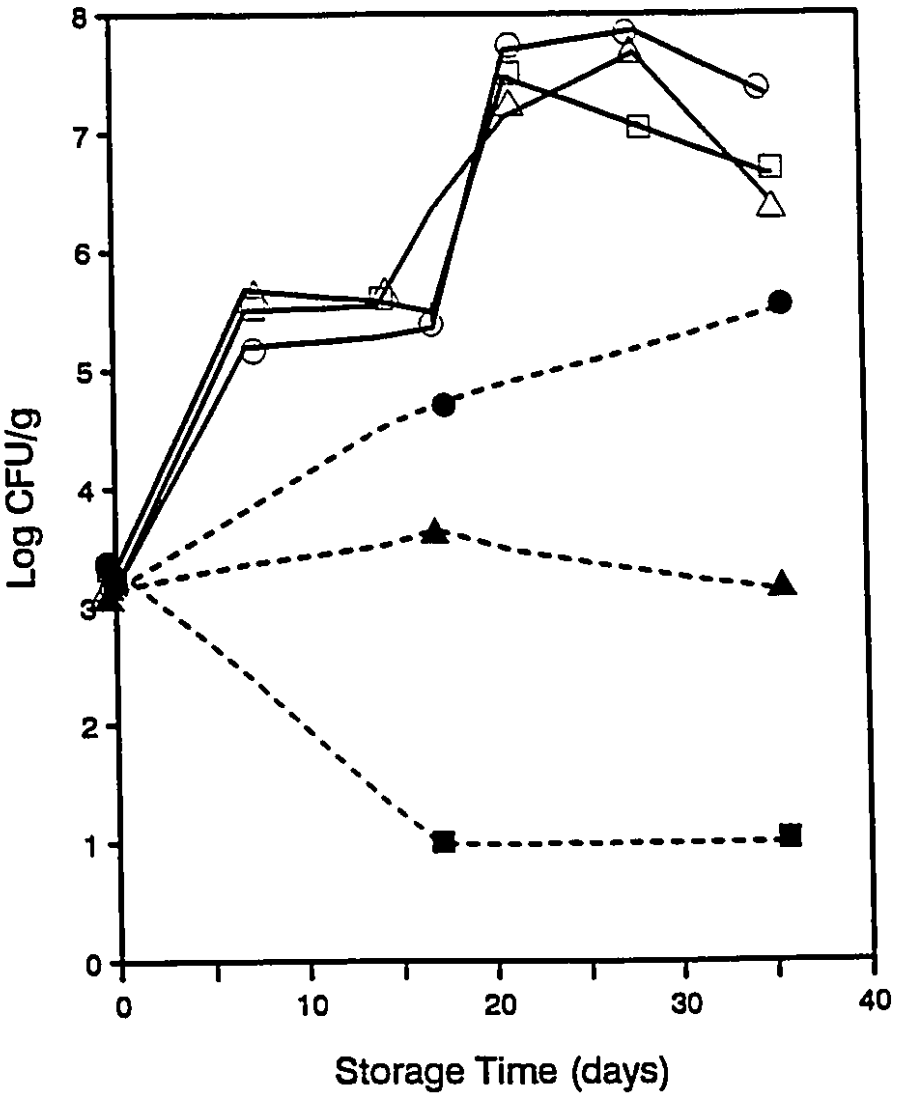


Fig. 12 : Total aerobic, anaerobic and LAB plate counts in sous-vide spaghetti and meat sauce product given a 5D process at 75°C and stored at 5°C and 15°C

▲ Aerobic (5°C) ; ■ Anaerobic (5°C) ; ● LAB (5°C)

Δ Aerobic (15°C) ; ❑ Anaerobic (15°C) ; ○ LAB (15°C)

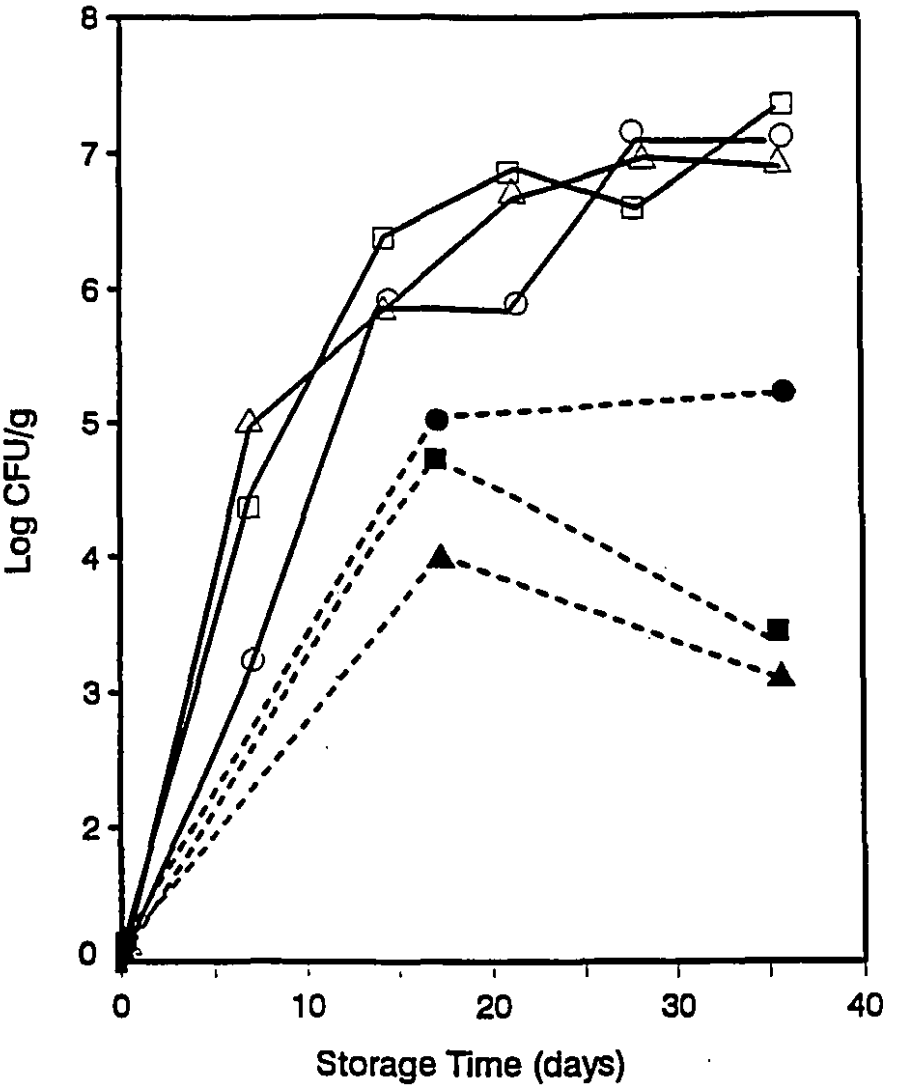


Fig. 13 : Total aerobic, anaerobic and LAB plate counts in sous-vide spaghetti and meat sauce product given a 13D process at 75°C and stored at 5°C and 15°C

▲ Aerobic (5°C) ; ■ Anaerobic (5°C) ; ● LAB (5°C)

Δ Aerobic (15°C) ; ❑ Anaerobic (15°C) ; ○ LAB (15°C)

However when products were subjected to a 13D process at the same temperature, a further 2 - 3 log reduction in the initial microbial load was observed (numbers reduced to undetectable levels) (Figs. 10 - 13).

Although the initial counts were significantly less in products subjected to a more severe heat processing treatment (13D at 65°C or 75°C), total plate counts gradually increased throughout storage, particularly at 15°C, and eventually reached similar spoilage levels (10^6 - 10^7 CFU/g) as in products subjected to a less severe heat processing treatment (5D at 65°C or 75°C) at the end of the 35 day storage period. This would indicate that a large population of the cells were not totally inactivated at 75°C, but only thermally injured, and were able to grow in high numbers in products stored at 15°C. It further confirms the observation that storage temperature has an effect on the growth of lactic acid bacteria and other bacteria numbers (Lee & Solberg, 1983). According to Allen and Foster (1960), spoilage of vacuum packed sliced meats commences when lactic acid bacteria counts are > 7 log CFU/g. The higher growth rates of microorganisms processed at 65°C and stored at 15°C make the difference between non-injured and injured cells. The significance of these results are that minimally processed foods may contain a large proportion of thermally injured cells which are able to undergo repair throughout storage, particularly at temperature abuse conditions, and reach levels of public health concern. However, the final total plate counts were significantly lower ($P < 0.05$) in all products stored at 5°C compared to products stored at 15°C for all processing treatments, again emphasizing the importance of storage temperature to ensure the quality and safety of minimally processed food products (Figs. 10 - 13). The microbial population of the

minimally processed sous-vide products comprised mainly of Gram-positive bacteria which were subdivided on the basis of their catalase reactions into *Bacillus* species and lactic acid bacteria. *Bacillus* isolates on all plates were of two main types - a small greyish white mucoid colony and a small greyish rhizoid colony. These isolates were tentatively identified, based on their gram stain and spore stain reactions as *B. sphericus* and *B. cereus*, with the latter organism accounting for about 90% of all the isolates. These results were not entirely unexpected since *B. cereus* is a spore forming organism and a common contaminant of cereals and cereal products (Kramer & Gilbert, 1989). Lactic acid bacteria isolates on all plates were tentatively identified on the basis of cultural and morphological characteristics as *Leuconostoc* and *Lactobacillus* species with *Leuconostoc* species being the predominant isolates in products stored at 15^{o}C, while *Lactobacillus* species were the main isolate observed in plates stored at 5^{o}C.

It was shown by cultural isolation and sample identification procedures that there was a shift in bacterial population throughout storage, particularly at 15^{o}C. Initial total plate counts, aerobic and anaerobic plate counts comprised mainly of *Bacillus* species. However, after ~ 15 days, *Bacillus* species decreased and the predominant microorganisms comprised mainly lactic acid bacteria, specifically *Leuconostoc* species. Lactic acid bacteria have been reported to be the predominant spoilage microorganisms in vacuum packed meat products in studies with MAP/irradiated pork (Lambert et al., 1992).

Korkeala et al., (1990), and Zurera-Cosano et al., (1988) found LAB to be the predominant spoilage microorganisms in vacuum packaged meat products, although other organisms have also been isolated (Schillinger & Lucke, 1987). Smith et al., (1983) also found that lactic acid bacteria, specifically *Leuconostoc mesenteroides* were responsible for terminating the shelf life of gas packaged bakery products. Ingram and Simonsen (1980), also demonstrated that packaging food products under vacuum produces an ecosystem that encourages growth of carbon dioxide sensitive organisms.

The fact that lactic acid bacteria became the predominant spoilage microorganisms in packaged products is not surprising due to their microaerophilic/anaerobic nature and short generation time and hence their ability to outgrow their competitors. However, the fact that they became the predominant spoilage microorganisms in minimally processed foods, particularly products heated at 75^{o}C for a time period equivalent to a 13D process is somewhat surprising. However, both *Lactobacillus* and *Leuconostoc* species have been frequently isolated from pasteurized fruit juices and soft drinks. Most lactic acid bacteria isolated from these products were strongly heterofermentative and had the ability to withstand and grow at high concentrations of sugar (30% w/w; i.e., medium a_w), and at temperatures used for fruit juice processing (Marshall & Walkley, 1952). Furthermore, these authors reported higher D values for *Leuconostoc* species and *Lactobacillus* isolates in pasteurized orange juice concentrate compared to pasteurized single strength juice. Therefore, the lower moisture content and hence lower a_w of the formulated product may explain the higher heat resistance of the lactic acid bacteria isolates observed in this study.

Changes in total acidity (expressed as % lactic acid), and hence pH for products given a 5D or 13D heat processing treatment at 65^{o}C or 75^{o}C are summarized in Figs. 14 - 17. The initial pH was similar in all products, i.e., ~ 5.15 $\pm$ 0.05. For products stored at 5^{o}C, pH remained fairly constant throughout storage irrespective of the heat treatment. However, for all processed products stored at 15^{o}C, the percentage lactic acid increased throughout storage with a concomitant decrease in product pH (Figs. 16 & 17). This change was more pronounced and statistically significant ($P < 0.05$) for products receiving a 13D heat processing treatment at 65^{o}C compared to products receiving similar treatment at 75^{o}C. These results were in agreement with those of Kempton and Bobier (1970), Mol et al., (1971), Paradis and Stiles (1978), Simard et al., (1983) and Zurera-Cosano et al., (1988).

The decrease in the pH of the products can be again attributed to the increase in lactic acid bacteria throughout storage, although *Bacillus* species have the ability to produce lactic acid and contribute to the increased lactic acid concentration found in stored products (Smith et al., 1983).

Throughout the storage period, marked changes were noted in package volume, with most packages stored at 15^{o}C being visibly swollen after 14 days for product processed at 65^{o}C (5D and 13D) and after 21 days for products receiving a more severe heat processing treatment. No changes in package volume were observed for any processed product stored at 5^{o}C.

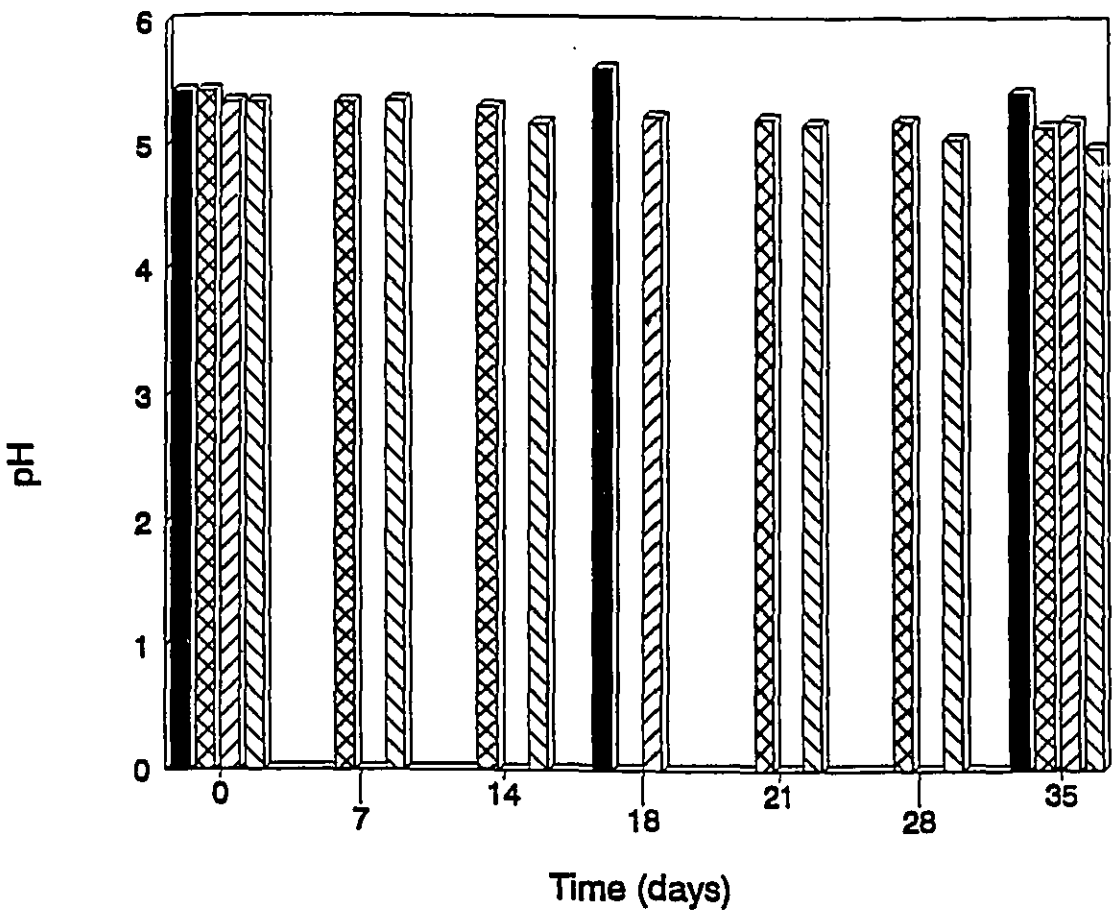


Fig. 14 : Changes in pH in a sous-vide spaghetti and meat sauce product, 5D & 13D processed at 65°C and stored at 5°C or 15°C

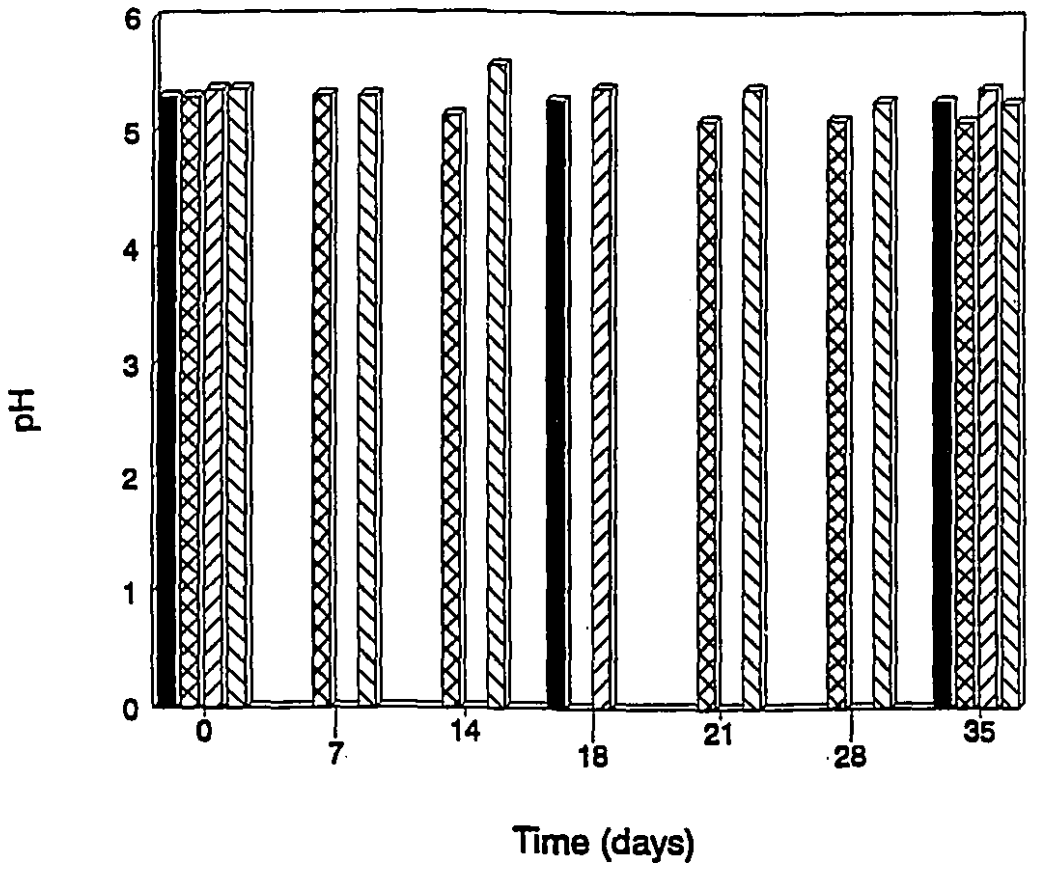


Fig. 15 : Changes in pH in a sous-vide spaghetti and meat sauce product, 5D & 13D processed at 75°C and stored at 5°C or 15°C

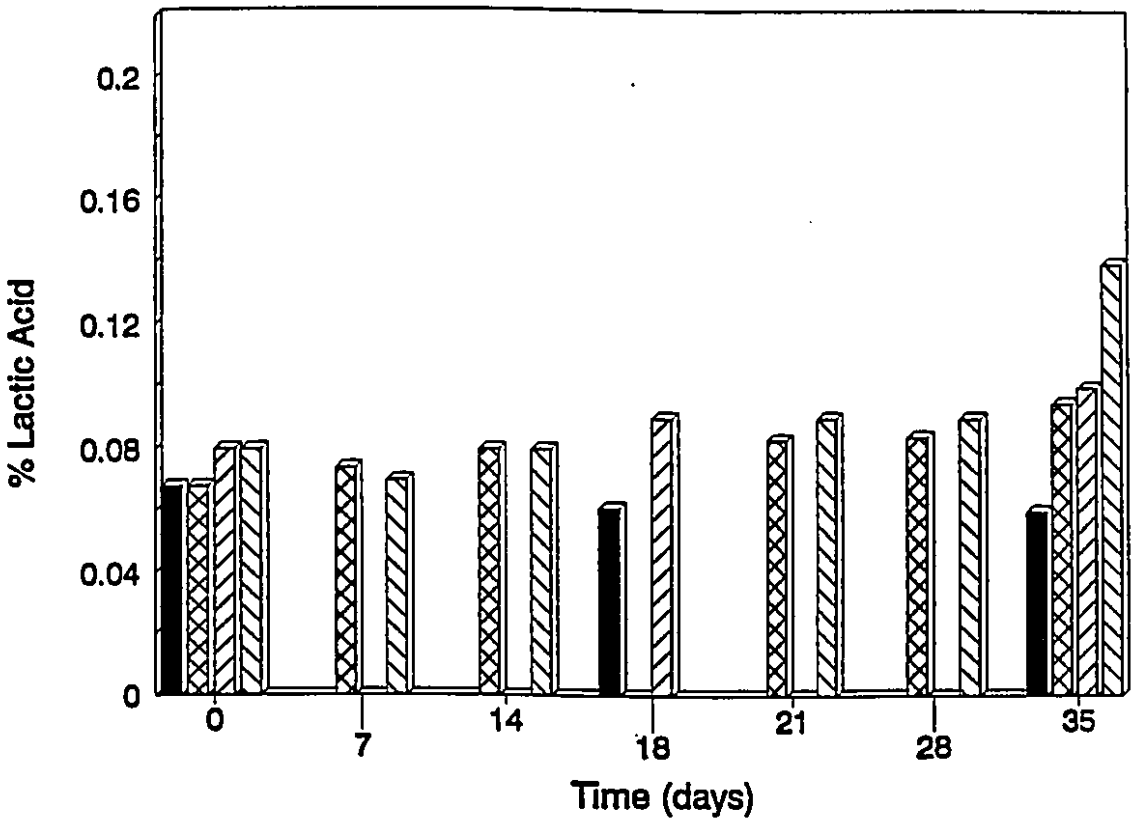


Fig. 16 : Changes in total acidity in a sous-vide spaghetti and meat sauce product, 5D & 13D processed at 65°C and stored at 5°C or 15°C

