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The efficacy of weak acid salts for the reduction or prevention of growth of Listeria monocytogenes in processed meat products

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Summary

L. monocytogenes poses a health threat to consumers of foods that have long, refrigerated shelf lives and that are eaten without further cooking prior to consumption. Such foods include vacuum-packed (VP), or modified atmosphere packed (MAP), ready-to-eat (RTE) processed meats.

Methods to reduce the risk of *L. monocytogenes* in smallgoods include minimisation of contamination and minimisation of its growth in products. In practice, minimising the growth of *L. monocytogenes* in RTE processed meats could most readily be achieved by including growth inhibitors in the product. Permitted food additives such as weak acid salts, e.g. sodium diacetate or sodium lactate or potassium lactate, can prevent the growth of *Listeria monocytogenes* under some conditions of pH, water activity and temperature.

This project, following on from PRMS.071, comprised two elements. The first, and larger, component was to prepare two smallgoods products with various levels of lactates and diacetates and to assess their potential, by challenge studies, to prevent *L. monocytogenes* growth in those products. The second component was sought to develop a model system that could be used to efficiently determine product formulations and storage conditions that would preclude *L. monocytogenes* growth without the need for extensive product challenge tests. The value of the knowledge that could be obtained lies in being able to formulate a product in which it can be demonstrated that *L. monocytogenes* growth is not possible. This would considerable reduce regulatory burdens or producers for *L. monocytogenes* testing and potential recalls if levels of *L. monocytogenes* of <100 CFU/g are detected on the product.

Part 1

In the first part of the study experiments were conducted to examine the influence of two variables on the development of populations of *Listeria monocytogenes* in MAP sliced ham and MAP shaved chicken:

- Presence of preservatives, namely weak acid salts (either a potassium lactate-diacetate blend, or sodium lactate),
- storage temperature (either 4 or 8°C).

The influence of total viable bacteria on the potential for growth of *L. monocytogenes* was also considered.

Ham was prepared by a commercial processor and sliced and packed in modified atmosphere containers typical of those used for consumer retail packs. Hams were either prepared and processed normally, or were prepared with 3% PURASAL®S/SP 60 (sodium lactate) or 3% PURASAL®P Opti.Form 4 (potassium lactate-diacetate blend). Similarly, shaved chicken was prepared by a commercial processor and sliced and packed in modified atmosphere containers typical of those used for consumer retail packs. Chicken was either prepared and processed normally, or prepared with 3% PURASAL®P Opti.Form 4 (potassium lactate-diacetate blend). The containers were appropriately labelled and forwarded to the University of Tasmania, Hobart, using refrigerated transport.

Samples were inoculated with a cocktail of five strains of *L. monocytogenes* and incubated at either 4 or 8°C. At appropriate intervals samples were removed from refrigerated storage and *L. monocytogenes*, total viable count (TVC) and lactic acid bacteria (LAB) enumerated. Water activity and pH of the samples were also determined from duplicate samples at the commencement of the trial, and at intervals throughout. Lactate and acetate content was determined on samples taken at the beginning of, and throughout, the trial.

Collectively, the results indicate that the addition of lactate or lactate and diacetate salts can reduce the risk of listeriosis from smallgoods by reducing the potential for growth of *L. monocytogenes*. While significant inhibition of growth of *L. monocytogenes* was observed in either chicken or ham samples stored at 4°C, the inhibition achieved at 8°C was more modest. In almost all product and treatments growth of *L. monocytogenes* was observed within the nominal shelf life typical of MAP, sliced, processed cooked meat products, *viz*. 6- 8 weeks. The exception was Opti.Form 4 in ham stored at 4°C for which the suppression of *L. monocytogenes* growth was complete.

The effect of other microorganisms on the potential growth of *L. monocytogenes* was also highlighted. Despite the similarity in the physico-chemical composition of the two products tested (i.e. chicken, ham), *L. monocytogenes* grew to much higher levels in the chicken samples. The microbial load on the chicken at the time of inoculation of the product with *L. monocytogenes* was much less than in the ham challenge trials. Thus, the difference in the results of the ham trials with lactate and the chicken trials with lactate is probably an example of the role of lactic acid bacteria, and the Jameson Effect in general, on the potential risk from listeriosis in processed meats.