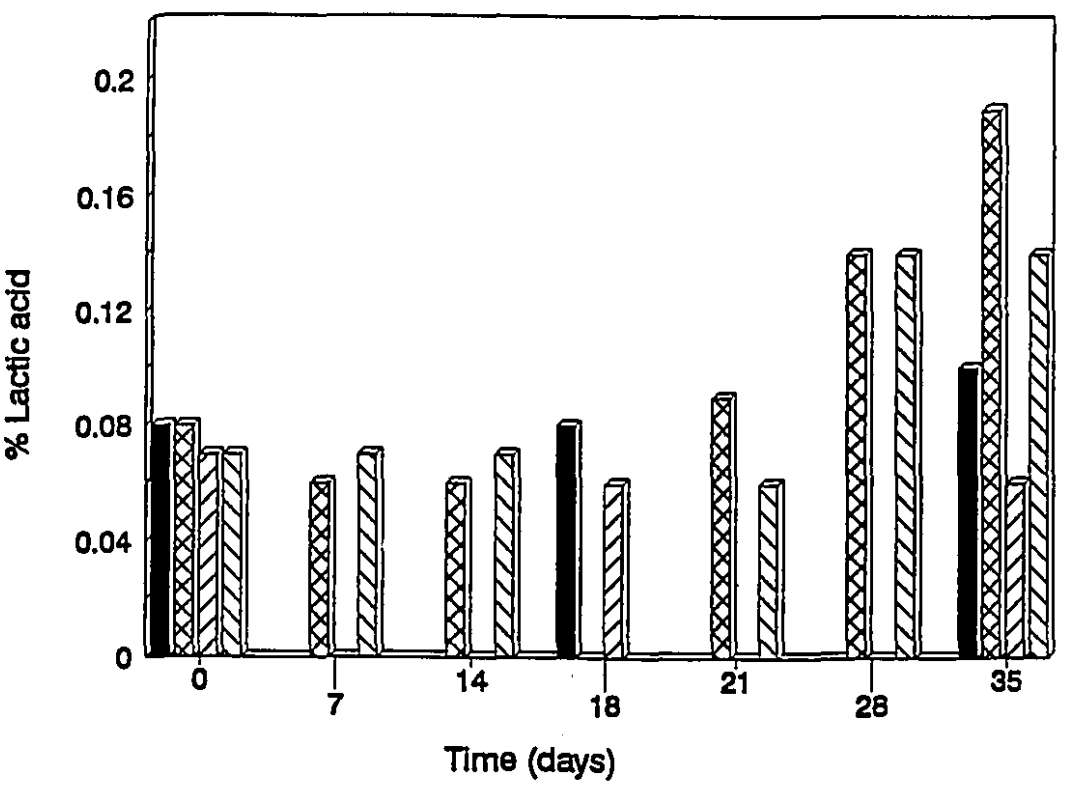


Fig. 17 : Changes in total acidity in a sous-vide spaghetti and meat sauce product, 5D & 13D processed at 75°C and stored at 5°C or 15°C

Headspace gas analysis of swollen packages (Table 20) showed that it consisted of mainly carbon dioxide (~97%), produced presumably by heterofermentative strains of lactic acid bacteria. Indeed changes in headspace carbon dioxide correlated with increase in LAB counts, i.e., ~ 8 log CFU/g in processed products. Korkeala and Lindroth (1989) reported that LAB were responsible for increases in carbon dioxide during storage of vacuum packed sausages. However, *Bacillus* species have been shown to be heterofermentative, and may have also contributed to the increase in headspace CO_2 during the early stages of storage.

Sensory evaluation of products upon opening, showed that all products stored at 5^{o}C had very good (+1) odor scores and good (+2) color scores and were almost as fresh at day 35 as at day 0. However, for products stored at 15^{o}C, there was a distinct fruity odor when packages were opened and were discolored (creamish yellow) compared to the fresh product. Unacceptable odor/color scores, (i.e., >3) were observed after 14 days and 21 days respectively, for products processed at 65 and 75^{o}C, equivalent to a 5D and 13D process and stored at 15^{o}C. These sensory scores correlated well with increase in both LAB and headspace CO_2 concentration throughout storage. The typical odor of packages which were visibly swollen can be attributed to the presence of lactic acid bacteria. Similar observations have been reported in gas packaged crumpets (Smith et al., 1983) and MAP/irradiated meat (Lambert et al., 1992; Shamsuzzaman et al., 1992).

Table 20 : Headspace Gas Composition of Selected Sous-vide Spaghetti and Meat Sauce Product - after 25 Days Storage at 15°C

CO_2	N_2	O_2
97.04	1.80	0.27
96.48	1.79	0.29
96.46	2.44	0.24

5.5. Conclusions

This study has shown that physical, chemical and microbiological changes can be used as indicators of shelf life acceptability in minimally processed sous-vide products. Changes in package volume, odor/color and bacterial counts, particularly lactic acid bacteria, can all be used as indicators of spoilage. Based on the number of days for LAB to reach > 10^6 CFU/g and odor / color score of 3, a minimally processed spaghetti and meat sauce product has a shelf life of approximately 14 days when processed at 65^{o}C and stored at mild temperature abuse conditions (15^{o}C). This shelf life could be extended to 21 days at 15^{o}C if the product were minimally processed at 75^{o}C equivalent to a 5D or 13D heat treatment. However, a shelf life of ~ 35 days is possible for minimally processed products if stored at 5^{o}C or less. The higher plate counts, CO_2 production and sensory scores in products stored at 15^{o}C emphasize the need for strict temperature control throughout distribution and storage of minimally processed products. The survival and growth of spores in minimally processed products is of concern since it implies that *C. botulinum* spores, if present, may grow and produce toxin. However, at the processing time/temperatures used in this study, the public health concern would appear minimal since *C. botulinum* may be outgrown by LAB and the products would be visually spoiled and rejected by the consumer. Nevertheless, this concern is justified if products were processed at slightly higher temperatures sufficient to inactivate the lactic acid bacteria, or other microbiological indicators of spoilage in sous-vide products. Challenge studies with *C. botulinum* spores are thus, necessary to assess the public health safety of minimally processed products, particularly at temperature abuse conditions.

CHAPTER 6

CHALLENGE STUDIES WITH *CLOSTRIDIUM BOTULINUM* IN A SOUS-VIDE SPAGHETTI AND MEAT SAUCE PRODUCT

6.1. Summary

Challenge studies were carried out to evaluate the safety of a reformulated *sous-vide* spaghetti and meat sauce product (a_w 0.992 - 0.972 ; pH 4.5 - 6) inoculated with *Clostridium botulinum* types A and B spores. Following processing at 75^{o}C for 39 min, (equivalent to a 13D process for *Streptococcus faecium*), samples were stored at 5^{o}C or 15^{o}C and analyzed at selected time intervals for toxin production and visible signs of spoilage. Toxin was not detected in any samples stored at 5^{o}C throughout the 42 day storage period. However, for products stored at 15^{o}C, toxin was detected in samples of pH $\geq$ 5.5 at days 14 & 35 for samples of pH 6.0 and 5.5 respectively. Toxin was not detected in any samples of pH $\leq$ 5.25 during 42 days storage at 15^{o}C. All products of pH 5.75 and 6 were visibly spoiled, i.e., swollen due to CO_2 production, prior to toxigenesis. However, for the product of pH 5.5, toxigenesis preceded spoilage. Subsequent studies were done to test the effect of additional salt (1 - 3% ; w/w) on the safety of a product of pH 5.5 at mild temperature abuse storage conditions (i.e., 15^{o}C). Toxin production was not delayed in samples containing < 1.5% added salt, while $\geq$ 1.5% salt (w/w) prevented toxin production throughout the 42 day storage at 15^{o}C. Mathematical methods used to quantify individual effects of a_w, pH, and storage time further substantiated and confirmed these results. Heating of products for 5 - 10 min at full or half power in a domestic microwave oven (800 watts) inactivated the pre-formed toxin in all samples.

6.2. Introduction

Over the past few years, there has been a tremendous increase in minimally processed vacuum packed (*sous-vide*) refrigerated foods on the market as a result of consumer demands for foods that offer greater convenience and time savings in preparation. Experts predict that these minimally processed refrigerated foods (MPRF) will replace canned and frozen foods in the supermarkets by ~25% within the next 10 years, due to their fresher taste and the fact that they are "preservative-free" (Folkenberg, 1989).

"*Sous-vide*" means "under vacuum", and it describes a processing technique whereby freshly prepared foods are vacuum sealed in individual packages and subsequently pasteurized at time/temperature combinations sufficient to destroy all vegetative pathogens (Kramer, 1988). Under good manufacturing practices, *sous-vide* processed products have a shelf life of 20 to 30 days when stored at refrigeration temperatures (Schafheitle, 1988). However regulatory authorities recognize that minimally processed foods may be a potential public health risk due to the growth of food borne pathogens which may survive the heat process (Baird, 1990; Beauchemin, 1989).

The heat treatment applied in *sous-vide* processing results primarily in the destruction of vegetative cells, while spores, if present, will often survive the mild heat processing conditions. Of most concern in such commercially processed foods is the survival and growth of *C. botulinum*. *Sous-vide* products contain sufficient nutrients, low salt concentration, high pH and an oxygen free environment conducive for the growth and toxin production by *C. botulinum*.

C. botulinum species is very heterogeneous, and two main groups causing human botulism are recognized : viz., Group I - the proteolytic types A, B, & F; and Group II - the non-proteolytic types B, E, & F. The proteolytic strains have a minimum growth temperature of $10^{o}C$, are more acid tolerant and produce very heat resistant spores (Smelt & Maas, 1978; Hauschild, 1989). Therefore, if *sous-vide* foods are stored at temperatures higher than $10^{o}C$, there is a risk of growth of *C. botulinum* with concomitant production of toxin. As well, if the heat treatment is insufficient to destroy Group II spores, even proper refrigeration may not be sufficient, as these strains may grow at $4^{o}C$.

The purpose of this study was to determine the risk of survival of, and toxin production by proteolytic strains of types A and B spores in a *sous-vide* spaghetti and meat sauce product at mild temperature abuse storage conditions. The effects of additional barriers, salt and pH, to control the growth of *C. botulinum* in the minimally processed product were also studied, as well as the effect of microwave heating on the pre-formed botulinum toxin.

6.3. Materials and Methods

6.3.1. *Production of Spores*

The strains of *C. botulinum* used were proteolytic types A6, 62A, 17A, 317121A, CK2-A, MRB, 1B, 13983-IIB, 368B and 426B obtained from the Bureau of Microbial Hazards, Health Protection Branch (HPB,), Health and Welfare, Ottawa. Spore suspensions of the individual strains were prepared by growing

the strains in trypticase-peptone-glucose-yeast extract (TPGY) broth at 35°C for 10 days according to methods outlined by Health and Welfare Canada (HBP, 1989). Spores were harvested with sterile distilled water, centrifuged at 17,500 xg for 20 min at 2 to 5°C, resuspended in gelatin-phosphate buffer (pH 6.6) and heat shocked at 75°C for 20 min. The number of spores/mL in each suspension was enumerated on Wynne's agar (Difco) as outlined by Hauschild and Hilsheimer (1977). For inoculation, spores were pooled from each strain into a gelatin-phosphate buffer to give a final spore concentration of 10^8/mL.

6.3.2. *Product Inoculation & Packaging*

The spaghetti and meat sauce product was prepared by adding 200 g of previously cooked spaghetti to 175 g of commercially produced meat sauce. The spaghetti with meat sauce was weighed directly into a pre-tared stomacher bag and inoculated with 0.5 mL of prepared spore inoculum to give a final inoculum of 10^3 spores/g of sample. The contents of the bag were then thoroughly massaged by hand to mix the sample. The mixture was then carefully transferred into a thermoformed oriented polypropylene tray and vacuum packaged with a top film of Wallo peel (polyester/polyamide/EVOH, O_2 transmission rate, 12cc/m^2 atm at 23°C) using a vacuum skin/thermoforming packaging system (Trigon Model # RM331 MMIS Inc., Toronto, Ontario, Canada).

6.3.3. *Effect of pH and Salt*

The effects of pH and salt on the growth of, and toxin production by, *C.*

botulinum were investigated in reformulated products. Samples were adjusted to various pH levels (i.e., 5.0, 5.25, 5.5, 5.75 and 6.0) by adding appropriate amounts of 10 N NaOH to the meat sauce. The products were then inoculated with *C. botulinum* types A and B spores, packaged, heat processed, stored at 15°C and assayed for toxin.

The pH of each sample was measured in duplicate by inserting the probe of a calibrated Acumet pH meter (Fisher Scientific, Montréal, Québec, Canada) directly into the samples.

The effect of salt was studied at pH 5.5 only. Appropriate amounts of salt to give final salt concentrations of 0.5, 1, 1.5, 2, 2.5 and 3% w/w respectively, were added to the meat sauce. The a_w of the final products was measured using a Decagon water activity meter (model CX-II, Hotpack Refrigerated Bath Circulator, Decagon devices, Inc., Pullman, WA, U.S.A). The salt concentration was measured in the reformulated products using a Horiba compact salt meter C-121 (Horiba Instruments Inc., Irvine, Calif.) previously calibrated with 1% and 5% standard NaCl solutions.

6.3.4. *Thermal Processing and Sampling of Product*

The prepared samples were processed at time/temperature conditions previously determined by Simpson et al., (1993a) to achieve a 13D reduction in *S. faecium*, i.e., heating in a water bath maintained at 75°C for 39 min. Products were then cooled rapidly to 4°C, and then stored at 5°C or 15°C for up to 42 days. Triplicate samples were withdrawn and analyzed for toxin after the first 14

days and subsequently at 7 day intervals until the end of the storage period. For products stored at 5^{o}C, samples were analyzed after 0, 21 and 42 days only.

6.3.5. *Toxin Assay*

At each sampling time, 20 g of sample from triplicate packages in each treatment were weighed into stomacher bags with 20 g of gelatin-phosphate buffer, homogenized in a stomacher for 1 min, then centrifuged at 17,500 xg for 20 min. The supernatant was then filter-sterilized and 0.5 mL samples were injected intraperitoneally in duplicate into mice (20 - 25 g) and the animals observed for 3 days for symptoms of botulism (pinched waist, labored breathing, and/or death). Animals showing typical symptoms were euthanized using CO_2 inhalation.

To confirm the presence of *C. botulinum* toxin, randomly selected samples that killed mice were re-tested with antitoxin. For neutralization and toxin typing, 0.4 mL of botulinum antisera A and B were added to 1.4 mL of a 1 : 1 dilution of the supernatant, and left at room temperature for 45 min, and then injected into mice and monitored as described previously for symptoms of botulism.

The time until toxin production was defined as the earliest time at which toxin was detected in any given specific treatment. For example, if for a specific treatment, two out of three samples were toxic on day 14 and then two out of three were again toxic after 21 days in another set of replicates, the reported time until toxin production was recorded as 14 days.

6.3.6. *Sensory Evaluation*

Samples were visually examined for swelling due to gas production, as well as changes in color and odor. For unswollen packages, presence of toxin was used to indicate spoilage.

6.3.7. *Headspace Gas Analysis*

Samples which were found to be swollen were analyzed for headspace gas composition by withdrawing gas samples using a 0.5 mL gas-tight pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, LA) through silicone seals attached to the outside of each package. Headspace gas was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector and using Porapack Q with molecular sieve 5A (80 - 100 mesh) columns in series. Helium was used as carrier gas at a flow rate of 20 mL min^{-1}. The column oven was set at 80^{o}C. The injector and detector were set at 100^{o}C. Peaks were recorded and analyzed with a Hewlett-Packard integrator (model 3390A, Hewlett-Packard Co., Avondale, PA).

6.3.8. *Calculation of Probability*

The number of toxic samples was converted to the **"Most Probable Number"** (MPN) of spores able to initiate growth and toxin production in each set of conditions. The MPN was calculated from the Halvorson-Ziegler equation (Halvorson & Ziegler, 1933) as : **Ln (n/q)**

where **"n"**, is the number of samples analyzed, and **"q"** the number of non-toxic samples as described by Hauschild, (1982). The probability of toxigenesis from a

single spore was then defined as : **P (%) = (MPN X 100)/inoculum**

When none of the samples was toxic at 15°C, P(%) was taken as 10^{-4} (Dodds, 1989). The P(%) values calculated were plotted against storage time, a_w, or pH. Using the predictive equations, predicted time of toxin production was calculated for the various a_w and pH values.

6.3.9. *Statistical Analyses*

The effects of the experimental factors (i.e., pH, a_w) on P(%) and on the time until first toxin production (lag time) were further investigated by a simple regression technique which was used to select the best model for predicting P(%) and lag time. All statistical calculations were performed by the general linear models procedure of the STAT graphics statistical package (Release 3.0, GSC*CGI graphics for Personal Computers, GSS Inc. 1988; Cary, NC).

6.3.10. *Microwave Treatment*

Samples in which toxin was detected were individually microwave heated in their original containers (opened, but contained in another bag to prevent moisture loss) at either full or half power for 5 or 10 min, using a conventional domestic microwave oven (Kenmore brand, Sears Inc. Montréal, Québec, Canada) with 800 Watts power. Immediately after microwave heating, the temperature of each product was measured at 5 points using a calibrated digital thermometer. The products were th re-examined for presence of toxin as described previously.

6.4. Results and Discussion

This work extends previous studies on the microbiological changes that occurred in sous-vide spaghetti and meat sauce product which indicated the survival of spores and heat resistant lactic acid bacteria (Chapter 5). The products used in this study were prepared to be as similar in formulation to previously prepared products and the commercially produced product in terms of their proximate composition, water activity and pH. However, it was necessary to raise the initial pH of the product used in this study from ~4.4 to pH 5.5, i.e., the average pH of samples used in earlier thermal processing/storage studies. The variations in the pH values were attributed to changes in the production/formulation of commercially produced meat sauce, e.g., different batches of tomatoes.

A storage temperature of 15^{o}C was used to represent mild temperature abuse since surveys of retail cases in supermarkets and domestic refrigerators indicate that 20% exceeded temperatures of 10^{o}C (Davidson, 1987; Harris, 1989; Van Garde & Woodburn, 1987).

The inoculum level of *C. botulinum* used in this study was substantially higher than the contamination level which has been found in either fresh or processed meat products. Several authors have shown that the concentration of spores range from 4.0 x 10^{-5} to 1.67 x 10^{-3}/g in raw and processed meat products (Abrahamsson & Riemann, 1971; Greenberg et al., 1966; Hauschild, 1989; Insalata et al., 1969; Taclindo et al., 1967). Therefore the inoculum level and the storage conditions used in this study represent the worst case scenario for this kind of *sous-vide* product.

It is evident that the probability of toxigenesis increased with storage time, but decreased as either the a_w (increased salt concentration) or pH was decreased (Table 21, Figs. 18 & 19).

The effect of pH on the growth of and toxin production by *C. botulinum* is shown in Table 21, and the corresponding observed probability of toxigenesis by one *C. botulinum* spore is shown in Fig. 18. Toxin was not detected in any control samples (pH 4.4 or pH 5.0) at day 42 of storage at 15°C, while toxin was detected in samples at pH 6 as early as at day 14 for the same storage temperature. For products reformulated to pH 5.5 or 5.75, toxin was detected at day 21, while in the products at pH 5.25, toxin was not detected until day 35 at 15°C. Toxin was not detected in any samples (results not shown) stored at 5°C throughout the storage period, emphasizing the importance of storage temperature to ensure the public health safety of minimally processed products. Furthermore, this is in agreement with literature data which shows that storage at 5°C is well below the reported cut-off growth temperature for proteolytic types A and B of *C. botulinum* (Hauschild, 1989).