It was also observed that lactate salts can reduce the growth rate of other bacteria present on processed meat products and, as such, can extend their shelf life. This was observed most clearly in lactate-treated chicken samples, but was not significant in the lactate treated ham samples. The reason for the difference is not known but, while the initial microbiota of the ham products was dominated by lactic acid bacteria, this was not the case for the chicken product. It is possible that these organisms on processed chicken were more inhibited by lactate than are lactic acid bacteria.

Part 2

In the second part of the study, the publication by Mejholm and Dalgaard (2007) of a comprehensive and validated model for *L. monocytogenes* growth rate and growth potential confirmed the importance of various environmental factors not included in the PRMS.071 model for *L. monocytogenes* growth limits. As such, it appears that the objectives of this part of the project have been obviated, by highlighting factors important to the prediction of *L. monocytogenes* growth potential in processed meats. These factors include nitrite concentration, smoke compounds (measured as phenol) and, to a lesser extent, dissolved CO_2 concentration in the case of modified-atmosphere packed products. That model was incorporated into 'user-friendly' spreadsheet-based software and is an output of the project.

Experience with the model highlights the strong contribution of the interaction of pH and undissociated lactic acid (in realistic product formulations) in determination of the limits to growth. Other factors also contribute, but at realistic levels are not as influential in dictating whether growth is possible or prevented. With levels of added lactic acid in the range 2.5 to 3% (w/w), pH in the range 6.0 to 6.05 is predicted to preclude growth. As such, studies to investigate the combined pH and lactic acid concentration, in the presence of realistic levels of nitrite, phenol, CO2 etc and at ideal and at ideal, and slightly abusive storage temperature would yield useful information. Studies to validate the model, in particular at factor combinations predicted to be at, or near, the limits of growth of *L. monocytogenes* in the multi-factor space relevant to processed meat products, are underway at the time of reporting.

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CONTENTS

OVERVIEW

This project, following on from PRMS.071: "Modelling growth prevention of *Listeria monocytogenes* by weak acid salts and nisin", comprised two elements. The first, and larger, component was to prepare two smallgoods products with various levels of lactates and diacetates and to assess their potential, by challenge studies, to prevent *L. monocytogenes* growth in those products. The second component was sought to develop a model system that could be used to efficiently determine product formulations and storage conditions that would preclude *L. monocytogenes* growth without the need for extensive product challenge tests. The value of the knowledge that could be obtained lies in being able to formulate a product in which it can be demonstrated that *L. monocytogenes* growth is not possible. This would considerable reduce regulatory burdens or producers for *L. monocytogenes* testing and potential recalls if levels of *L. monocytogenes* of <100 CFU/g are detected on the product.

PART 1 - CHALLENGE TRIALS

Introduction

Listeria monocytogenes is a foodborne pathogen that is not unusually resistant to heat but, unlike most foodborne pathogens, can grow at refrigeration temperature in the presence of >10% salt and in the absence of oxygen. These characteristics mean that *L. monocytogenes* poses a health threat to consumers of foods that have long, refrigerated shelf lives and that are eaten without further cooking prior to consumption. Such foods include vacuum-packed (VP), or modified atmosphere packed (MAP), ready-to-eat (RTE) processed meats.

Methods to reduce the risk of *L. monocytogenes* in smallgoods include minimisation of contamination and minimisation of its growth in products. In practice, minimising the growth of *L. monocytogenes* in RTE processed meats could most readily be achieved by including growth inhibitors in the product. Permitted food additives such as weak acid salts, e.g. sodium diacetate or sodium lactate or potassium lactate, can prevent the growth of *Listeria monocytogenes* under some conditions of pH, water activity and temperature. Organic acids and their salts, applied singly and in combination, have been shown by many studies (*cited below*) to prevent, delay or greatly retard the growth of *L. monocytogenes* in processed meats under both recommended temperatures of storage and mild temperature abuse (e.g. up to 10°C). Most studies (e.g. Weaver and Shelef, 1993; Qvist *et al*., 1994; Blom *et al.*, 1997; Islam *et al.*, 2000; Juncher *et al*., 2000; Stekelenberg and Kant-Muermans, 2001; Bedie *et al.*, 2001; Goode 2001; Mbandi and Shelef, 2001; Glass *et al*., 2002; Mbandi and Shelef, 2002; Samelis *et al*., 2002; Choi and Chin, 2003) have focused on the