Products reformulated to pH 6 and 5.75 were visibly spoiled, i.e., visibly swollen at 14 and 21 days respectively, when toxin production was detected. Swelling was due to headspace CO_2 production and confirms spoilage profiles by LAB in previous shelf life studies of the product (Chapter 5). However, of most concern was the fact that there was no swelling or discoloration in samples of pH 5.25 and 5.5, when toxin was detected at day 21 at 15°C, i.e., toxigenesis preceded spoilage.

Table 21 : Time of Botulinum Toxin Production in Formulated Sous-vide Spaghetti and Meat Sauce Stored at 15°C as Influenced by pH and Water Activity (a_w).

Barrier Used in Sample		Days to toxin Detection
A. ***Effect of pH*** [1]		
Control (pH 4.4)		>42
pH 5		>42
pH 5.25		35
pH 5.5		21
pH 5.75		21
pH 6		14
B. ***Effect of salt or a_w*** [2]		
[NaCl]	a_w	Days to toxin Detection
nsa (control)	0.992	21
1.0 %	0.986	28
1.5 %	0.983	42
2.0 %	0.976	>42
2.5 %	0.975	>42
3.0 %	0.972	>42

[1] (a_w of all samples was 0.992) ;

[2] pH of all samples was 5.5 ; nsa => no salt added

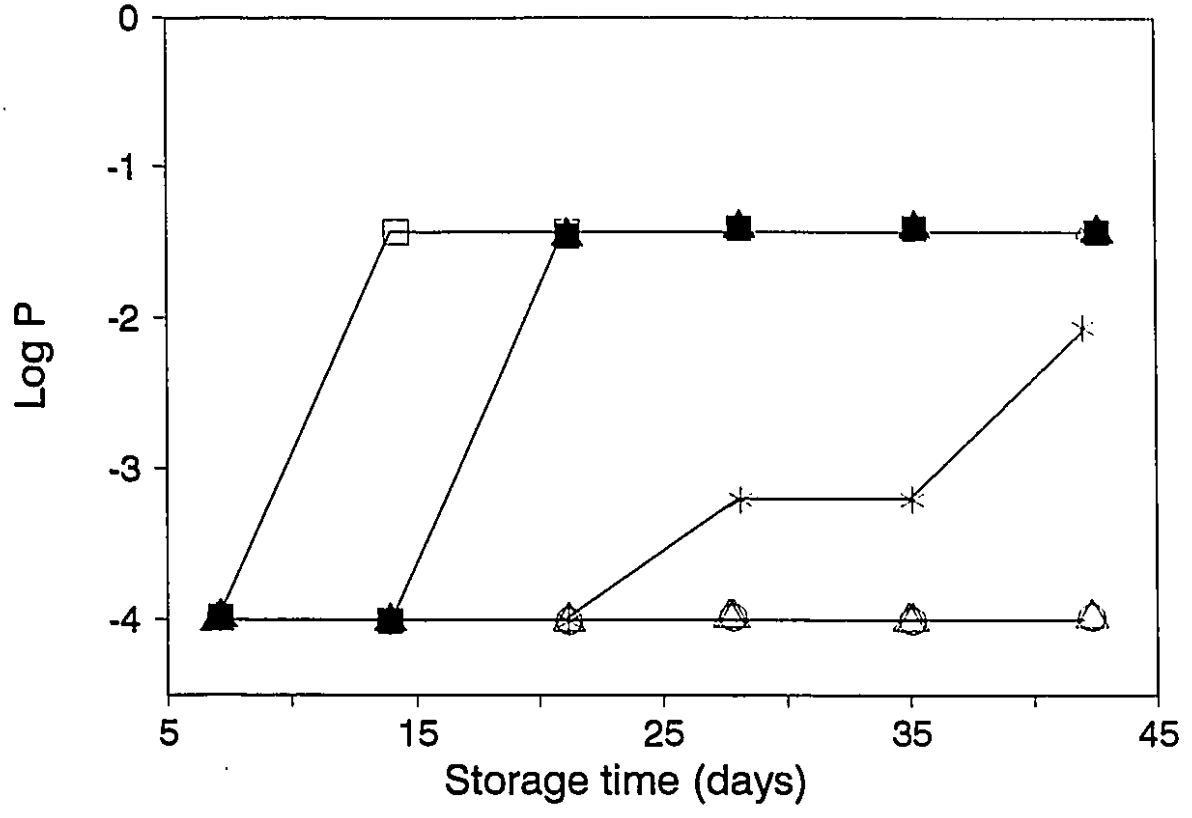


Fig. 18 : Effect of pH on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide spaghetti and meat sauce product during storage at 15°C for up to 42 days.

○ Control (pH 4.4) ; △ pH 5 ; ✳ pH 5.25 ; ▲ pH 5.5 ; ■ pH 5.75; □ pH 6 .

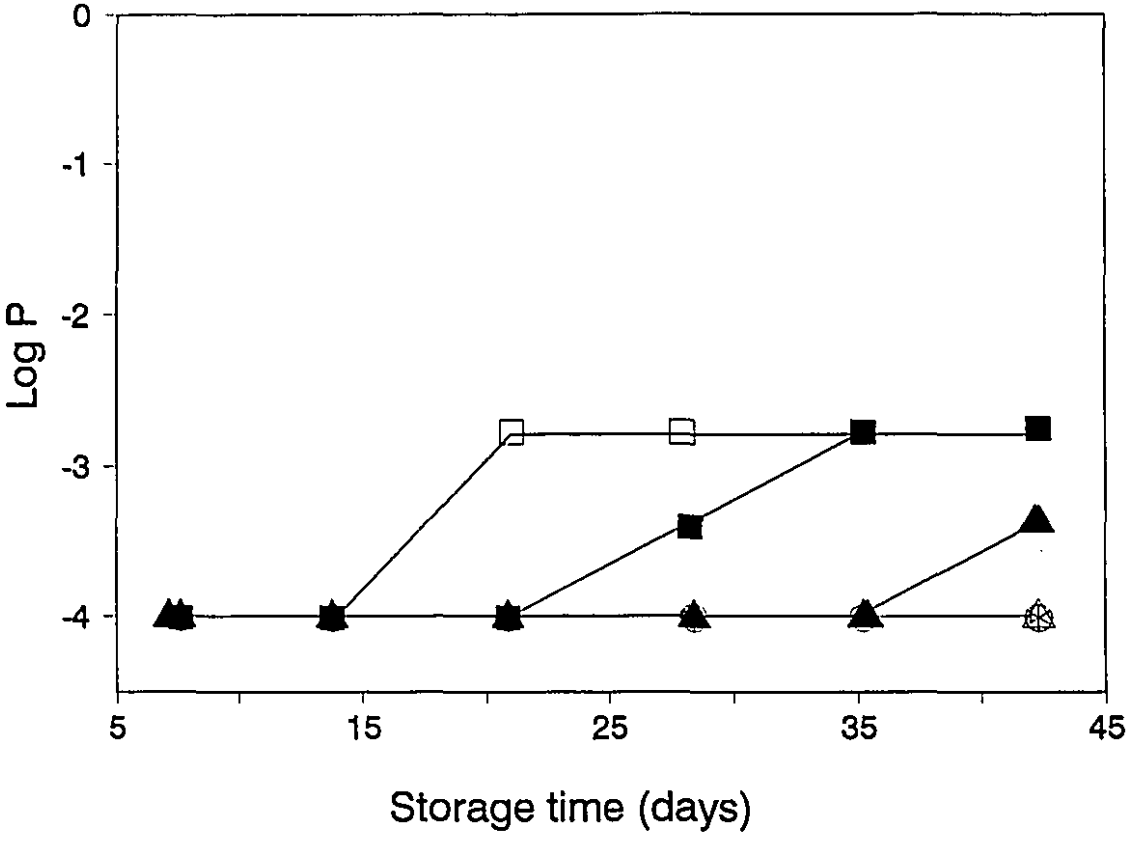


Fig. 19 : Effect of a_w on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide spaghetti and meat sauce product incubated at 15°C for up to 42 days.

□ Control (0.992) ; ■ 0.986 ; ▲ 0.983 ; ✳ 0.976 ; △ 0.975 ; ○ 0.972

The minimum pH requirement for growth of proteolytic *C. botulinum* is in the range 4.6 - 4.8, although for many strains it may be well over 5.0 (Hauschild, 1989; Odlaug & Pflug, 1978; Townsend et al., 1954). The results of this study confirm the effect of pH in controlling toxin production by proteolytic *C. botulinum* types A and B spores in minimally processed sous-vide products with pH $\leq$ 5.0. However, products with pH > 5.0 could obviously pose a public health hazard and additional barriers, e.g., a_w reduction, are necessary in medium acid products (pH 5 - 6) to prevent growth of, and toxin production by *C. botulinum* particularly at mild temperature abuse conditions.

To test the effect of additional barriers, i.e., salt concentration, on toxin production by *C. botulinum*, the spaghetti and meat sauce product was re-formulated to contain an additional 1 - 3% salt. A pH of 5.5 was chosen since, (i) toxigenesis preceded spoilage in these products, (ii) the average pH of the commercially produced pasta/meat sauce products was 5.3 $\pm$ 0.2.

The effects of decreasing a_w through variation in salt concentration, on toxin production in products reformulated to pH 5.5 is shown in Table 21 & Fig. 19. Adding salt decreased the product's a_w from 0.992 (control) to 0.972 for products containing an additional 3% salt. This addition of salt and reduction in a_w had a significant effect on toxin production by *C. botulinum*. In control samples (pH 5.5, no added salt), toxin was detected at day 21, while in product containing an additional 1% salt, toxin was not detected until day 28 when samples were stored at 15°C.

However, at salt concentrations greater than 1.5%, toxin was not detected in any samples throughout the 42 day storage period at 15^{o}C. The results of this study confirm the importance of salt (NaCl) in controlling food borne *C. botulinum*. The decrease in water activity by salt and the delay in toxin production at higher salt levels is consistent with previous observations (Sperber, 1982; Beuchat, 1987; Baird-Parker & Freame, 1967). These authors reported minimum water activities of 0.94 - 0.96 for *C. botulinum* types A and B spores in NaCl-containing food media. Although a lower minimum water activity was observed for growth of, and toxin production by *C. botulinum* in NaCl-adjusted substrates, the inhibitory effect of lower salt concentrations, and hence higher a_w values in this study can be attributed to the combined synergistic effect of reduced a_w and storage temperature (Mossel 1983; Leistner & Rodel, 1976; Scott, 1989; Baird-Parker & Freame, 1967).

Mathematical modelling has been used in several studies to assess the safety of food products with respect to *C. botulinum* (Dodds, 1989; Ikawa & Genigeorgis, 1987; Jensen et al., 1987). In this study, a logarithmic transformation of the probability data resulted in a better fit. The relationship between the time until first toxin production (lag time) and the variables (salt, a_w and pH) was determined in a similar manner. Only terms which were statistically significant by analysis of variance were included in the models. The resulting models and the predicted time until toxin production, as well as the actual time observed are shown in Tables 21 - 23. A good correlation was observed between predicted and observed times until toxin production.

Table 22 : Predictive Equations which individually relate pH and Water Activity (a_w) to Time (days) of Toxigenesis of *C. botulinum* in Sous-vide Spaghetti and Meat Sauce.

Model No.			Equation	r^2
1	1/Time	=	-0.23139 + 0.0500 pH	0.89
2	1/Time	=	-2.89110 + 2.35335 a_w	0.88

Table 23 : Comparison of Time until Toxin Production by *C. botulinum* in an Inoculated Sous-vide Spaghetti and Meat Sauce Stored at 15°C, as Predicted by Derived Models Relating pH, Water Activity (a_w) and Time (days) of Toxin Production

Treatment	Model No.	Time until toxin production (Days) Predicted	Observed
pH 6.0	1 [a]	15	14
pH 5.75	1	18	21
pH 5.5	1	23	21
pH 5.25	1	32	35
a_w (0.992)	2 [b]	22	21
a_w (0.986)	2	32	28

[a] 1 / Time = -0.23139 - 0.05000 pH ($r^2 = 0.89$)

[b] 1 / Time = -2.28911 + 2.35335 a_w ($r^2 = 0.88$)

These findings are consistent with observations made by previous investigators that mathematical models can be used to quantify the risk of *C. botulinum* growth in foods affected by different food and environmental parameters (Chirife & Favetto, 1992; Dodds, 1989; Hauschild, 1982; Ikawa & Genigeorgis, 1987; Jensen et al., 1987).

Several methods have been examined to inactivate botulinum toxin in foods without destroying nutrients or the sensory qualities of the products. These include heating, freezing, and more recently the use of low dose ionizing irradiation (Shamsuzzaman et al., 1992). The consumer appeal of many ready-to-eat convenience foods, such as sous-vide products, is that these products can be re-heated quickly by microwave energy. However, there is a paucity of data on the effect of microwave energy on the stability of pre-formed toxin in sous-vide products, especially products which appear acceptable to the consumer from the sensory point of view. The effect of microwave heating on toxic sous-vide products in a domestic microwave (800 Watts, full power) for either 5 or 10 min at both full and half power was investigated (Table 24). No attempt was made to measure the initial or final concentration of toxin prior to, or after microwave heating. However, it was evident that microwave energy denatured pre-formed toxin in the sous-vide spaghetti and meat sauce product.

Table 24 : Effect of Microwave Energy on Pre-formed *C. botulinum* Toxin in Inoculated Spaghetti and Meat Sauce During Storage at 15°C

pH of sample	Full Power[1] at		Half Power[2] at	
	5 min	10 min	5 min	10 min
5.25	0/3	0/3	0/3	0/3
5.75	0/3	0/3	0/3	0/3
6.0	0/3	0/3	0/3	0/3

[1] Full power at 800 watts; [2] 50% full power

No toxin was detected after microwave heating the toxic samples of pH 5.25, 5.75 and 6.0 at either full or half power for either 5 or 10 min. The internal temperature of the microwave heated products measured by inserting a digital thermometer directly into product after microwave heating showed the product temperature ranged from 95 to 100^{o}C in products heated at full power for 5 and 10 min respectively, and from 85 - 99^{o}C for products heated at half power for the same time period. These results confirm previous studies which showed that *C. botulinum* types A and B toxins can be inactivated at 60 - 85^{o}C for time periods up to 20 min (Woodburn et al., 1979). However, the complete inactivation of toxin after microwave heating for 5 min is contrary to the observations of Notermans et al., (1990) who reported that complete inactivation of pre-formed toxin in products required heating in a domestic microwave for, at least, 10 min. The differences in these results may be attributed to lower power of the microwave, i.e., 700 watts vs 800 watts, used in the two studies. It may also relate to differences in the food composition used in the two systems, although, Notermans et al., (1990), used inoculum levels of 10^5 spores/2g sample as compared to 10^3 spores/g used in this study. If the latter is the case, then it may be inferred that higher spore levels probably resulted in higher toxin production, and hence the degree of inactivation achieved would depend on the duration of the heating time.

6.5. Conclusion

The study indicates that both decreased pH and increased salt exerted antimicrobial / antibotulinal effects, resulting in the retardation of growth and toxin production by *C. botulinum* types A and B spores in sous-vide spaghetti and meat sauce. Since a worst case scenario was presented, it would be expected that if lower levels of *C. botulinum* spores are present in the raw ingredients used in product formulation of sous-vide products, a reduction in pH or a_w (through the addition of salt) could be used either alone, or in combination, to innibit the growth of clostridial spores. Although microwave heating inactivated pre-formed toxins in the product, it would not be judicious to recommend it as a safety factor to reduce the botulism risk in minimally processed food products. This can best be achieved through proper refrigerated storage of products and/or product reformulation to suitable a_w and pH levels to ensure the public health safety of products subjected to temperature abuse at any stage of its storage/distribution prior to consumption of the product.

CHAPTER 7

STORAGE STUDIES ON A *SOUS-VIDE* RICE AND SALMON PRODUCT

7.1. Summary

Sous-vide rice and salmon products were subjected to a 5D and 13D heat processing treatments at 65^{o}C and 75^{o}C and then monitored for quality changes during storage at 5^{o}C or mild temperature abuse storage conditions (15^{o}C). The formulated products stored at 5^{o}C were stable for $\geq$35 days, irrespective of the processing treatment. However, for products stored at 15^{o}C some were visibly spoiled after 21 days depending on the severity of the heat processing treatment. Spoilage was usually accompanied by carbon dioxide production, and swelling of packages which had a distinct fruity odor when opened. The predominant spoilage organisms at the initial stages of storage comprised *of Bacillus* species, while lactic acid bacteria became the predominant spoilage microorganisms after 7 - 12 days at 15^{o}C storage. The growth of lactic acid bacteria in the products was accompanied by an increase in carbon dioxide and lactic acid concentration, resulting in a slight decline in pH of the products. This study shows that physical, chemical and microbiological changes during storage can be used as indicators of temperature abuse for minimally processed food products.

7.2. Introduction

Sous-vide products are attractive to consumers for various reasons such as their closer to fresh characteristics, and convenience. In addition, the minimal

heat treatment applied in conjunction with storage under vacuum at refrigeration temperatures has potential to slow down microbial growth and metabolism, and certain undesirable chemical changes in the food product. Properly formulated and processed sous-vide products have a shelf life ranging from 20 to 30 days (Schafheitle & Light, 1989; Young et al., 1988). These advantages have stimulated considerable interest in the use of the technology to extend the quality, marketable shelf life and safety of various fresh foods (Light & Walker, 1990a,b; Smith et al., 1990). *Sous-vide* products are formulated from top quality raw ingredients and pre-cooked (if necessary), and packaged under vacuum in heat-stable air-impermeable trays, under stipulated conditions prior to storage at $4^{o}C$ as described in section 5.2. of Chapter 5. The products are then stored or distributed under refrigeration to curtail growth and muitiplication of heat resistant foodborne pathogens that survive the minimal heat treatment used in sous-vide processing (Raffael, 1984 & 1985; Light et al., 1988). In North America, commercial application of sous-vide technology has lagged behind Europe and Japan mainly because of concerns about the safety of the process (Shamsuzzaman et al., 1992). These public health concerns are related to: the fact that *sous-vide* products are not "commercially sterile"; the ability of spores of foodborne pathogens as well as heat resistant non-spore formers to survive the mild heat processing treatment; the anaerobic conditions created in the product as a result of vacuumization which is conducive to the growth and toxin production by *Clostridium botulinum*; and the potential for temperature abuse along the food distribution chain. Thus far, very few studies have been done to examine the physical, chemical and microbiological changes occurring in

minimally processed *sous-vide* products under various storage conditions. This information would be useful in formulating or rationalizing proper handling procedures to assure the high quality shelf life and microbiological safety of these products.

Thus, the present study was aimed at investigating the physical, chemical, microbiological, and sensory changes that take place in a minimally processed rice and salmon product stored at mild temperature abuse storage conditions.

7.3. Materials and Methods

7.3.1. *Preparation of Sous-Vide Product*

The food materials used in the study viz., rice, salmon and white sauce, were obtained from a local supermarket and packaged by weighing 200 g of pre-cooked rice with 87.5 g of clover leaf canned salmon and 87.5 g of "Cordon Bleu" white sauce into a thermoformed oriented polypropylene (OPP) tray (8cm x 12cm x 3cm) as described under section 5.1. of Chapter 5.

7.3.2. *Proximate Analysis*

The formulated sous-vide rice and salmon product was analyzed for moisture, ash, crude protein, and crude fat, using standard AOAC methods (A.O.A.C. 1980), and carbohydrate was determined by difference as described under section 5.3.2. of Chapter 5. Water activity (a_w), pH and salt content were all determined as described previously (section 5.3.2., Chapter 5).

7.3.3. *Thermal Processing of Samples*

The prepared samples were heated at 65°C and 75°C for a specific time equivalent to either a 5D or a 13D reduction in the number of *S. faecium* according to procedures described by Simpson et al., (1993a). The thermally processed products were then immediately cooled under running tap water for 30 min and stored in a low temperature incubator (Fisher Scientific, Model 307, Montréal, Québec, Canada), maintained at either 5°C or 15°C. Samples stored at 5°C were analyzed after 0, 18 and 35 days while those stored at 15°C were examined after 0, 7, 14, 21, 28 and 35 days for physical, chemical and microbiological changes.

7.3.4. *Physical and Chemical Analysis*

The sous-vide rice and salmon products were analyzed after various time intervals for changes in pH, total acidity (expressed as % lactic acid), and headspace gas composition using the procedures described in section 5.3.4. of Chapter 5.

7.3.5. *Microbiological Analysis*

The formulated sous-vide rice and salmon products were analyzed for microbial counts, viz., total aerobic, anaerobic, and lactic acid bacteria counts, using standard procedures described previously under section 5.3.5. (Chapter 5).

7.3.6. *Sensory Evaluation*

The formulated sous-vide rice and salmon packages were opened and evaluated for changes in odor and color. A fresh sample was used each time as control for both color and odor. Packages were assigned a score of between 0 (fresh smell, excellent color) to +5 (foul smell, highly discolored) in a random sequence.

7.3.7. *Statistical Analysis*

A one way analysis of variance (ANOVA) comparison of means was done using Statistical Analysis System (SAS release 6.04, 1991).