activity of sodium lactate but several also consider potassium lactate or potassium lactate in combination with sodium diacetate (Porto *et al*., 2002; Seman *et al*., 2002; Stekelenberg, 2003). Most studies have considered the activity of organic acid salts in sausage products (e.g. bratwurst, frankfurters, saveloys) but Blom *et al*. (1997) and Stekelenberg and Kant-Muermans (2001) also studied their effectiveness in cooked ham. Mbandi and Shelef (2001) considered their effectiveness in sterile uncooked comminuted beef emulsion. A previous Australian study (AFSCoE, 2004) also indicated the efficacy of salts of organic acids as listeriostatic agents in sliced, MAP, ham. Preparations of salts of lactic and acetic acids, either singly or in combination intended for food preservation and their listeriostatic potential are commercially available (Purac, 2005).

In this study experiments were designed to examine the influence of two variables on the development of populations of *Listeria monocytogenes* in two commercial smallgoods (MAP sliced ham and shaved chicken):

- Presence of preservatives, namely weak acid salts (either a potassium lactate-diacetate blend, or sodium lactate),
- storage temperature (either 4 or 8°C).

The influence of total viable bacteria on the potential for growth of *L. monocytogenes* was also considered.

Materials and Methods

Overview of Trial

Hams were prepared under commercial conditions by Primo Smallgoods and sliced and packed in modified atmosphere containers typical of those used for consumer retail packs. Hams were either prepared and processed normally, or were prepared with 3% PURASAL®S/SP 60 (sodium lactate) or 3% PURASAL®P Opti.Form 4 (potassium lactate-diacetate blend). The containers were appropriately labelled and forwarded to the University of Tasmania, Hobart, using refrigerated transport.

Shaved chicken was prepared under commercial conditions by MQF Smallgoods and sliced and packed in modified atmosphere containers typical of those used for consumer retail packs. Chicken was either prepared and processed normally, or prepared with 3% PURASAL®P Opti.Form 4 (potassium lactate-diacetate blend). The containers were appropriately labelled and forwarded to the University of Tasmania, Hobart, using refrigerated transport.

Upon receipt, all samples were labelled and stored at 2°C until commencement of the trial. Samples were then either inoculated with a cocktail of five strains of *L. monocytogenes* or a diluent 'blank' and incubated at either 4 or 8°C in walk-in refrigerators. At intervals appropriate to each treatment duplicate samples were removed from refrigerated storage and *L. monocytogenes*, total viable count (TVC) and lactic acid bacteria (LAB) enumerated. Water activity and pH of the samples were also determined from duplicate samples at the commencement of the trial, and at intervals throughout. Lactate and acetate content was determined on samples taken at the beginning of, and throughout, the trial.

The experimental design, involving twelve treatment combinations for ham and eight for chicken, is summarised in Tables 1(a) and (b), overleaf.

Table 1(a) Experimental design: treatment and variable combinations for sliced ham

Sample Code	Treatment			*Listeria monocytogenes* inoculum	Storage temperature	
	Untreated	PURASAL®P Opti.Form 4	PURASAL® S/SP 60		4°C	8°C
HCU4	✓				✓	
HCU8	✓					✓
HCL4	✓			✓	✓	
HCL8	✓			✓		✓
HOU4		✓			✓	
HOU8		✓				✓
HOL4		✓		✓	✓	
HOL8		✓		✓		✓
HSU4			✓		✓	
HSU8			✓			✓
HSL4			✓	✓	✓	
HSL8			✓	✓		✓

Table 1(b) Experimental design: treatment and variable combinations for shaved chicken

Sample Code	Treatment		*Listeria monocytogenes* inoculum	Storage temperature	
	Untreated	PURASAL®P Opti.Form 4		4°C	8°C
CCU4	✓			✓	
CCU8	✓				✓
CCL4	✓		✓	✓	
CCL8	✓		✓		✓
COU4		✓		✓	
COU8		✓			✓
COL4		✓	✓	✓	
COL8		✓	✓		✓

Materials

Reagents and Test Substrates

PURASAL®P Opti.Form 4, consisting of 54.5 to 57.5% potassium lactate and 3.7 to 4.3% potassium diacetate, and PURASAL®S/SP 60, consisting of 58.8 to 61.2% sodium lactate, were prepared by the manufacturer and added to product under commercial conditions.

Microorganisms

Listeria monocytogenes

Five strains of *L. monocytogenes* were used in combination for all "*L. monocytogenes* inoculated" samples:

- Scott A (type strain)
- L5/22 (isolated in Tasmania from cold smoked salmon)
- Strains 20425, 20432 and 20423, all isolated from a smallgoods factory and supplied by Silliker Microtech Pty., Ltd., Melbourne, Victoria.

Culture Media

Listeria monocytogenes were enumerated by spread plating of suspensions of appropriately diluted samples on PALCAM agar (Oxoid CM 877, with SR150 antibiotic supplement). Confirmation of *Listeria* spp. was undertaken using Sheep Blood Agar (Oxoid PP2133), Brain Heart Infusion Agar (Oxoid CM 225 and Oxoid L13) and Brain Heart Infusion Broth (Oxoid CM 225).

Total viable aerobic counts were determined on APHA Standard Plate Count Agar (PCA; Oxoid CM463).

Lactic acid bacteria were enumerated by spread plating on DeMan, Rogosa, Sharpe Agar (MRS; Oxoid CM361).

Dilutions of homogenised food samples were prepared in 0.1% Bacteriological Peptone (Oxoid L37), hereafter simply referred to as 'diluent' unless otherwise noted.

Test Kits

Confirmed *Listeria* spp. were further identified using Listeria API kits (bioMérieux Vitek, Australia, Pty Ltd, Upper Mount Gravatt, QLD, Australia). Confirmed *L. monocytogenes* isolates were serotyped using Listeria Antisera Test Kits (Denka Seiken, Cat # 294616, Tokyo, Japan).

Equipment

pH meter: Orion 250 with flat tip probe (Orion Research, Inc., Boston, USA).

Spiral plater: AutoPlate 4000 (Spiral Biotech, Bethesda, USA)

Water activity meter: Aqualab CX2 (Decagon Devices, Pullman, USA)

The pH meter was calibrated on each day of use against commercial pH reference solutions. The water activity meter calibration was checked, and adjusted if required, on each day of use against distilled water and saturated salt (NaCl) solutions (i.e. water activities of 1.000 and 0.755, respectively, at 25°C).

Methods

Product preparation, Transport and Receipt

Lactate and diacetate treatments were applied to ham and chicken during normal commercial processing according to the manufacturer's recommendations.

For hams a treatment of either 3% (w/w) of PURASAL®P Opti.Form 4 or 3% PURASAL®S/SP 60 was added during processing. 100g lots were dispensed into thermoformed ridge bottom packs, composed of PET and PE and produced on horizontal form fill. The packages were then sealed under MAP conditions, the gas atmosphere comprising 50% CO_2 and 50% N_2.

For chicken a treatment of 3% (w/w) of PURASAL®P Opti.Form 4 was applied to uncooked batter. The product was then filled into waterproof casing and steam cooked, then cooled prior to slicing. Approximately 50g lots were dispensed into packs composed of a base of PET with PE barrier and a top nylon film with peelable sealant laminate. The packages were then sealed under MAP conditions, the gas atmosphere comprising 30% CO_2 and 70% N_2.

Material from both manufacturers were shipped to Hobart by commercial transport operators under refrigerated storage (Note: no temperature records were supplied). Immediately upon receipt all samples were labelled (for sample codes see Tables 1 a and b) and the site to be used for injection of the inoculum was prepared as follows. The corner of each packet was sanitised with ethanol spray, dried with a tissue and then a 2x2cm piece of self-adhesive rubber septum attached. Samples were then placed at 2°C until the inoculum was ready to be added. Time spent at 2°C prior to inoculation was 2 days for ham and 1 day for chicken. The time between production and receipt at our laboratories was 11 days for ham samples, and 9 days for chicken samples.