7.4. Results and Discussion

A summary of the proximate analysis of the formulated rice and salmon product is presented in Table 25. Thus, the formulated rice and salmon product had ~70% moisture, 10% crude protein, ~3% fat and ~1% ash (Table 25). The ash and moisture levels were similar to that of a previously formulated sous-vide pasta product. However, the rice salmon product had relatively higher levels of crude fat, and crude protein, but lower carbohydrate content than the spaghetti and meat sauce product (Tables 19 - Chapter 5, & 25), and these differences were found to be statistically significant ($P < 0.05$) (Simpson et al., 1993b). The higher fat content associated with the rice and salmon product could be attributed to the salmon (a fatty fish) used in the product formulation.

Table 25 : Proximate Composition of Sous-vide Rice and Salmon product

Sample	Formulated rice and salmon sous-vide product
% Moisture	69.52 ± 0.62
Ash	0.94 ± 0.03
% Crude fat	2.83 ± 0.10
% Crude protein	9.63 ± 0.06
% Carbohydrate[1]	17.08 ± 0.20
pH	6.30 ± 0.10
% Salt	0.50 ± 0.01
a_w	0.99 ± 0.01

[1] Carbohydrate was calculated by difference

The higher protein content of the rice and salmon product may also be due to a relatively higher protein content derived from the salmon as compared with the sauce used in formulating the spaghetti based product.

The results of the microbiological analyses for the sous-vide rice and salmon products subjected to various heat processing treatments, then stored at 5 or 15^{o}C for up to 35 days, are shown in Figs. 20 - 23. Each data point in the Figures represents the means of three packages of each treatment sampled in duplicate. Generally, total aerobic, anaerobic and lactic acid plate counts increased in all products throughout storage, particularly at 15^{o}C.

The total plate counts were influenced by the heat treatment and storage temperature. Heat processing at 65 and 75^{o}C equivalent to a 5D process resulted in a reduction in the initial microbial load to ~ 4 log CFU/g. However, when products were subjected to a 13D process at the same temperature, a further 4 - 5 log reduction in the initial microbial load was observed (Figs. 21 & 23). Overall, the microbial growth pattern was similar to that observed in the study with sous-vide spaghetti and meat sauce product (section 5.4., Chapter 5), although the initial bacterial counts were generally higher in the sous-vide rice and salmon product. The slight difference in the initial microbial load in the rice based product is possibly due to the differences in the initial pH values in the two products.

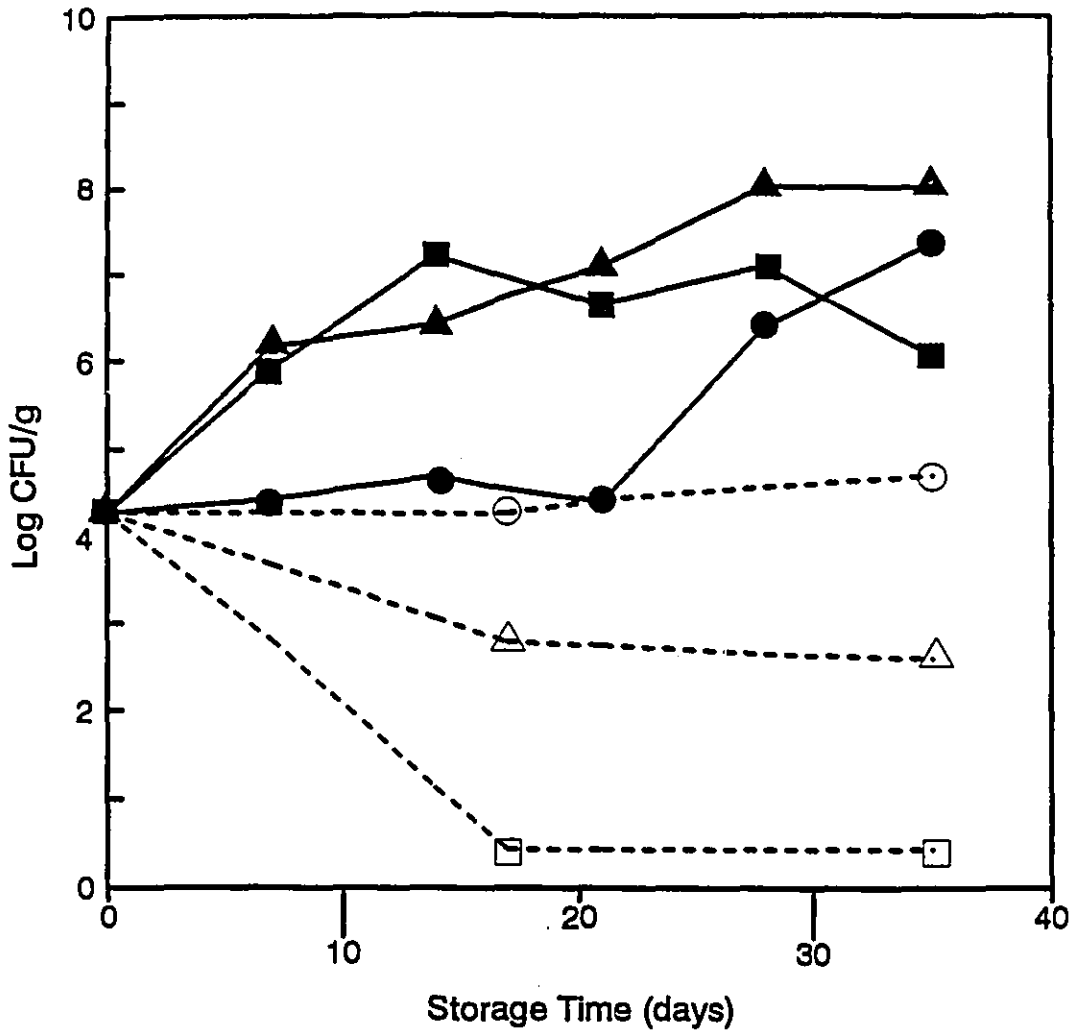


Fig. 20 : Total aerobic, anaerobic and LAB plate counts in rice and salmon product given a 5D process at 65 C and stored at 5 C or 15 C

▲ Aerobic (15 C) ; ■ Anaerobic (15 C) ; ● LAB (15 C)

△ Aerobic (5 C) □ Anaerobic (5 C) ○ LAB (5 C)

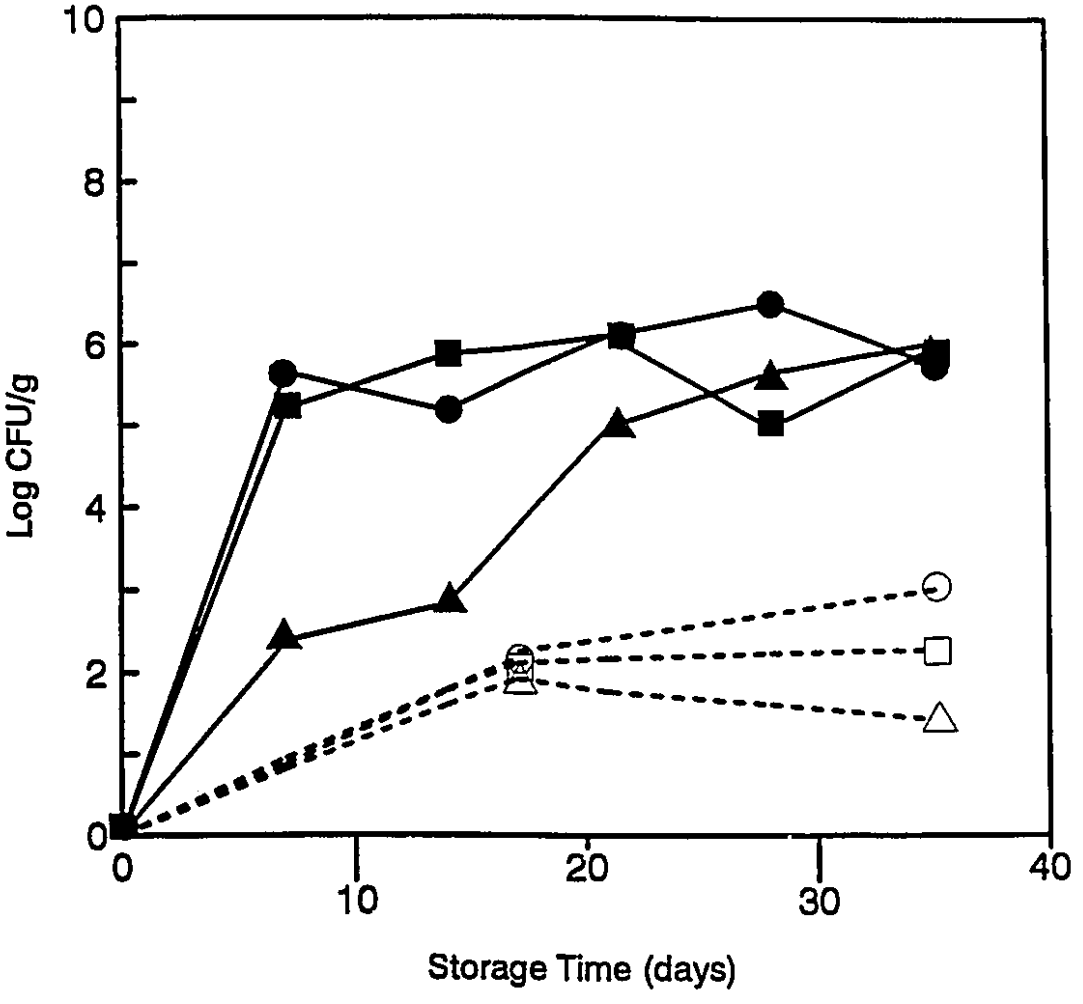


Fig. 21 : Total aerobic, anaerobic and LAB plate counts in a rice and salmon product given a 13D process at 65 C and stored at 5 C or 15 C

▲ Aerobic (15 C) ; ■ Anaerobic (15 C) ; ● LAB (15 C) ;
△ Aerobic (5 C) ; □ Anaerobic (5 C) ; ○ LAB (5 C)

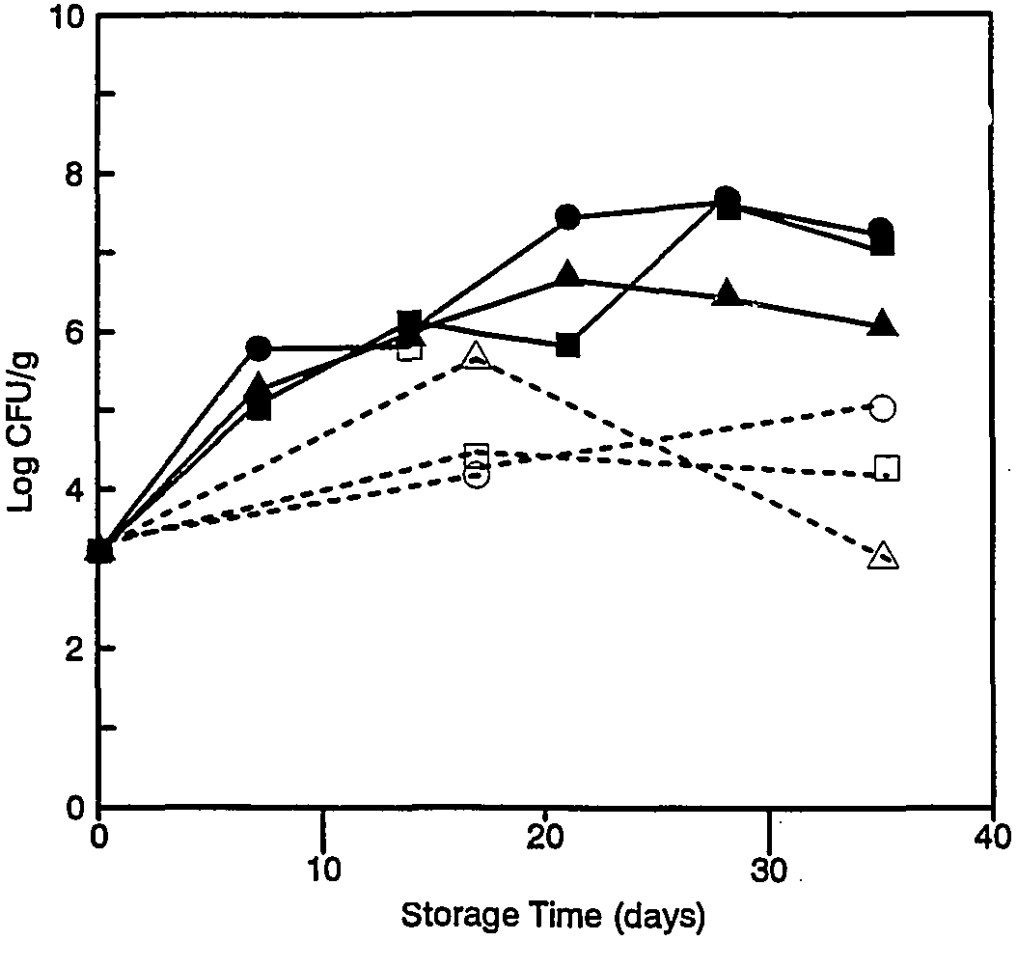


Fig. 22 : Total aerobic, anaerobic and LAB plate counts in a rice and salmon product given a 5D process at 75 C and stored at 5 C or 15 C

▲ Aerobic (15 C) ; ■ Anaerobic (15 C) ; ● LAB (15 C) ;
△ Aerobic (5 C) ; □ Anaerobic (5 C) ; ○ LAB (5 C)

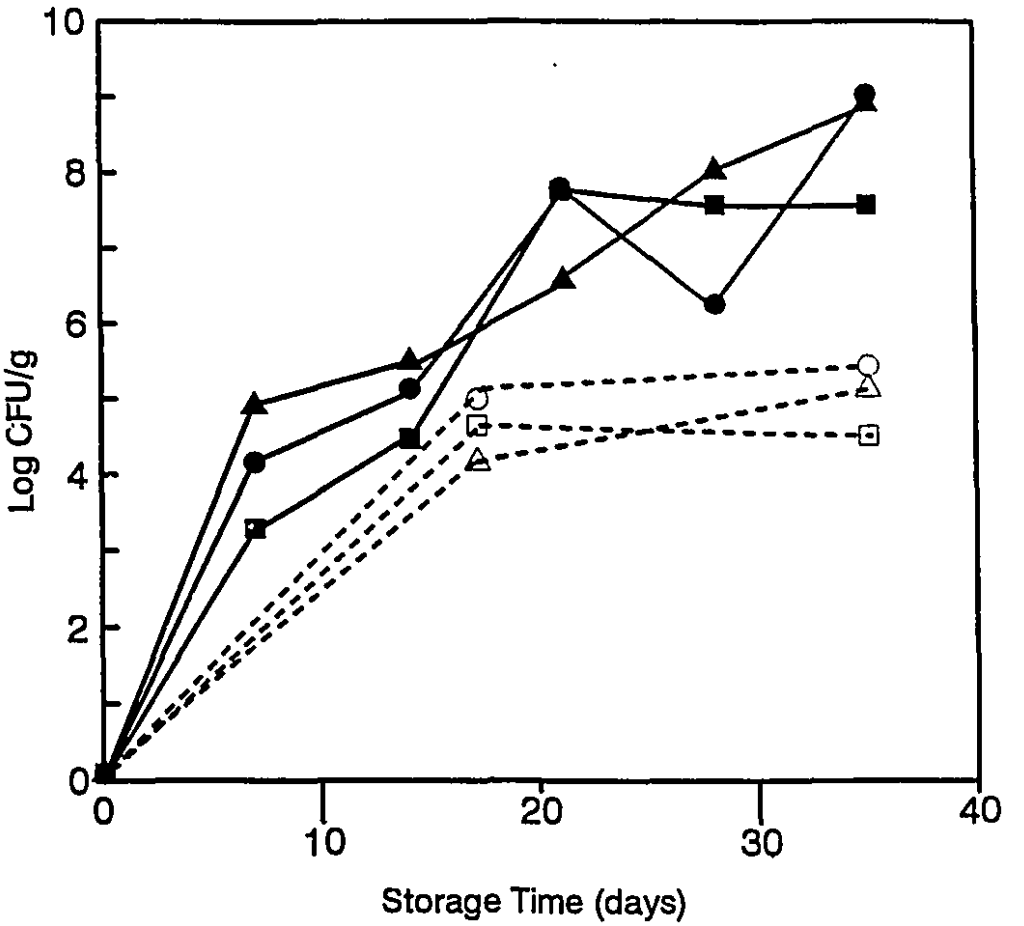


Fig. 23 : Total aerobic, anaerobic and LAB plate counts in a rice and salmon product given a 13D process at 75 C and stored at 5 C or 15 C

▲ Aerobic (15 C) ; ■ Anaerobic (15 C) ; ● LAB (15 C) ;
△ Aerobic (5 C) ; □ Anaerobic (5 C) ; ○ LAB (5 C)

Possibly, the organisms became less susceptible to thermal destruction by the relatively higher pH (6.3) of the rice and salmon product compared to the spaghetti and meat sauce product (5.1) (Tables 19 & 25). Furthermore, the organisms that survived may have been protected by the higher fat content of the rice and salmon product.

As with the sous-vide spaghetti and meat sauce product, the initial plate counts in the formulated rice and salmon products were significantly less in products subjected to a 13D process at 65^{o}C or 75^{o}C, versus samples receiving a 5D treatment at the same temperatures. Furthermore, total plate counts gradually increased throughout storage, similar to observation made with the spaghetti and meat sauce product (Figs. 20 - 21). This microbial growth pattern was similar to previous findings reported by Hollingworth et al., (1991), for pasteurized crab meat. A plausible explanation to this observation would be that a fraction of the cells were not totally inactivated by the heat treatment but only thermally injured, and were able to grow to high numbers after resuscitation in products, during storage at 15^{o}C. Again, the significance of these results is that minimally processed foods may contain thermally injured cells which are able to undergo repair, growth and multiplication during storage, particularly at temperature abuse conditions and reach levels of spoilage of public health concern. However, the final total plate counts were significantly lower ($P< 0.05$) in all products stored at 5^{o}C compared to products stored at 15^{o}C for all processing treatments emphasizing the importance of storage temperature to ensure the quality and safety of minimally processed food products (Figs. 20 - 23). The stability of the product at 5^{o}C is consistent with the literature (Gittleson et al.,

1992), and was probably due to the combined effects of heat treatment and storage temperature, which inactivated or inhibited the microbial growth and metabolism.

The microorganisms of the minimally processed sous-vide rice and salmon product comprised mainly of Gram-positive bacteria which were subdivided on the basis of their catalase reactions into *Bacillus* species and lactic acid bacteria. The *Bacillus* isolates were tentatively identified, based on their Gram stain reaction and spore stain as *Bacillus sphericus* and *Bacillus cereus*, with the latter organism accounting for approximately 90% of all the isolates (similar to the observation made with the spaghetti and meat sauce product, under section 5.4, Chapter 5). Lactic acid bacteria isolates on all plates were tentatively identified on the basis of cultural and morphological characteristics as *Leuconostoc* and *Lactobacillus* species with *Leuconostoc* species being the predominant isolates in products stored at 15^{o}C while *Lactobacillus* species were particularly the only isolate observed in plates stored at 5^{o}C.

The microorganisms associated with fish are influenced by environmental factors such as temperature and salt, and range from predominantly psychrotrophic or psychrophilic Gram-negative rods (for cold water fish), to predominantly Gram-positive mesophilic bacteria for tropical and sub-tropical fish (Kraft, 1992). The major spoilage bacteria in fish include members of the genera *Pseudomonas, Acinobacter, Flavobacterium, Streptococcus, Bacillus, Micrococcus* and *Achromobacter* (Kraft, 1992; Castell & Greenough, 1958; Lerke et al., 1965; Schewan, 1961; Schewan et al., 1960; Frazier & Westhoff, 1988).

However, since the ingredients used in formulating the sous-vide products were either canned (i.e., commercially sterilized) or pre-cooked, it is unlikely that they determined the type(s) of microorganisms encountered in these products during storage. It is more likely due to cross-contamination during formulation, transfer into containers and/or packaging of the products. This would explain the similarities in the types and growth patterns of the microorganisms in the spaghetti and meat sauce product (section 5.4., Chapter 5).

S. faecium was chosen as the target organism in establishing the pasteurization values for the products because it has been reported to have the highest thermal resistance of the vegetative organisms (Banwart, 1989; Magnus et al., 1988). However, the D value of ~1.5 at 70^{o}C and z - value of 10 obtained for *S. faecium* was lower than *S. faecalis* which had a reported D value of ~2.95 min at the same temperature and z - value (Rosset et al., 1986; Mossel and Thomas, 1988). The differences in D values were perhaps due to differences in the strains selected and also the conditions under which the value was obtained, which was not stated in the case of *S. faecalis*. The processing times for the two products were based on a D value of *S. faecium* and not *S. faecalis*, i.e., 1.5 min versus 2.95 at 70^{o}C, suggesting that both the spaghetti and meat sauce as well as the rice and salmon products received a less severe heat treatment. The time and temperature combination may still be desirable for sous-vide products from the sensory quality as well as microbiological point of view since microbial numbers were reduced to undetectable levels after a 13D heat treatment. The significance of this study is, that although the products received ~2/3 heat processing compared to *S. faecalis*, both products remained shelf-stable at 5^{o}C

but not at 15°C. The growth of microorganisms in the products confirms that pasteurized products are not commercially sterile and even vegetative bacteria, especially those present in higher fat foods may survive the heat treatment. This would explain the survival of organisms in the products especially the rice and salmon (fatty fish) even after receiving a 13D thermal treatment. However, the heat processing at pasteurization values based on the D value of *S. faecalis* would probably have inactivated virtually all spoilage microorganisms, possibly creating a more dangerous scenario since there would be no indicators of spoilage.