Inoculum preparation

The *L. monocytogenes* inoculum was prepared by first inoculating 5 colonies of each strain into individual Tryptone Soya broths (Oxoid CM 129) containing 0.6% Yeast Extract (Oxoid L21), TSB-Ye, and incubating at 37°C for 24 hours. Each culture was serially diluted and added to TSB-YE broths containing 0.5% PURASAL®P Opti.Form 4 and adjusted to pH 6.2, to achieve a population density of ~10^6 CFU.mL^{-1}. The cultures were then incubated at 8°C until cell density had increased ~100-fold, assessed by turbidimetry. The temperature, pH and antimicrobial acclimated cultures were considered to be at the required level of exponential growth, ~10^8 CFU.mL^{-1}, when transmittance reached 30%. Due to difficulties in determining the arrival time of samples, the process of diluting to ~10^6 CFU.mL^{-1} was repeated daily, as required, to ensure that cultures were in exponential growth at the time of inoculation.

To prepare the cocktail for inoculation, two millilitres of each of the five strains was added to a sterile McCartney bottle and vortexed for one minute. This "cocktail" suspension was then diluted

in pre-chilled (8°C) diluent to a level of ~200000 cells per mL (i.e. a 10^{-3} dilution). The actual concentration of *L. monocytogenes* in the inoculum "cocktail" was determined by spread-plating appropriate dilutions onto PCA and incubating at 37°C for 24 hours.

The steps described above generated an exponentially growing inoculum that was acclimated to conditions considered to be representative of a smallgoods processing facility, specifically:

- chill temperature (8°C)
- low level of weak acids salts (i.e. potassium lactate and sodium diacetate levels found in product prepared with PURASAL®P Opti.Form 4)
- pH 6.2-similar to that observed for smallgoods

Inoculation of Samples with L. monocytogenes *and Incubation*

As described previously, upon receipt at the Hobart laboratories of the Australian Food Safety Centre of Excellence, samples were stored at 2°C for approximately 2 days for ham and 1 day for chicken, prior to inoculation with the *L. monocytogenes* cocktail and commencement of the challenge trial. This delay arose because, once prepared, the inoculum could not be held for more than a few hours without jeopardising control of the physiological state of the inoculum and cell density and because the time of arrival of the samples was not known with certainty.

An inoculum of a *L. monocytogenes* "cocktail" prepared as described above, or sterile diluent, was aseptically injected into the MAP sliced or shaved products using a hypodermic syringe. Sterile diluent was added to 'control' samples (i.e. not inoculated with *L. monocytogenes*). An inoculum volume of 0.1mL was added to ham samples, and 0.5mL was added to chicken samples. To preserve the integrity of the gas mixture, the syringe was inserted into the pack through the self-adhesive rubber septum applied to each sample package upon receipt (as described above). The inoculum was added to the base of the tray in an area not containing any product. The injection site was then covered with a piece of adhesive tape. Immediately after inoculation (or addition of sterile diluent) the ham packages were inverted then gently shaken (by hand) for approximately 30 seconds so that the slices of ham moved across the inner surfaces of the container, and each other. Due to the fragile nature of the shaved chicken it was only possible to invert the packages after inoculation and ensure that the inoculum 'bled' across the chicken pieces, followed with a little gentle shaking. These steps were undertaken to maximise the likelihood of even distribution of *L. monocytogenes* cells and diluent throughout the sample. Inoculated samples were incubated standing upright (to simulate position in a retail cabinet) at either 4 or 8°C.

Untreated samples not inoculated with *L. monocytogenes* were also incubated at 4 and 8°C for the duration of the trial and sampled at regular intervals to assess whether *L. monocytogenes* may also have been present in the samples prior to inoculation with the challenge strains.

Sampling

At appropriate intervals two samples from each treatment, or control treatment, were assessed for levels of *L. monocytogenes*, LAB and TVC as described above. Samples were diluted 1:1 in diluent and further 10-fold dilutions prepared as required. Spread plates were prepared using either the 50µL exponential deposition mode on a spiral plater or 250µL aliquots spread by hand. The maximum sample volume plated was 250 μl of the 1:1 dilution on quadruplicate plates. This permitted a maximum test sensitivity of 2 CFU.g^{-1}. PALCAM plates were incubated at 37°C for 48h. PCA plates were incubated at 20°C for 72h for ham samples and 72-96h for chicken samples. MRS plates were incubated at 25°C for 72h for ham samples and 72-96h for chicken samples, selected based on standard practice as well as suitability for reading of results in order to plan the next set of dilutions and plating. It should be noted that the results presented are reflection of viable counts based strictly on the stated combination of time and temperature of incubation. It was observed that on occasion very small colonies appeared on PCA and MRS plates if the incubation time was extended by several days. Additionally, difficulties were encountered in determining viable counts for samples containing few microorganisms due to the opacity of, and debris contained in, the initial dilution (referred to as a 'neat' sample).