With the sous-vide rice and salmon products, there was a shift in bacterial population throughout storage particularly at 15°C. Initial total plate counts, aerobic and anaerobic plate counts comprised mainly of *Bacillus* species. However, after ~ 15 days, *Bacillus* species decreased and were replaced by lactic acid bacteria, specifically *Leuconostoc* as the predominant species. This observation was not unexpected due to their microaerophilic/anaerobic nature and short generation time of lactic acid bacteria and hence their ability to outgrow competitors. What was surprising was the fact that they became the predominant spoilage microorganisms in minimally processed foods given a 13D treatment at 75°C. It must be noted however that both *Lactobacillus* and *Leuconostoc* species have been frequently isolated from pasteurized fruit juices and soft drinks (Marshall & Walkley, 1952). These authors reported higher D values for *Leuconostoc* species and *Lactobacillus* isolates in pasteurized orange juice concentrate compared to pasteurized single strength juice.

The pH, and total acidity (expressed as % lactic acid) profiles for the reformulated sous-vide rice and salmon products during storage at various temperatures are summarized in Figs. 24 and 25, respectively. The initial pH was similar in all products, i.e., ~ 6.3 $\pm$ 0.1, and for products stored at 5^{o}C, pH remained fairly constant throughout storage irrespective of the heat processing treatment (Figs. 26 & 27). However, for the reformulated products stored at 15^{o}C, pH decreased slightly throughout storage as a result of increased lactic acid production throughout storage. This change was more pronounced and statistically significant ($P<0.05$) for products receiving a 13D heat processing treatment at 65^{o}C compared to products receiving similar treatment at 75^{o}C. The slight decrease in pH can be attributed to the production of lactic acid that flourished in the products throughout storage, although *Bacillus* species also have the ability to produce lactic acid and may have also contributed to the increased lactic acid concentrations found in stored products (Smith et al., 1983).

Even though the lactic acid content in the sous-vide rice and salmon product generally increased throughout storage, i.e., similar to the trends observed with the spaghetti based product (Figs. 16 & 17 - Chapter 5, vs Figs. 26 & 27), the lactic acid concentration were relatively higher in the rice and salmon products. At first glance, this observation would be unexpected since it would be predicted from the higher carbohydrate content of the spaghetti based product (Table 19 - Chapter 5, vs. Table 25), that more lactic acid should form in the spaghetti based product.

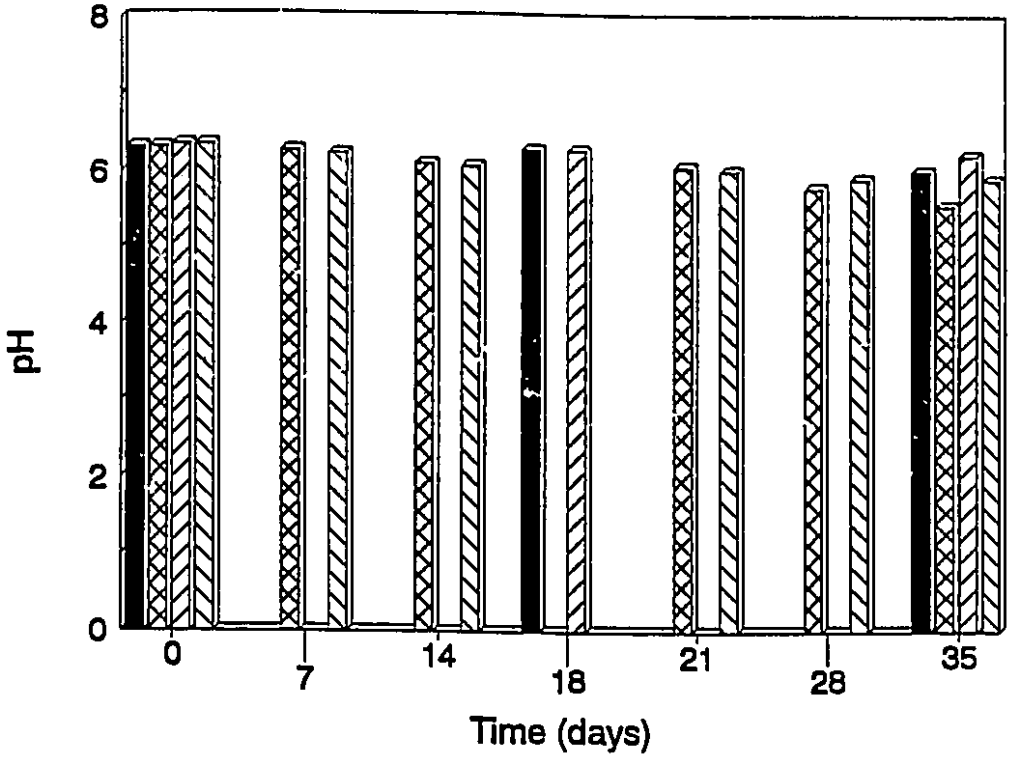


Fig. 24 : Changes in pH in sous-vide rice and salmon product, 5D or 13D processed at 65°C and stored at 5°C or 15°C for 35 days

5C (5D) ; 15C (5D) ; 5C (13D) ; 15C (13D)

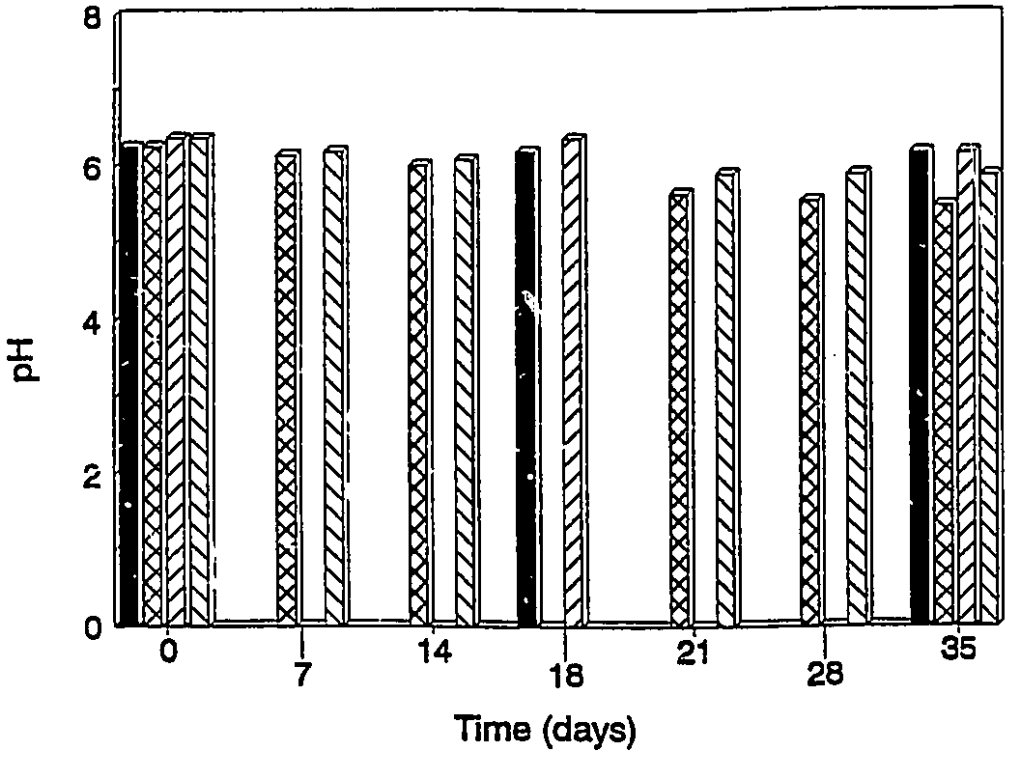


Fig. 25 : Changes in pH in sous-vide rice and salmon product, 5D or 13D processed at 75°C and stored at 5°C or 15°C for 35 days

5C (5D) ; 15C (5D) ; 5C (13D) ; 15C (13D)

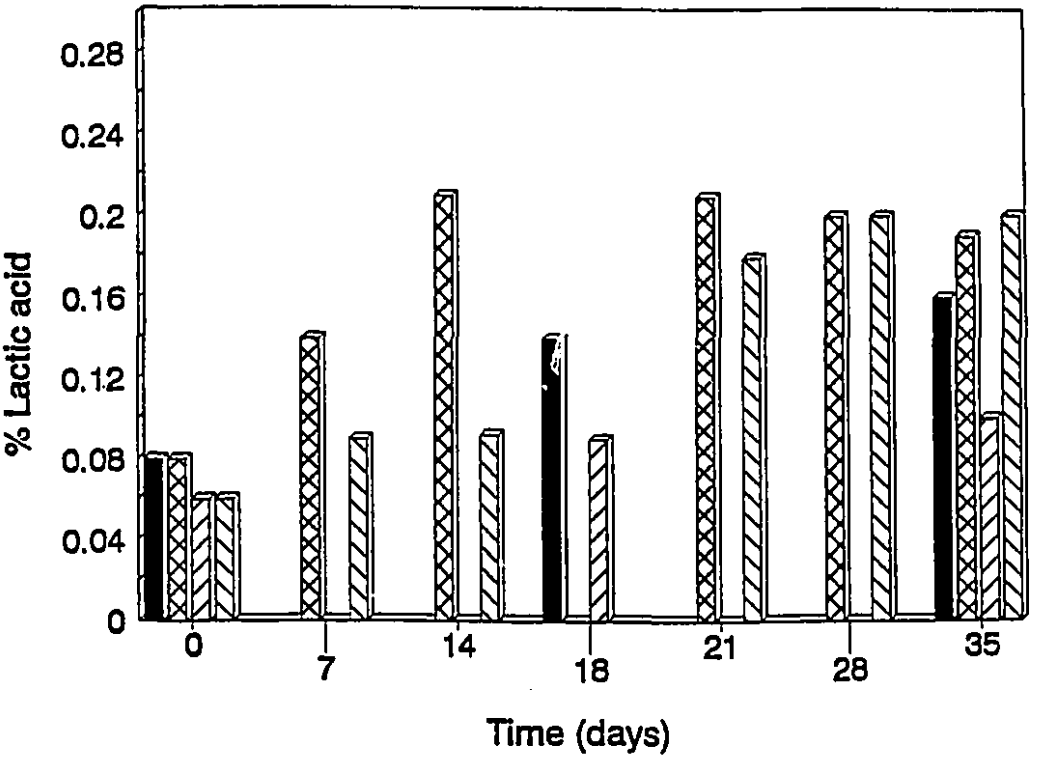


Fig. 26 : Changes in total acidity in sous-vide rice and salmon product, 5D or 13D processed at 65°C and stored at 5°C or 15°C for 35 days

5C (5D) ; 15C (5D) ; 5C (13D) ; 15C (13D)

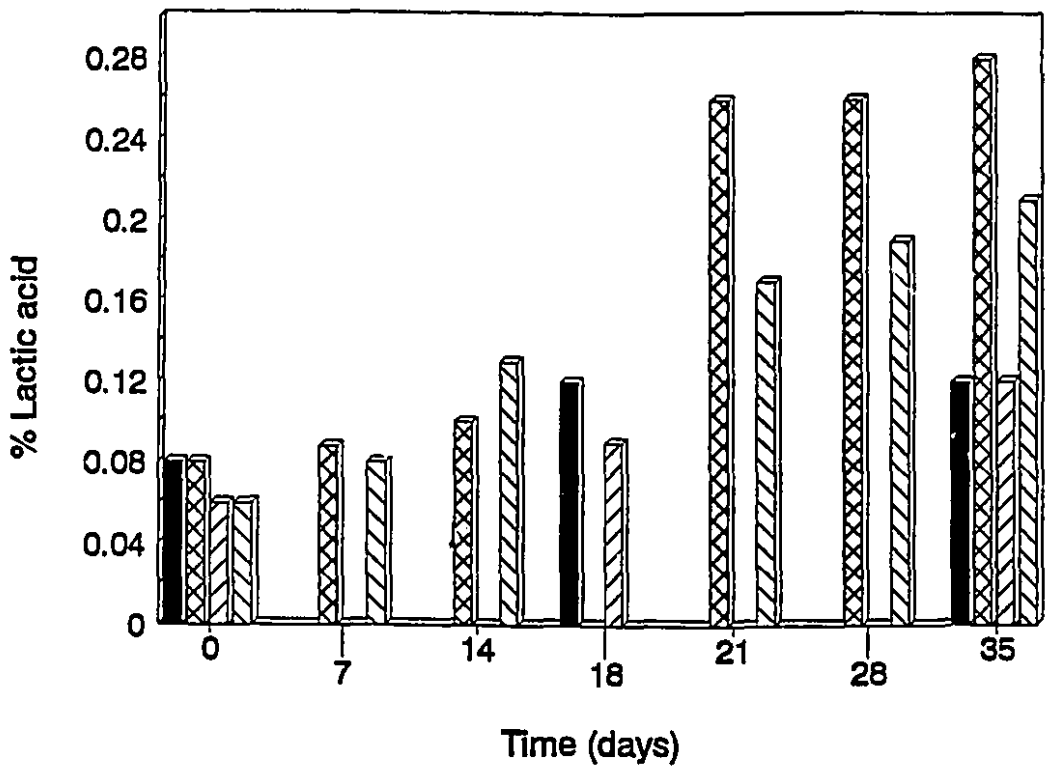


Fig. 27 : Changes in total acidity in sous-vide rice and salmon product, 5D or 13D processed at 75°C and stored at 5°C or 15°C for 35 days

5C (5D) ; 15C (5D) ; 5C (13D) ; 15C (13D)

That higher lactic acid levels formed in the rice and salmon product suggests this product provided a better medium for the growth and metabolism of lactic acid bacteria. This may be attributed to the synergism between the amylase produced by *Bacillus* species to provide sufficient sugars for fermentation by the lactic acid bacteria.

Throughout the storage period, marked changes were noted in package volume with most packages being visibly swollen after day 14 for products processed at 65°C (5D and 13D) and after day 21 for products receiving a more severe heat processing treatment (5D or 13D at 75°C). No changes in package volume were observed for products processed and stored at 5°C. Headspace gas analysis of swollen packages showed that it consisted mainly of carbon dioxide (~97%) produced presumably by heterofermentative strains of lactic acid bacteria (Table 26). Some *Bacillus* species are also heterofermentative and may have also contributed to the increase in headspace CO_2 during the early stages of storage. The changes in headspace carbon dioxide correlated with the increase in lactic acid bacteria counts, i.e., ~ 8 log CFU/g in processed products again similar to observations made with the spaghetti and meat sauce product (section 5.4., Chapter 5). This observation is not unusual and is similar to others made by Ingram and Simonsen (1980), who reported that packaging products under vacuum produces an ecosystem that encourages the growth of microaerophilic bacteria such as lactic acid bacteria.

Table 26 : Headspace gas composition of selected sous-vide rice and salmon product stored at 15^{0}C

CO_2	N_2	O_2
96.98	1.51	0.24
97.65	1.10	0.11
96.45	1.78	0.24

Sensory evaluation of products upon opening showed similarities with the spaghetti and meat sauce product (section 5.4., Chapter 5). The products stored at 5^{o}C had very good (+1) odor scores and good (+2) color scores and were almost as fresh at day 35 as at day 0. However, the products stored at 15^{o}C had a distinct fruity odor when packages were opened and they were discolored. The sensory scores correlated well with increase in both lactic acid bacteria numbers and headspace CO_2 concentration throughout storage. The typical odor of packages which were visibly swollen can be attributed to the presence of lactic acid bacteria.

7.5. Conclusion

This study has shown that physical, chemical and microbiological changes can be used as indicators of shelf life acceptability in minimally processed sous-vide products. Changes in package volume, odor/color and bacterial counts, particularly lactic acid bacteria, can all be used as viable indicators of spoilage. Based on the number of days for lactic acid bacteria to reach > 10^6 CFU/g and an odor/color score of 3, a minimally processed rice and salmon product has a shelf life of approximately 14 days when processed at 65^{o}C and stored at mild temperature abuse conditions. This shelf life could be extended to 21 days at 15^{o}C if the product were minimally processed at 75^{o}C equivalent to a 5D or 13D heat treatment. However, a shelf life of ~ 35 days is possible for minimally processed products if stored at 5^{o}C or less. The higher plate counts, CO_2 production and sensory scores in products stored at 15^{o}C emphasize the need

for strict temperature control throughout distribution and storage of minimally processed products. The survival and growth of *Bacillus* spores in minimally processed products is of concern, since it implies that *C. botulinum* spores, if present, may grow and produce toxin. However, at the processing time/temperatures used in this study, the public health concern would appear minimal since *C. botulinum* may be outgrown by lactic acid bacteria competitors and the products would be visually spoiled and rejected by the consumer. Nevertheless, this concern is justified if products were processed at slightly higher temperatures sufficient to inactivate the lactic acid bacteria, or other microbiological indicators of spoilage in sous-vide products. Challenge studies with *C. botulinum* spores are thus necessary to assess the public health safety of minimally processed products, particularly at temperature abuse conditions.

CHAPTER 8

CHALLENGE STUDIES OF A SOUS-VIDE RICE AND SALMON PRODUCT WITH *CLOSTRIDIUM BOTULINUM*

8.1. Summary

The microbiological safety of a processed *sous-vide* rice and salmon product was evaluated by "challenging" the product with *Clostridium botulinum* type E spores at a level of 10^3 spores/g sample. Prior to storage, samples were processed at 75°C for 43 min (i.e., process time equivalent to 13 decimal reductions of *Streptococcus faecium*). The effect of various levels of pH and salt (NaCl) on time until toxin detection in the samples were studied during storage at 5, 10 and 15°C. Toxin was not detected in samples at pH $\leq$ 5.3 during 42 days of storage at 5°C. However, toxin was detected in samples of pH $\geq$ 5.3 as early as day 14 and 7 during storage at 10 and 15°C, respectively. Mathematical transformation of the data showed a combination effect between pH and storage temperature. As the storage temperature and product pH increased, the log probability of toxin production also increased. Salt levels $\geq$ 2% delayed toxin formation even in products stored at 15°C. Increasing concentrations of sodium lactate also delayed toxin formation in the product, but not to the same extent as common salt. A combination of pH, salt (NaCl) and lactic acid bacteria also delayed toxin formation in the product. Microwave heating of products containing toxin at varying levels of power and time inactivated the pre-formed botulinum toxin.

8.2. Introduction

Consumer demands for foods that offer greater convenience and time savings in preparation has generated considerable interest in sous-vide technology in the food industry.

"*Sous-Vide*" products were first introduced in France over 20 years ago (Leadbetter, 1989) and today it represents one of the most common types of refrigerated processed foods with extended durability (REPFEDS). While the shelf life specified for these foods in France was originally not more than 6 days, this could be extended by a ministerial decision based on tests of microbiological criteria and sensory properties in specified laboratories (Lund & Notermans, 1993). In sous-vide processing, the food is packaged in heat-stable, air impermeable bags under vacuum, the bags are then sealed followed by cooking or pasteurization at time/temperature combinations sufficient to destroy all vegetative pathogens (Kramer, 1988). The prepared products are then cooled immediately to below $4^{o}C$ and stored under refrigeration. Although quality deterioration of sous-vide products may be due to a number of different factors, microbial activity is by far the most important factor. Therefore, control of microbial growth is the key to the extension of product shelf life during transit and subsequent storage. Produced under good manufacturing practices, *sous-vide* processed products have a shelf life of 20 to 30 days when stored at proper refrigeration temperatures (Schafheitle & Light, 1989). However, regulatory authorities recognize that these minimally processed foods may be a potential public health risk due to the growth of psychrotrophic food borne pathogens which may have survived the heating process (Baird, 1990; Beauchemin, 1991).

Since the heat treatment applied in *sous-vide* processing results primarily in the destruction of vegetative cells, bacterial spores if present, could survive the mild processing condition. Of most concern in such commercially processed foods is *Clostridium botulinum*. *Sous-vide* products contain sufficient nutrients, low salt level, high pH and an oxygen free environment conducive for the growth and toxin production by *C. botulinum* - a very heterogenous group of bacteria with two main groups recognized as causing human botulism; viz., groups I & II, representing the proteolytic types (A, B & F), and the non-proteolytic types (B, E & F), respectively. Non-proteolytic types have been associated with outbreaks involving mainly fish and fish products. The sous-vide products in addition to the mild heat treatment may have high a_w (0.90 - 0.95) and therefore require additional barriers or hurdles to ensure their microbiological stability (Leistner, 1987 & 1992). This process relies on the application of combined parameters which may act synergistically to inhibit or retard microbial growth resulting in a shelf stable product during refrigeration of the products. A proper combination of various parameters, or hurdles such as slight reduction in a_w, lowered pH, addition of either single or combined safe antimicrobial agents, pasteurization etc., will help preserve the products.