For control hams inoculated with *L. monocytogenes* and 3% PURASAL®S/SP 60 treated hams inoculated with *L. monocytogenes* and incubated at 8°C, an additional set of samples was processed on day 23 to determine the incidence and variability of counts of *L. monocytogenes* in the samples. Similarly, upon completion of each trial, where possible, all remaining samples, i.e. either uninoculated or those inoculated with *L. monocytogenes,* were sampled.

Colonies were counted manually and $\log_{10}$(viable cell count) plotted against time.

Calculation of generation time

Generation times, defined as the time for cells to 'double in number' were estimated by linear regression on the natural logarithm of the viable count growth curves as follows. Briefly, a straight line was fitted to those points that appeared to best represent exponential growth. Generation time was calculated by dividing 0.301 (equivalent to $\log_{10}2$) by the slope of the regression line, *m*.

Proximate Analysis

Proximate analyses for lactate and acetate content were undertaken by the Department of Primary Industries Research Victoria, Werribee, Victoria, Australia. Samples were withdrawn from 4 or 8°C storage at various times throughout the trial and stored at - 20°C until the conclusion of the experiment. All samples were then forwarded to the consulting laboratory for analysis. In addition to the above, a single sample of each type of ham and chicken was analysed for lactate and acetate content at the commencement of the trial, i.e. they had been stored at 2°C for 2 and 1 day(s) respectively.

Results and Discussion

Due to the time taken to ship the ham samples from Sydney to Hobart and to prepare the inoculum in Hobart, the time between preparation of the hams and commencement of the challenge trial was 11 days. For chicken samples the time between preparation and trial commencement was 9 days. Unless otherwise stated, all time measurements reported below are related to the time of preparation of the samples.

Ham samples underwent 51 days of incubation at 8°C and 92-101 days at 4°C. Chicken samples underwent 78-80 days of incubation at both 4 and 8°C.

Proximate analyses-Lactate and acetate assay of treatments

Table 2 presents the means and standard deviations of results of analyses of untreated controls and 45 ham and 32 chicken samples that were treated with organic acid salts and were subjected to storage at either 4 or 8°C. Raw data for all analyses is presented in Appendix 1. The results indicate that levels id not change significantly during storage with the exception of slight increases in lactic acid. This probably arises from the growth and metabolism of lactic acid bacteria and was also evident in untreated ham samples. These results also indicate that the organics acid salts were present at approximately the expected levels[1].

Table 2 Average percentage lactate and acetate content of treated and untreated ham and chicken samples either upon receipt or after storage at 4 or 8°C (H. Lindsay, *pers. comm.*, 2006). Standard deviations are shown in brackets.

		Upon receipt			4°C			8°C	
	Sample type	***n***	**Lactate (%)**	**Acetate (%)**	***n***	**Lactate (%)**	**Acetate (%)**	***n***	**Lactate (%)**	**Acetate (%)**
Ham	**Untreated Control**	1	0.31	<0.05	9	0.44 (0.17)	<0.05	6	0.56 (0.15)	<0.05
	3% PURASAL®P OptiForm 4	1	0.90	0.053	9	1.14 (0.11)	0.07 (0.01)	6	1.17 (0.08)	0.07 (0.01)
	3% PURASAL®S/SP 60	1	1.10	<0.05	9	1.21 (0.11)	<0.05	6	1.18 (0.13)	<0.05
Chicken	**Untreated Control**	1	0.26	<0.05	8	0.25 (0.04)	<0.05	8	0.26 (0.04)	<0.05
	3% PURASAL®P OptiForm 4	1	1.00	0.074	8	1.05 (0.05)	0.08 (0.00)	8	1.05 (0.05)	0.08 (0.00)

* minimum level of detection = 0.05 % w/w

[1] [Expected lactate levels: 3% (w/w) Purasal Optiform 4 =3%*0.55 (% sodium lactate in powder) * 90/112 (mol wt lactate/ mol wt sodium lactate) = 1.32% + (0.3) lactate initially present = 1.6%; a similar calculation leads to an estimate of 1.75% for Purasal S/SP 60; acetate from Purasal OptiForm 4: =3%*0.04 (% sodium diacetate in powder) * 119/142 (2* mol wt acetate/ mol wt sodium diacetate) = 0.10%].