The objectives of this study were to determine (i) the potential of growth and toxin production of non-proteolytic strain of type E spores in a *sous-vide* rice and salmon product during storage at mild temperature abuse conditions, (ii) the effect of varing levels of pH during storage at 5, 10 and 15^{o}C, (iii) the levels of additional barriers of NaCl, sodium lactate and bacteriocin-producing lactic acid bacteria to control the growth of *C. botulinum* in the minimally processed product, (iv) the use of ¯ - 2 macroglobulin, & (v) the effect of microwave heating on the

pre-formed botulinum toxin.

8.3. Materials and Methods

8.3.1. *Production of Spores*

The strains of *C. botulinum* used were non-proteolytic types E 8550, Gorden, Bennett and E Russ obtained from Microbial Hazards Bureau (HPB), Health and Welfare Canada, Ottawa. Spore suspensions of the individual strains were prepared by growing the strains in trypticase-peptone-glucose-yeast extract (TPGY) broth at 35°C for 10 days according to methods outlined by Health and Welfare Canada, Ottawa (HPB, 1989). The spores were harvested by three successsive centrifugations with sterile distilled water at 17,500 xg for 20 min at 4°C, and resuspended in gelatin-phosphate buffer (pH 6.6) and stored refrigerated at ~4°C until used.

8.3.2. *Enumeration of Spores*

Spores were heat shocked at 60°C for 10 min and decimally diluted with gelatin phosphate buffer. Aliquots of 0.1 mL of the diluted sample was enumerated on Wynne's agar (Difco) as outlined by Hauschild and Hilsheimer (1977). For product inoculation, spores were pooled from each strain into a gelatin-phosphate buffer to give a final concentration of 10^8/mL.

8.3.3. *Experimental Design*

The experimental design used was that of a factorial design. The effect of

5 levels of pH (5.0, 5.3, 5.6, 5.9 and control 6.3) at 3 different temperatures was investigated. Other factors studied included sodium chloride (salt) (1 - 3%), sodium lactate (1 - 3%) and lactic acid bacteria (10^3, 10^6 CFU/g).

8.3.4. *Product Inoculation & Packaging*

The rice and salmon product was prepared by adding 200 g cooked rice to 87.5 g Clover Leaf brand canned salmon and 87.5 g of "Cordon Bleu" brand white sauce, and all these food products were purchased from a local supermarket. The ingredients were weighed directly into a pre-tared stomacher bag and inoculated with 0.5 mL of prepared spore inoculum to give a final inoculum of 10^3 spores/g of sample. The contents of the bag were then thoroughly massaged by hand to mix the sample. The mixture was then carefully transferred into a packaging tray and vacuum packaged using a vacuum skin machine. This was transferred to a thermoformed oriented polypropylene tray and vacuum packaged with a top film of Wallo peel (polyester/polyamide/EVOH, O_2 transmission rate, 12cc/m^2 atm at 23^{o}C) using a vacuum skin/thermoforming packaging system (Trigon Model # RM331 MMIS Inc., Toronto, Ontario), according to the method of Simpson et al., (1993a).

8.3.5. *Effect of Additional Barriers*

The additional barriers investigated on growth of and toxin production by *C. botulinum* were pH, NaCl, Na-lactate, bacteriocin producing lactic acid bacteria and α-2-macroglobulin.

8.3.6. *Effects of pH and Salt*

The effect of pH on growth of and toxin production by *C. botulinum* was investigated in the reformulated products by first adjusting the sauce to various pH levels (5.0, 5.3, 5.6, and 5.9) using appropriate amounts of lactic acid (BDH), followed by inoculation with *C. botulinum* type E spores, and heat processing prior to storage at 5, 10 and 15^{o}C. The pH of the samples was measured by inserting the probe of Acumet pH meter directly into the samples.

The effect of common salt (NaCl), hence water activity (a_w) on toxin production was studied at pH 6.3 by adding appropriate amounts of salt to the sauce to give final salt concentration of 1, 2 and 3% w/w, respectively. The salt concentrations were measured using a Horiba compact salt meter C-121 (Horiba Instruments Inc., Irvine, CA) which has been previously calibrated with standard salt solutions. The a_w of the final products were measured using a water activity meter, Decagon, model CX-II.

The effect of sodium lactate (Purasal S), {Purac America Inc. Lincolnshire Corporate center, Lincolnshire, IL} on toxin production was determined at pH 6.3 by incorporating the Na-Lactate into the product, to give varying final concentrations (i.e., 0.5, 1.0, 1.5, 2.0, 2.5, & 3.0%), one set of which was stored at 10^{o}C and the other at 15^{o}C.

The effect of α-2-macroglobulin on toxin production was studied at pH 6.3. Alpha-2-macroglobulin, purchased from Boehringer Mannheim (Laval, Québec, Canada), was weighed into the sauce such that the final concentration of the product was 1 mg/375 g sample (2.7 ppm). This was added to products prior to

packaging and processing.

8.3.7. *Effect of Lactic Acid Bacteria*

The strains of lactic acid bacteria used for the study were *Pediococcus pentasaceous* ATCC 43200, and *Lactococcus lactis* ATCC 11454. Lactic acid bacteria cultures were grown overnight in MRS broth in a candle jar incubator at 30°C. The cells were harvested and washed twice by centrifugation at 4°C and 10,000g for 20 min. The cell pellets were resuspended in gelatin phosphate buffer and standardized to a turbidance (600 nm) of 0.40, which corresponded to a viable cell count of ~10^8 CFU/mL. Two levels of inoculum (10^3 & 10^6 cells/g sample) of each strain were used at two pH levels (6.3 and 5.9), and at 1% salt concentration.

8.3.8. *Thermal Processing and Sampling of Product*

The prepared samples processed at time-temperature condition previously established by Simpson et al., (1993a), to achieve a 13D reduction in *Streptococcus faecium*, i.e., heating in a water bath maintained at 75°C for 43 min. Products were then cooled rapidly in another water bath under running cold tap water and then stored at 5, 10 or 15°C for a 42 day period. After the first 7 days and subsequently at 7 day time intervals, samples were withdrawn in triplicate and analyzed for toxin.

8.3.9. *Toxin Assay*

At each sampling time, 20 g of sample from triplicate packages in each treatment were weighed into stomacher bags with 20 g of gelatin-phosphate buffer and homogenized for 2 min, then cetrifuged at 17,500 xg for 20 min. The supernatant was then filter-sterilized and 0.5 mL samples were injected intraperitoneally into duplicate mice and they were observed for 3 days for symptoms of botulism, such as pinched waist, laboured breathing and or death.

The time until toxin production was defined as the earliest time at which toxin was detected in any given specific treatment. For example, if for a specific treatment, two out of three samples were toxic on day 14 and then two out of three were again toxic after 21 days in another set of replicates, the reported time until toxin production was recorded as 14 days.

For toxin confirmation and toxin type identification, samples that killed mice were retested with antitoxin by adding 0.4 mL of botulinum antisera E to 1.4 mL of a 1 : 1 dilution of the supernatant. This mixture was left to stand 45 min at room temperature and then injected into mice and monitored for symptons of botulism.

8.3.10. *Headspace Gas Analysis*

For headspace gas analysis, swollen samples were analyzed for headspace gas composition by withdrawing gas samples using a 0.5 mL gas-tight pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, LA) through silicone rubber seals attached to the outside of each package. Headspace gas

was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector and using Porapack Q, with molecular sieve 5A (80 - 100 mesh) columns in series. Helium was used as the carrier gas at a flow rate of 20 mL min^{-1}. The column oven was set at 80°C. The injector and detector were set at 100°C. Peaks were recorded and analyzed with a Hewlett-Packard integrator (model 3390A, Hewlett-Packard Co., Avondale, PA).

8.3.11. *Microwave Treatment*

Samples in which toxin was detected were individually microwave heated at either full or half power for 5 or 10 min, using a conventional domestic microwave oven (Sears Kenmore brand) with 800 Watts of power. At the end of microwave heating, the products were then retested for presence of toxin as described above.

8.3.12. *Calculation of Probability*

The number of toxic samples was converted to the **"Most Probable Number"** (MPN) of spores able to initiate growth and toxin production in each set of conditions. The MPN was calculated from the Halvorson-Ziegler equation (Halvorson & Ziegler, 1933) as :

$$\ln (n/q)$$

where **"n"**, is the number of samples analyzed and **"q"**, the number of non-toxic

samples as described by Hauschild (1982) and was used as the baseline. The probability of toxigenesis from a single spore was then defined as :

$$\mathbf{P\ (\%) = (MPN\ X\ 100)/inoculum}$$

When no samples were toxic at 15^{o}C, 10^{o}C and 5^{o}C P(%) was taken as 10^{-4} (Dodds, 1989). The P(%) values calculated were plotted against storage time, a_w, or pH as shown in the results and discussion section.

8.3.13. *Statistical Analysis*

The effects of the experimental factors (i.e., pH & a_w) on P(%) and on the time until first toxin production (lag time) were further investigated by a simple regression technique to select the best model for predicting P(%) and lag time. All statistical calculations were performed by the general linear models procedure of the STAT graphics statistical package (Release 3.0, GSC*CGI computer graphics for Personal Computers, Graphic software systems Inc. 1988; Cary, NC).

8.4. Results and Discussion

Growth and toxin production by *C. botulinum* type E occurred in all control rice and salmon products (pH 6.3, a_w 0.997) stored at 5, 10 and 15^{o}C (Figs. 28 - 30), indicating that the pasteurization process was not sufficient to inactivate the spores.

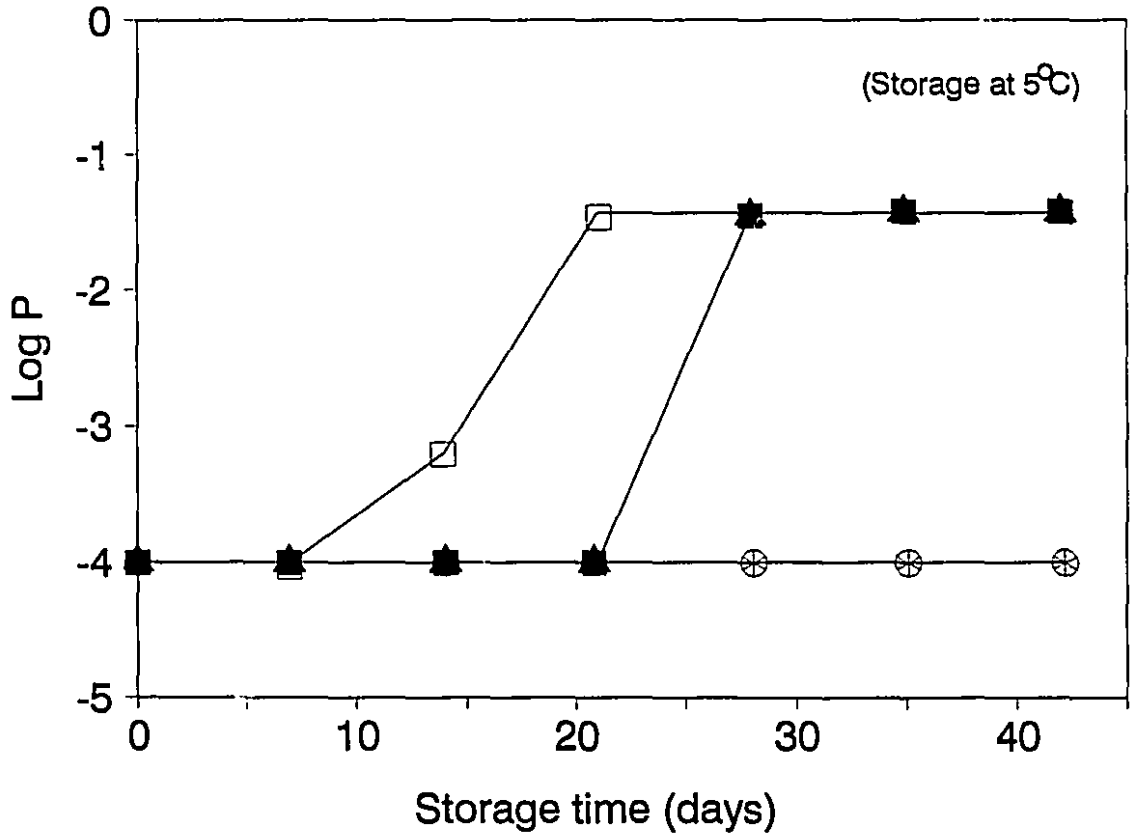


Fig. 28 : Effect of pH on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 5°C for up to 42 days.

□ Control (pH 6.3); ▲ pH 5.9; ■ pH 5.6; ✳ pH 5.3; ○ pH 5.

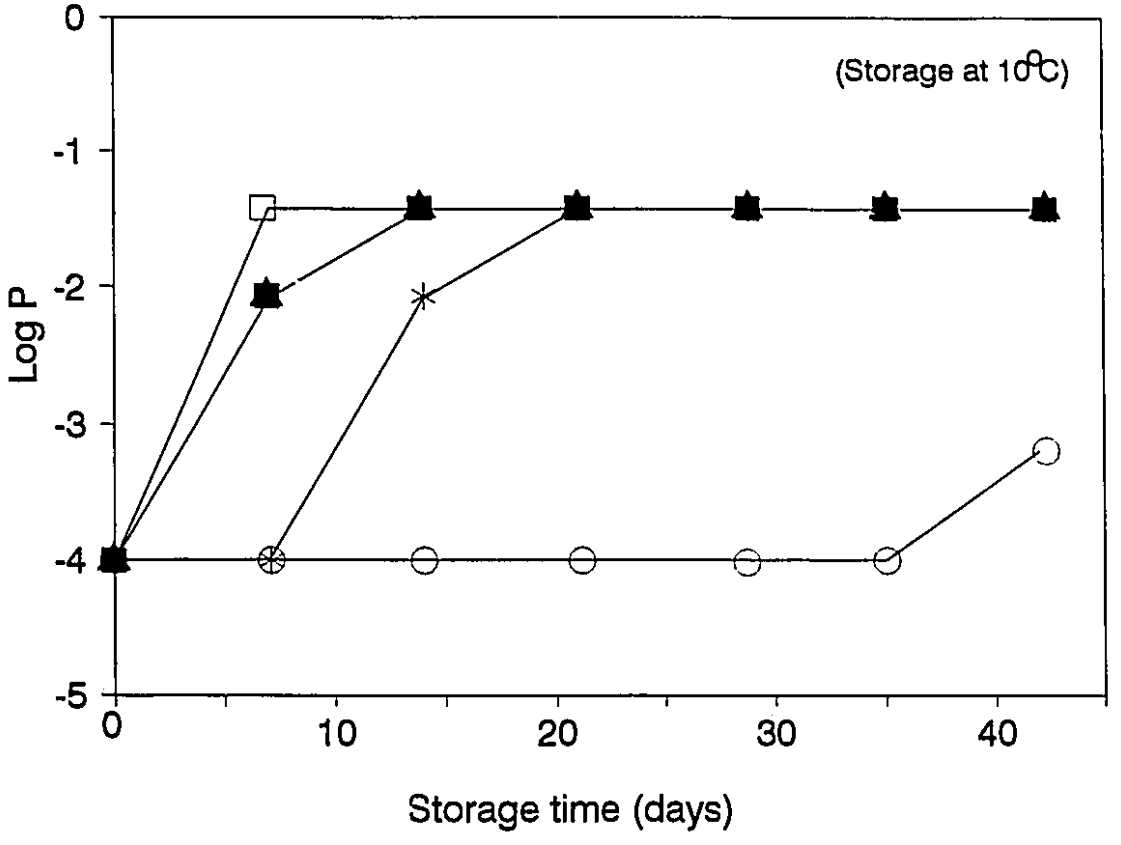


Fig. 29 : Effect of pH on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 10°C for up to 42 days.

□ Control (pH 6.3); ▲ pH 5.9; ■ pH 5.6; ✳ pH 5.3; ○ pH 5.

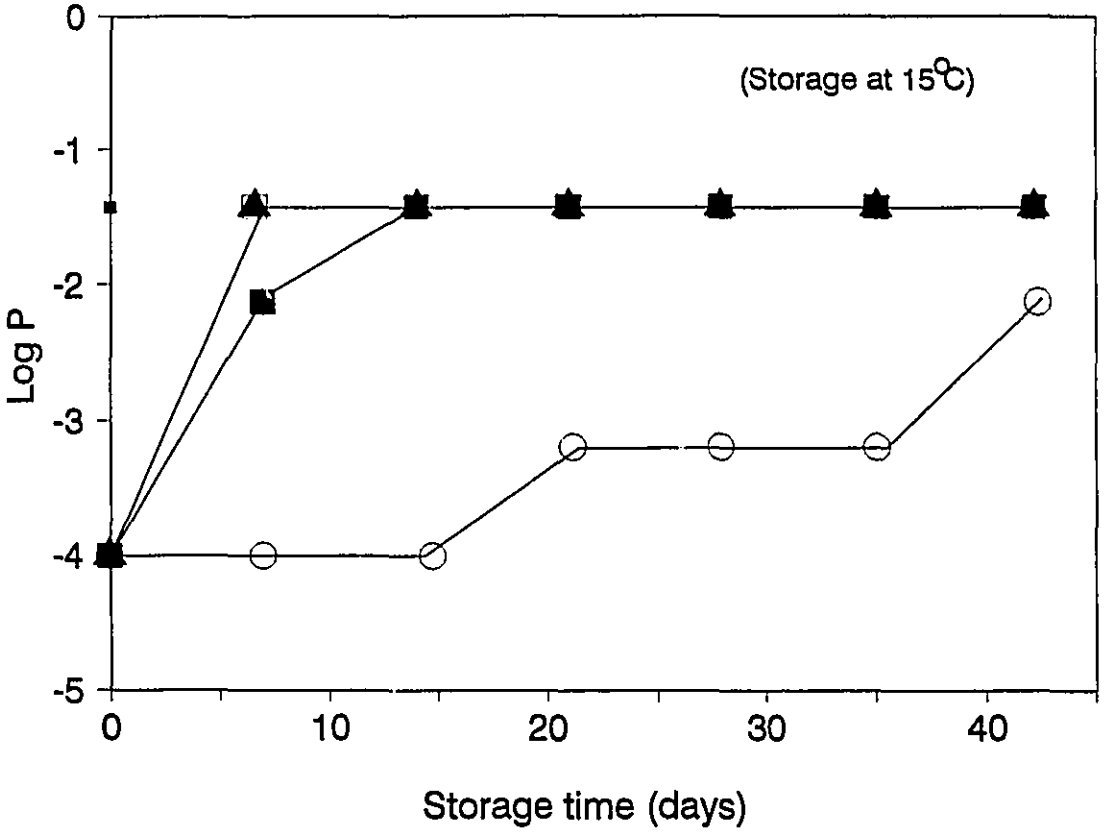


Fig. 30 : Effect of pH on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 15°C for up to 42 days.

□ Control (pH 6.3); ▲ pH 5.9; ■ pH 5.6; ✳ pH 5.3; ○ pH 5.

Thus, the mild heat processing heat shocked and stimulated the spores to germinate and grow. These results were expected since *C. botulinum* was not the target organism when pasteurization values were established for the sous-vide rice and salmon product. Sampling for toxin analysis was done at 7 days time intervals since preliminary tests indicated that, unlike the proteolytic types A and B, toxin production occurred earlier in this particular product during storage at 15^{o}C. Type E spores were used in the challenge study since it is the type most often encountered in fish and fish products. Several studies have shown that fresh fish may be contaminated with *C. botulinum* type E, either as a result of the organisms being present in the microbiota of the fish ecosystem or as a result of post-catching contamination during processing (Bott et al., 1966; Foster et al., 1965; Fantasia and Duran, 1969; Craig et al., 1968; Insalata et al., 1967). Food borne outbreaks caused by fish are predominantly due to non-proteolytic type E strains of *C. botulinum* in products which were consumed without further heat processing. According to Eklund (1982) and Hobbs (1976), the type E and non-proteolytic types B and F are able to grow and produce toxin at temperatures as low as 3.3^{o}C. Thus, it would be expected that maintaining the sous-vide fish products below this temperature would reduce the *C. botulinum* hazard. However, storage temperatures of 10 and 15^{o}C were used to represent mild temperature abuse conditions, since surveys of retail cases in supermarket and domestic refrigerators indicated that 20% exceeded temperatures of 10^{o}C (Davidson, 1987; Harris, 1989; Van Garde & Woodburn, 1987).

The inoculum level of *C. botulinum* used in this study was substantially higher than the contamination level which has been found in either fresh or

processed food products. For example, a maximum spore load of 17 spores/100g has been found in fillets of haddock caught from open waters (Eyles & Warth, 1981), and 5.3 spores/g in farmed trout (Huss et al., 1974).
Therefore, the inoculum level and the storage condition used in this study represent the worst case scenario for a *sous vide* product.

The effect of pH on the growth of and toxin production by *C. botulinum* type E during storage at various temperatures (5, 10 & 15^{o}C) is shown in Figs. 28 - 30. The data summarized in these figures indicate that during storage at 5^{o}C, toxin was not detected in any samples reformulated to pH 5.3 and 5.0 at 42 days, while toxin was detected at day 28 in samples of pH 5.9 & 5.6, with concomitant gas production and swelling in some of these packages. For the samples stored at 10^{o}C (Fig. 29), toxin was detected in the control and samples of pH $\geq$ 5.6 as early as day 7, while toxin was not detected until day 21 and day 42 in the pH 5.3 and pH 5.0 samples, respectively. However, in products stored at 15^{o}C, toxin was detected in all samples within 7 - 14 days with the exception of product of pH 5.0 in which toxin was detected at day 42 (Fig. 30). These observations indicate that low temperature storage is relatively more effective in slowing down toxin production as compared with mild temperature abuse conditions. Furthermore, it indicates that products reformulated to pH (5.0) can only be regarded as safe at low temperatures. Similar observations were made in studies with spaghetti and meat sauce where toxin was first detected after day 35 (1 out of 3 samples) in products of pH 5.25 during storage at 15^{o}C.

Lactic acid was used to lower the pH of the samples since it is a normal constituent of muscle tissue and has also been used as an antimicrobial agent in

the decontamination of meat (Smulders et al., 1986). Also, lactic acid has been found to be a more effective inhibitory acidulant (Young-Perkins & Merson, 1987; Juven, 1976).

The minimum pH requirement for growth of non-proteolytic *C. botulinum* is reported to be 5.0 (Hauschild, 1989; Odlaug & Pflug, 1978; Townsend et al., 1954). However, the results of this study show that pH alone is not sufficient in controlling toxin production at abusive storage temperature (15^{o}C) by non-proteolytic *C. botulinum* type E spores in a minimally processed sous-vide fish product. Products with a pH $\geq$ 5.0 could obviously pose a public health hazard and additional barriers, e.g., water activity reduction are necessary in medium acid products (pH 5 - 6) to prevent the growth of and toxin production by *C. botulinum*, particularly at mild temperature abuse conditions.

All products were visibly spoiled, i.e., visibly swollen at the respective times when toxin was detected during storage at 10 and 15^{o}C. Swelling was due to headspace carbon dioxide production and confirms spoilage profiles by lactic acid bacteria in previous shelf life studies of the product (Simpson et al., 1993b). The fact that there were visible signs of spoilage in these samples of varying pH values stored at 10 and 15^{o}C indicates that spoilage preceded toxigenesis. However, at 5^{o}C, gas production did not occur in some of the samples indicating that toxin formation preceded spoilage. Toxin production prior to spoilage at 10^{o}C or below has been observed by Eklund (1982) and Lee & Solberg (1983), although other researchers maintain that spoilage usually precedes toxigenesis in products stored at $\leq$ 10^{o}C, (Lindroth & Genigeorgis, 1986). These differences could be attributed to the combination effect of pH and temperature in further

extending the storage life of the product at 5°C.

Since toxin was detected in samples stored at 5°C (Fig. 28), low storage temperature alone is not sufficient to ensure the safety of minimally processed fish products. Also, as the storage temperature increased from 5 to 15°C, the time interval between obvious spoilage and the time until toxin detection decreased. This observation was similar to that made by other researchers on fish products (Cann et al., 1980; Eklund, 1982; Eyles & Warth, 1981; Lee & Solberg, 1983; Lindsay, 1983; Post et al., 1985).

The data from these studies were transformed into mathematical models (Table 27) and used to construct three dimensional response and two dimensional contour plots of the combination effect of pH and temperature on probability of toxin production (Figs. 31 - 33). Webster (1989) described a model as a "system of postulates, data and inferences" presented as a mathematical description of an entity, or state of affairs. Predictive modelling has been used in several microbiological studies, (Dodds, 1989; Chirife & Favetto, 1992; van de Voort & Stanley, 1984) inorder to assure the quality and safety of fish products (McMeekin et al., 1992).

Using the predictive models in Table 27 response surface curves and contour plots were drawn to illustrate the combination effect of pH and temperature on log probability of toxin production. Although the R^2 values obtained for the models appeared to be low, on a descriptive basis the curves did show the trend observed.

Table 27 : Predictive Equation Relating pH (p), Temperature (T), Storage Time (d), and Log Probability (P), of Toxin Production by *C. botulinum* in Sous-vide Rice and Salmon Product

Model No.	Equation	R^2
1	$\text{Log } P = 8.07 + 0.01\ d + 0.58\ T + 0.01\ d^2 - 0.02\ T^2 - 0.01\ T \times d$	0.75
2.	$\text{Log } P = -29.67 + 5.99\ p - 0.36\ p^2 + 1.49\ T - 0.02\ T^2 - 0.18\ p \times T$	0.69
3.	$\text{Log } P = -16.62 - 0.03\ d + 4.19\ p + 0.01\ d^2 - 0.36\ p^2 + 0.01\ p \times d$	0.65

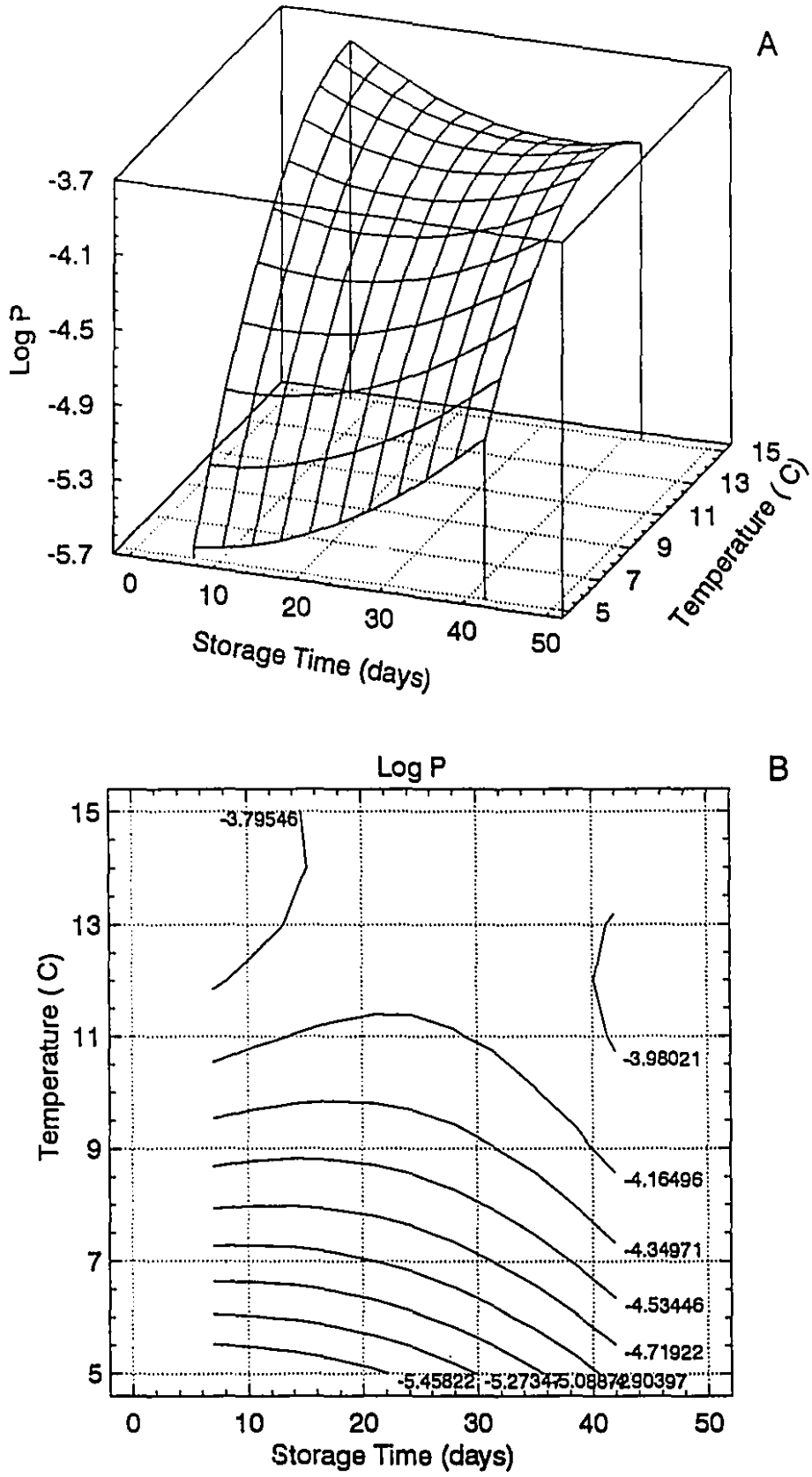


Fig. 31 : Response surface graph (A) and contour plot (B) generated with Model 1, showing the effect of time and temperature on Log P of *C. botulinum* toxin production in sous-vide rice and salmon product

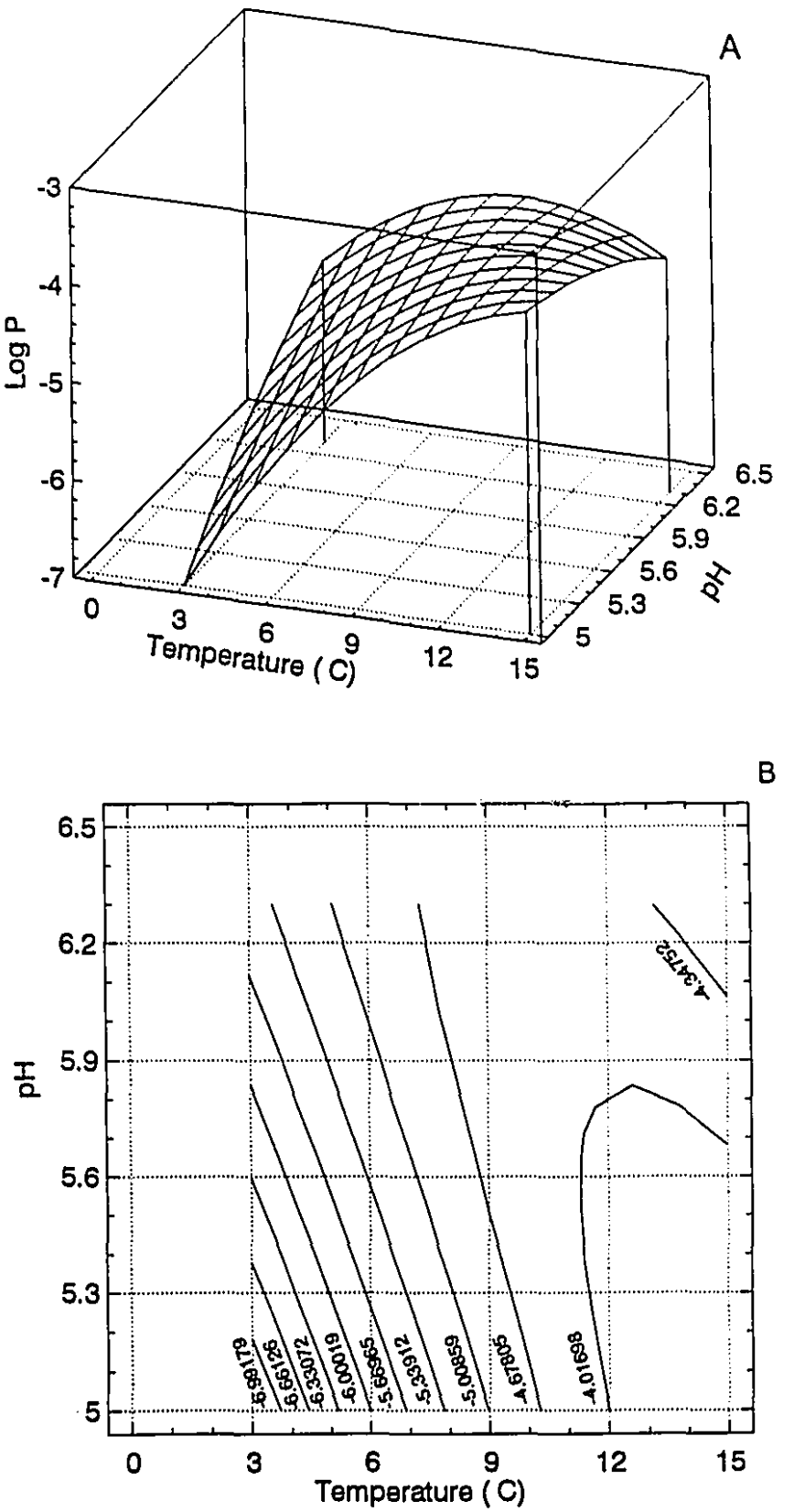


Fig. 32 : Response surface graph (A) and contour plot (B) generated with Model 2, showing the effect of pH and temperature on Log P of *C. botulinum* toxin production in sous-vide rice and salmon product

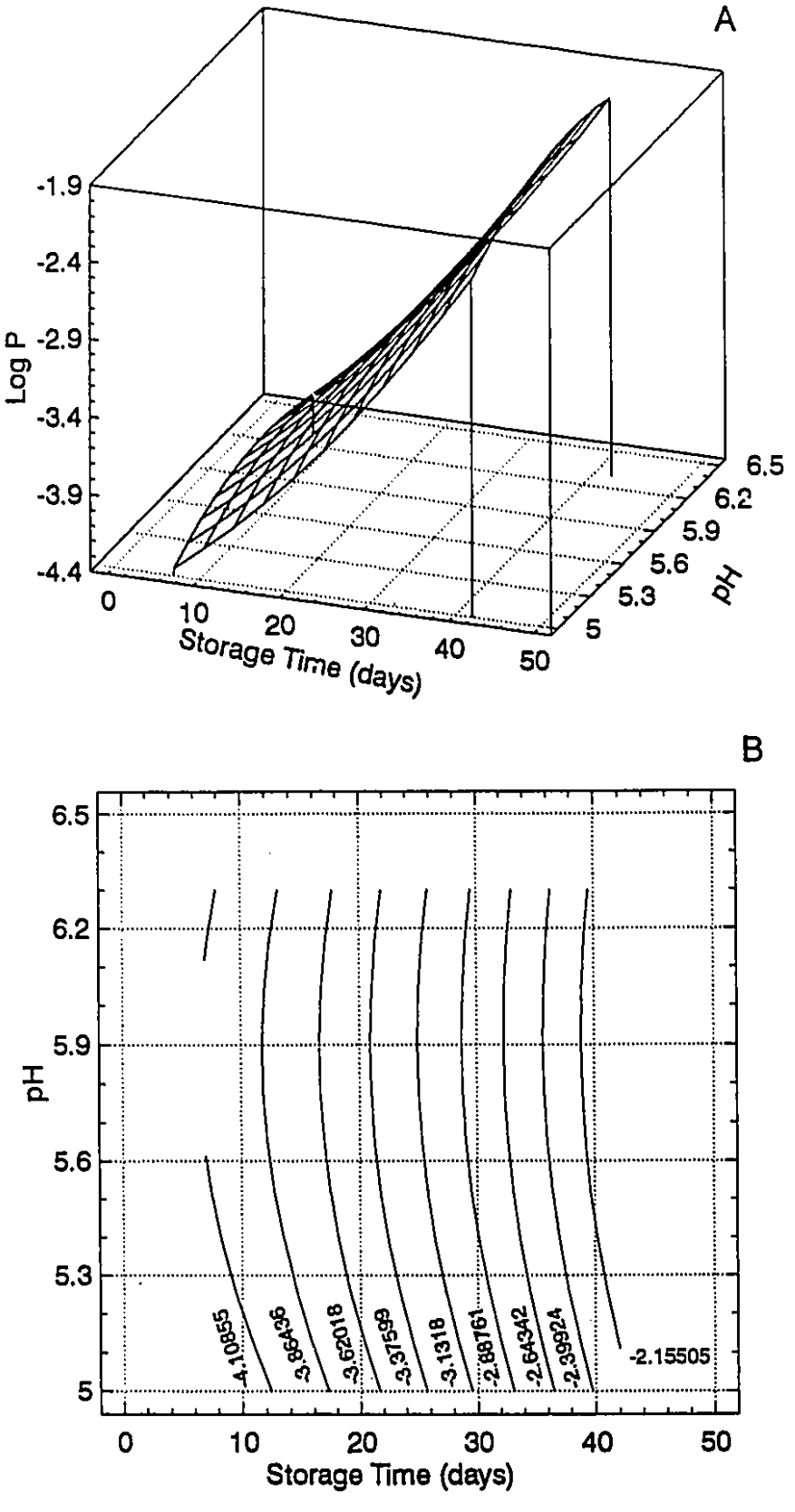


Fig. 33 : Response surface graph (A) and contour plot (B) generated with Model 3, showing the effect of storage time and pH on Log P of *C. botulinum* toxin production in sous-vide rice and salmon product

The log probability of growth and toxin production by one *C. botulinum* spore increased with increasing storage time and temperature (Fig. 31). As both pH and temperature decreased, the log probability of growth and toxin production decreased (Fig. 32). Consequently, when pH and storage time decreased, the log probability of growth and toxin production also decreased (Fig. 33). These observations are consistent with previous investigators that mathematical models can be used to quantify the risk of *C. botulinum* growth in foods affected by different food and environmental parameters (Chirife & Favetto, 1992; Dodds, 1989; Hauschild, 1982; Ikawa & Genigeorgis, 1987; Jensen et al., 1987). However, they are limited in that models apply only to discrete conditions used in each test and cannot be extrapolated to factor levels outside the range used in this study or to other food products (Baird-Parker & Freame, 1967; Emodi & Lechowich 1969; Ohye & Christian, 1967; Pivnick et al., 1969; Segner et al., 1966).

Since medium acid products may pose a greater public health risk, particularly at abuse storage conditions, studies were done to determine the at abusive storage temperature (15^{o}C) effects of additional barriers (NaCl, sodium lactate) on the growth and toxin production in these products stored at 10 and 15^{o}C. The effects of decreasing water activity through variation in salt (NaCl) levels on toxin production in reformulated rice and salmon product (pH 6.3) stored at 15^{o}C only is shown in Fig. 34. Adding salt decreased the product's a_w from 0.997 (control) to 0.977 for products containing an additional 3% salt. This addition of salt, and/or reduction in a_w, had a significant effect on toxin production by *C. botulinum*. At 3% salt concentration, toxin was not detected in any samples throughout the 42 day storage period at 15^{o}C.

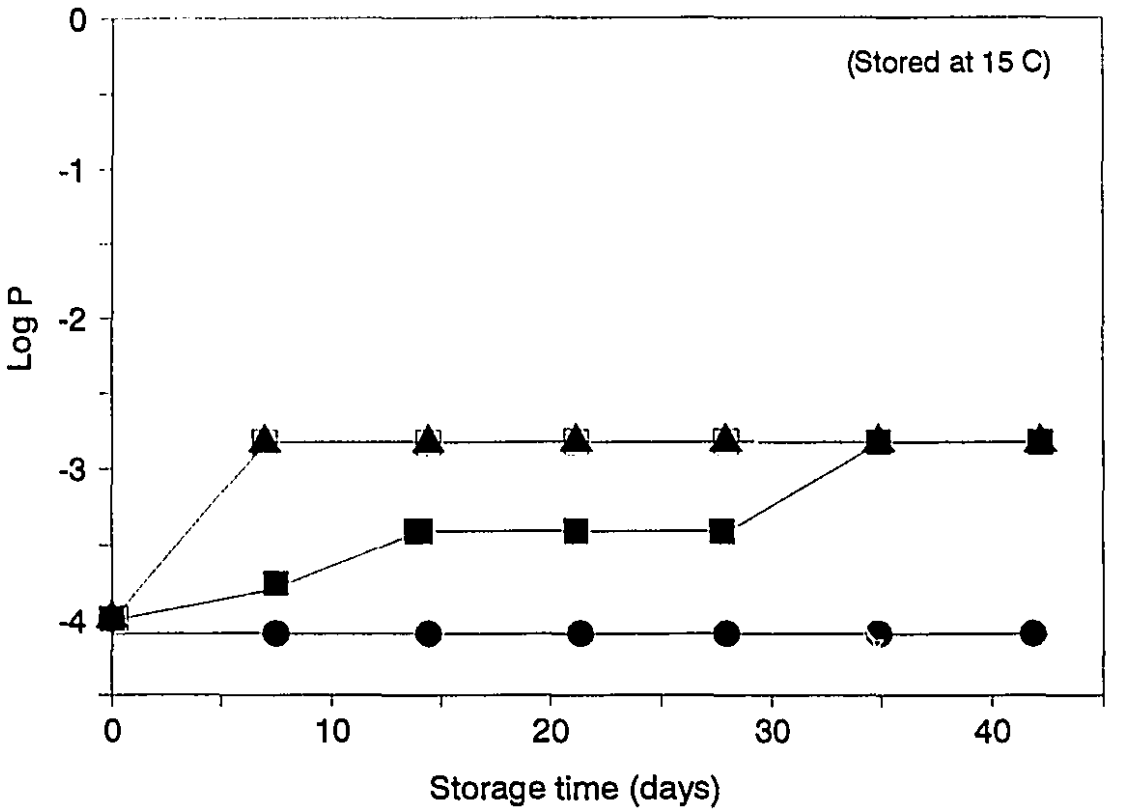


Fig. 34 : Effect of a_w (set with NaCl) on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 15°C for up to 42 days.

□ Control (Aw 0.997); ▲ Aw 0.989; ■ Aw 0.985; ● Aw 0.977.

The results of this study confirm the importance of salt (NaCl) in the control of food-borne *C. botulinum*. The decrease in water activity by salt and the delay in toxin production at higher salt levels is consistent with previous observations (Sperber, 1982; Emodi & Lechowich, 1969; Baird-Parker & Freame, 1967). These authors reported minimum water activities of 0.970 - 0.972 for *C. botulinum* type E spores in NaCl-containing foods as media. Although a lower minimum water activity was observed for growth of, and toxin production by *C. botulinum* in NaCl adjusted substrates, the inhibitory effect of lower salt concentrations and hence higher a_w values in this study can be attributed to the combined synergistic effect of reduced a_w and storage temperature (Mossel, 1983; Leistner & Rodel, 1976; Scott, 1989; Baird-Parker & Freame, 1967).

The effect of various concentrations of sodium lactate on the production of toxin by *C. botulinum* in products at 10 and 15°C is summarized in Figs. 35 and 36. The a_w of the products decreased with increasing Na-lactate concentration, and from 2% to 3%, toxin production was delayed. For example, toxin was detected in the rice and salmon product with no added sodium lactate after 9 days at 10°C, while toxin was detected in samples containing 1 - 2% at day 12. For samples containing 2.5 & 3% sodium lactate, toxin was detected at day 15. However, the pH of the samples was found not to be affected by the various levels of sodium lactate (results not shown). Similar trends were observed for products stored at 15°C, when toxin was detected in all re-formulated products after 14 days storage at this temperature. These results were similar to those of Maas et al., (1989) and Duxbury, (1990).

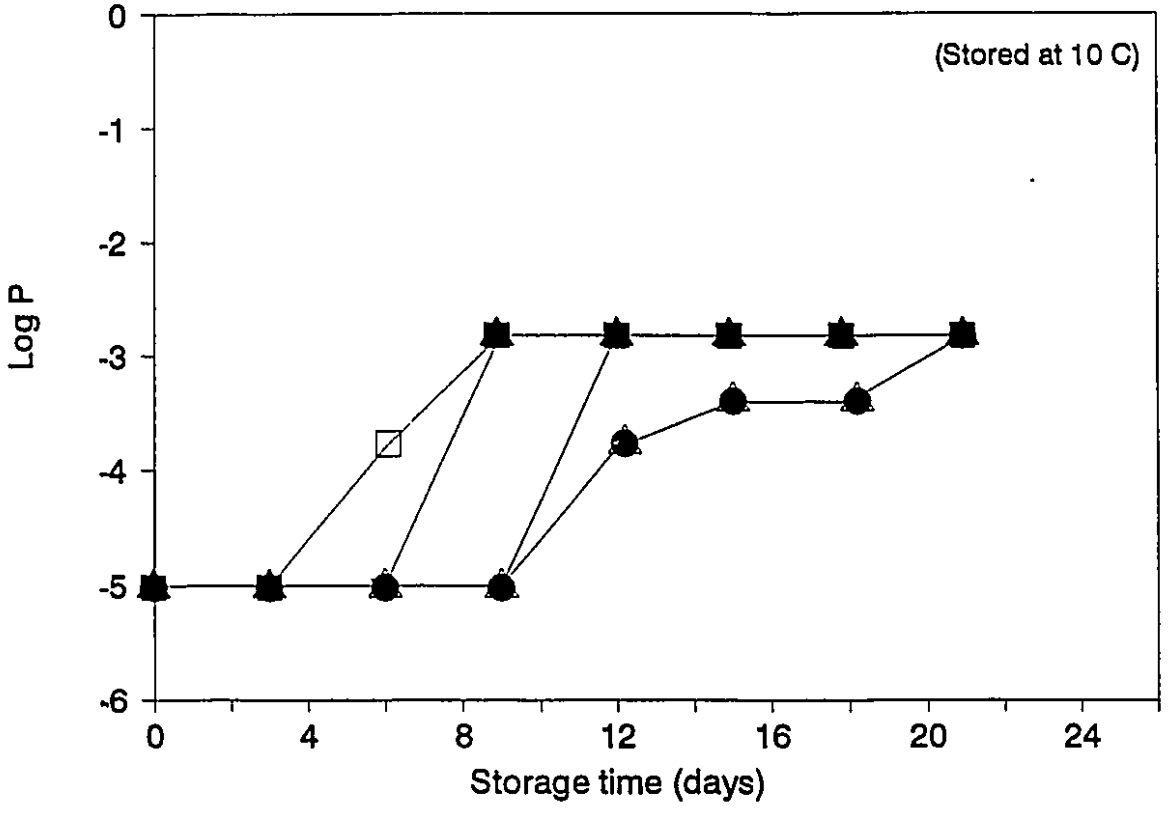


Fig. 35 : Effect of a_w (set with Na-lactate) on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 10^0C for up to 42 days.

□ Control (0.997); ▲ 0.994; ∗ 0.993; ■ 0.990; ● 0.989; △ 0.988.

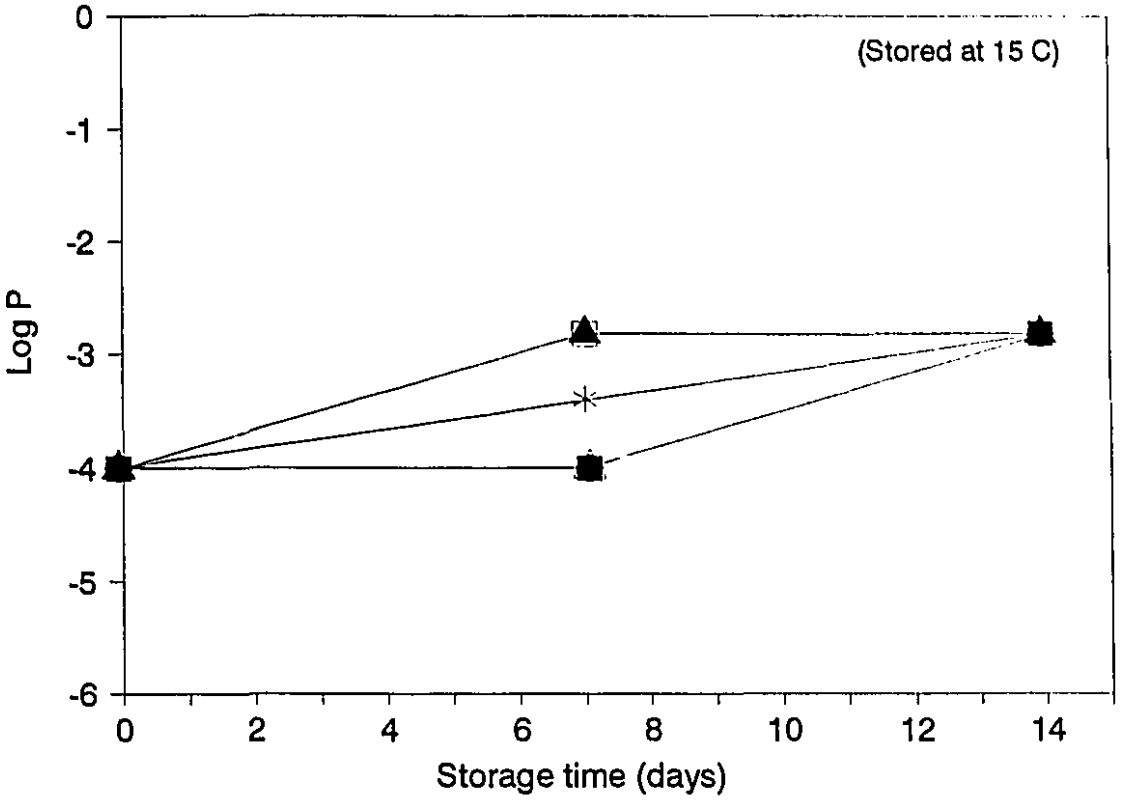


Fig. 36 : Effect of a_w (set with Na-lactate) on the observed probability of toxigenesis by one *C. botulinum* spore in a sous-vide rice and salmon product incubated at 15°C for up to 42 days.

□ Control (0.997); ▲ 0.994; ✳ 0.993; ■ 0.992; ● 0.991; △ 0.989;

The effect of sodium lactate on the water activity of the product and subsequently a delay in time of toxin production is consistent with the literature (Chirife & Ferro Fontan, 1980; de Wit & Rombouts, 1990). Sodium lactate is a normal constituent of muscle tissue and has been shown to demonstrate a wide antimicrobial activity against a broad range of microorganisms. These preservative characteristics can be attributed to various mechanisms including feedback inhibition, intracellular acidulation, interference with protein transfer across the cell membrane and lowering of the product water activity (Bacus and Bontendal, 1991). It is also evident, that on a w/w basis, sodium chloride gave greater a_w reduction than sodium lactate, and as such, is more effective in controlling the growth and toxin production by *C. botulinum*.

The effect of lactic acid bacteria on growth and toxin production by *C. botulinum* is shown in Tables 28 and 29. Addition of both *Pediococcus pentasaceous* and *Lactococcus lactis* at concentrations of 10^3 cells/g of sample and 10^6 cells/g of sample did not affect the time of toxin production in the products. However, lowering the pH to 5.9 delayed toxin production with 1 out of 3 samples being toxic at 7 days for *L. lactis*, but not for *P. pentasaceous*. Furthermore, a combination of pH, salt (1%) and *P. pentasaceous* (10^6/g) delayed time of toxin production from 7 days (1 out of 3) to 14 days, but this effect was not seen with *L. lactis*. Further confirmation of these results in products stored at 10°C is shown in Table 29. Bacteriocins are produced by naturally occurring foodborne organisms (Klaenhammer, 1988) and can inhibit many Gram-positive pathogens (Tagg et al., 1976).

Table 28 : Effect of Lactic Acid Bacteria on Toxin Production by *C. botulinum* in Rice and Salmon Stored at 15°C

Sample	*L. lactis*[1] Time in days 7	14	*P. pentasaceous*[2] Time in days 7	14
Control (pH 6.3)	3/3	ND	3/3	ND
Control + 1:1 LAB : Bot. spores / 100 g sample	3/3	ND	3/3	ND
Control + 2:1 LAB : Bot. spores / 100 g sample	3/3	ND	3/3	ND
Control + 1% NaCl + 2:1 LAB:Bot. spores / 100 g sample	3/3	ND	2/3	2/3
Control (pH 5.9)	3/3	ND	3/3	ND
pH 5.9 + 1:1 LAB : Bot. spores / 100 g sample	3/3	ND	3/3	ND
pH 5.9 + 2:1 LAB : Bot. spores / 100 g sample	1/3	3/3	3/3	ND
pH 5.9 + 1% NaCl + 2:1 LAB : Bot. spores / 100 g sample	3/3	ND	1/3	2/3

[1] => ATCC 11454 ; [2]ATCC 43200

Table 29 : Effect of Lactic Acid Bacteria on Toxin Production by *C. botulinum* in Rice and Salmon Stored at 10°C

	L. lactis[1]		*P. pentasaceous*[2]	
	Time in days		Time in days	
Sample	7	14	7	14
pH 6.3 + 2:1 LAB : Bot. spores / 100 g sample	2/3	3/3	3/3	ND
pH 5.9 + 1% NaCl + Bot. spores	3/3	ND	2/3	2/3
pH 5.9 + 1% NaCl + 2:1 LAB : Bot. spores / 100 g sample	3/3	ND	1/3	2/3

As such these organisms can be useful antimicrobial agents in minimally processed food particularly on *C. botulinum*. Both strains of LAB used in this study were bacteriocin producers (Okereke & Montville, 1991). Therefore, the slight increase in the time of toxin production by the added LAB, found in this work could be attributed to the synergistic effect of pH, salt and bacteriocin although the salt levels used in this study was lower than that suggested by Okereke and Montville (1991). Also, the antimicrobial effect was more pronounced at pH <6 which can be attributed to the fact that LAB prefer more acidic pH levels to grow in and produce bacteriocin.

Recently, enzyme inhibitors have gained considerable attention in the industrial community. One such enzyme inhibitor is α-2-macroglobulin which has been found to neutralize proteases contained in seafood. In this study, its capacity to prevent activation of *C. botulinum* protoxin was investigated at concentrations of 2.7 ppm. However, results obtained indicated that toxin formation was not delayed by the inhibitor. This observation was perhaps due to the low level of the enzyme inhibitor used or lack of sensitivity of the protoxin activating protease to the inhibitor.

Several methods have been examined to inactivate botulinum toxin in foods without destroying nutrients or the sensory qualities of the products. These include heating, freezing and more recently the use of low dose ionizing radiation (Shamsuzzaman et al., 1992). The consumer appeal of many ready-to-eat convenience foods, such as sous-vide products, is that these products can be re-heated quickly by microwave energy. However, there is a paucity of data on the effect of microwave energy on the stability/lability of pre-formed toxin in sous-vide

products, especially products which appear to be acceptable from a sensory standpoint to the consumer. Microwave heating of toxic sous-vide products in a domestic microwave oven (800 Watts of power) for either 5 or 10 min at both full power and half power inactivated the toxin (Table 30). No attempt was made to measure the initial or final concentration of toxin prior to or after the microwave treatments. However, it is evident from mouse toxicity tests that microwave energy has a denaturing effect on pre-formed toxin in the sous vide product. The internal temperature of the microwave heated products measured by inserting a digital thermometer directly into product after microwave heating showed the product temperature ranged from 95 - 100^{o}C in products heated at full power for 5 and 10 min, respectively, to a range of 85 - 99^{o}C for product heated at half power for the same time period. However, the complete inactivation of toxin after a 5 min microwave treatment is contrary to the finding of Notermans et al., (1990).

8.5. *Conclusion*

The present study has shown that appropriate combinations of pH, sodium chloride and sodium lactate delayed the growth and toxin production by *C. botulinum* type E spores in the reformulated minimally processed *sous-vide* rice and salmon product. Thus, one or more combinations of these barriers can be used at the appropriate level(s) to inhibit the growth of the clostridial spores in sous-vide products. For the use of *P. pentasaceous* and *L. lactis*, further investigation is warranted for any definite conclusion to be made.

Table 30 : Effect of Microwave Energy on Pre-formed *C. botulinum* Toxin in Inoculated Spaghetti and Meat Sauce During Storage at 15°C

pH of sample	Full Power[1] at		Half Power[2] at	
	5 min	10 min	5 min	10 min
pH 5.6	0/3	0/3	0/3	0/3
pH 5.9	0/3	0/3	0/3	0/3
pH 6.3	0/3	0/3	0/3	0/3

[1] Full power at 800 watts; [2] 50% full power

Since consumer acceptable products can be produced at ~pH 5.9 without any perceivable change in taste of the product, this pH or the other additional factors can be recommended as additional barriers to inhibit *C. botulinum* type E spores. Thus, safety of sous-vide products could be achieved by a combination of mild heat treatment or pasteurization and additional barriers to growth of *C. botulinum*, such as low pH, acidulants, NaCl, sodium lactate or other humectants, or natural preservatives such as bacteriocin producing lactic acid bacteria.

Although the microwave heating inactivated the pre-toxins in the product, this cannot be recommended as a safety factor to reduce the botulinum risk. This can best be achieved through proper refrigerated storage of products and/or product reformulation to suitable a_w, pH levels and perhaps by addition of bacteriocin producing organisms to ensure the public health and safety of these products particularly at temperature abuse conditions.

CHAPTER 9

GENERAL CONCLUSIONS

This study has shown that mild processing temperatures can be used in conjunction with combination treatments of reduced water activity, reduced pH, and low storage temperature, to extend the shelf life and safety of selected sous-vide products (as measured by inhibition of growth &/or toxin production by *C. botulinum* types A, B, and E spores in sous-vide spaghetti and meat sauce and rice and salmon products). Substantial extensions shelf life and safety of the products were achieved with combination treatments of pH $\leq$ 5.3 and storage at 5°C after products had been subjected to a 13D process at 75°C. The use of sodium chloride (to lower the water activity levels in the products) similarly inhibited growth of, and toxin production by *C. botulinum* spores in the products. When sodium chloride was added to the products at a level of 3%, substantial extension of shelf life and safety of the products were achieved even at 15°C, hitherto considered as **abuse temperature** storage condition. Addition of sodium lactate also exerted antimicrobial / antibotulinal effects in the sous-vide products, but not to the same extent as NaCl.

However, even though NaCl is universally acknowledged as a potent antimicrobial agent, its use for this purpose especially, at high levels, could be restricted by its potential impact on the overall flavor of the product, as well as concerns by consumers and regulatory agencies regarding the health implications of high levels of NaCl in food and heart related diseases.

This study has also shown that the thermal resistance characteristics of an appropriate target organism (e.g., *S. faecium*) can be used to adequately predict the time/temperature requirements needed to process various *sous-vide* products to assure the total destruction of heat-labile non-spore forming bacteria of public health concern.

This study has shown that physical, chemical and microbiological changes, viz., changes in package volume, odor/color and bacterial counts, particularly lactic acid bacteria, can all be used as reliable indicators of spoilage in minimally processed sous-vide products.

For products subjected to similar combination treatments, storage at relatively lower temperature (5°C) achieved greater antimicrobial / antibotulinal effects than storage at higher temperatures (10 or 15°C). Lactic acid levels, as well as numbers of lactic acid bacteria, produced in the samples increased with storage. Indeed, lactic acid bacteria, ultimately became the predominant species in the products. Since lactic acid bacteria are known to be capable of competitively inhibiting the proliferation of several pathogens, including *C. botulinum*, their growth in the products could be a significant safety mechanism.

Modelling has proven useful in predicting the time until toxin production in products formulated under mild heat treatments and stored under temperature abuse conditions (15°C), and in determining the conditions which enhance the probability of toxigenesis. Thus, these models can be used by processors to predict levels of factors in sous-vide products which could be used to control growth and proliferation of *C. botulinum*. They would also be of use to regulatory

agencies in the formulation of realistic guidelines to control growth of foodborne pathogens, especially *C. botulinum*.

The use of α-2-macroglobulin is quite a novel technique that can be studied further to evaluate its capacity to control toxigenesis by *C. botulinum*.

Sodium lactate has proven to be an effective antimicrobial / antibotulinal agent. Since this compound is naturally present in foods, its use can be encouraged as an additional barrier to safeguard against growth and toxin production by *C. botulinum*.

It is well known that certain strains of lactic acid bacteria are capable of producing bacteriocins like nisin, which can inhibit growth of, and toxin production by *C. botulinum* spores (Scott & Taylor, 1981a,b; Spelhaug & Harlander, 1989), and various researchers have attempted to exploit this property of LAB to control the botulinal effects of this pathogens in various food products, by co-inoculating the products with lactic acid bacteria (Tanaka et al., 1985). For example, these latter investigators reported the prevention of symptoms of botulism in bacon stored at 27°C by inoculating the product with LAB, and in the presence of sucrose and low levels of nitrite. In this study, inoculation of products with two strains of lactic acid bacteria, *L. lactis* and *P. pentasaceous* (both known to be nisin producers) at two different levels did not delay symptoms of botulism in the products (pH 6.5). Possibly, the levels of lactic acid bacteria cells used were too low. However, when the organisms were used together with reduced pH (5.9) and added NaCl (1%), botulinal effects were delayed somewhat, but not appreciably as compared with the combined effects of low pH ($\leq$ 5.3) & 5°C; or

3% NaCl at 15°C. Thus, the contention that inoculation of LAB could prevent botulinal effects must be used with caution. As would be expected from the hurdle concept, it is possible that the added nitrite in the study by Tanaka et al., (1985), though small, was still adequate to act synergistically with sucrose and the lactic acid bacteria to produce the overall antibotulinal effect observed. Further research in this area would provide useful information to ascertain the relative contribution of lactic acid bacteria to the prevention of botulism in foods, particularly by using the response surface methodology approach to optimize the appropriate levels of LAB, &/or combinations with pH, NaCl, storage temperature, etc., needed to achieve inhibition of growth and toxin production by *C. botulinum* spores in the sous-vide products.

Even though microwave heating effectively inactivated pre-formed botulinum toxins in the sous-vide products, this approach cannot be recommended as a safety factor to reduce the botulinum risk. However, further research is warranted in this area using microwave treatments of different power levels. Further studies are also warranted to establish whether the use of α-2-macroglobulin has the potential to prevent botulism in foods.

In conclusion, formulation of safe minimally processed sous-vide products of high quality and extended shelf life, can best be achieved through proper refrigerated storage of products and/or product reformulation to suitable a_w, pH levels and by addition of bacteriocin producing organisms to ensure the public health and safety of these products.

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