Inoculum densities

The *L. monocytogenes* "cocktail" was found to contain 8.2 x 10^5 CFU.mL^{-1} for the ham trial and 6.8 x 10^5 CFU.mL^{-1} for the chicken trial. To each ~100g sample of ham 0.1 mL of the 10^{-3} dilution of "cocktail" was added, resulting in expected levels of 1600 CFU.g^{-1} in the samples. To each ~50g sample of chicken 0.05 mL of the 10^{-3} dilution of "cocktail" was added, resulting in expected levels of 1300 CFU.g^{-1} in the samples.

Water activity and pH changes

Considering the precision of the water activity meter (±0.003 units), the a_w of control and treated ham and chicken samples did not alter as a function of storage time or temperature (Tables 3 and 4). Additionally, there was no significant effect of the addition of either antimicrobial treatment on the a_w of the product in comparison to untreated controls. Similarly, addition of *L. monocytogenes* to inoculated samples did not affect a_w.

For control ham and ham containing 3% (w/w) of PURASAL®P Opti.Form 4, with or without added *L. monocytogenes*, a_w remained at a_w ~0.965 at both 4 and 8°C (Table 3). Similarly, for ham containing 3% PURASAL®S/SP 60 a_w remained at ~0.968

Table 3 Water activity from duplicate samples of untreated ham and ham with either 3% (w/w) of PURASAL®P Opti.Form 4 or 3% (w/w) of 3% PURASAL®S/SP 60, either with or without added *L. monocytogenes* at 4 and 8°C (average and standard deviation in bold).

Temp. (°C)	Time (days)	Untreated Control		3% (w/w) PURASAL®P Opti.Form 4		3% PURASAL®S/SP 60	
		Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
4	14	0.970	0.967	0.968	0.968	0.970	0.967
	43	0.968	0.961	0.967	0.968	0.970	0.965
	58	0.962	0.967	0.966	0.965	0.966	0.967
	86	0.966	0.966	0.963	0.965	0.965	0.962
		0.966 (±0.004)	**0.965 (±0.003)**	**0.966 (±0.003)**	**0.967 (±0.002)**	**0.968 (±0.003)**	**0.965 (±0.003)**
8	14	0.966	0.966	0.965	0.964	0.969	0.970
	24	0.963	0.966	0.966	0.965	0.967	0.968
		0.964 (±0.002)	**0.966 (±0.001)**	**0.965 (±0.003)**	**0.964 (±0.001)**	**0.968 (±0.003)**	**0.969 (±0.001)**

For control chicken and chicken containing 3% (w/w) of PURASAL®P Opti.Form 4, with or without added *L. monocytogenes*, a_w remained at a_w ~0.967 (±0.003) at both 4 and 8°C (Table 4).

Table 4 Water activity from duplicate samples of untreated chicken and chicken with 3% (w/w) of PURASAL®P Opti.Form 4 either with or without added *L. monocytogenes* at 4 and 8°C (average and standard deviation in bold).

Temperature (°C)	Time (days)	Untreated Control		3% (w/w) PURASAL®P Opti.Form 4	
		Uninoculated	Inoculated	Uninoculated	Inoculated
4	10	0.967	0.971	0.964	0.965
	31	0.976	0.972	0.969	0.969
	56	0.970	0.962	0.961	0.965
	80	0.967	0.964	0.965	0.961
		0.970 (±0.004)	**0.967 (±0.006)**	**0.964 (±0.003)**	**0.965 (±0.003)**
8	10	0.970	0.971	0.963	0.965
	31	0.972	0.974	0.970	0.97
	56	0.969	0.968	0.964	0.962
	78	0.969	0.968	0.964	0.963
	79				0.965
		0.970 (±0.002)	**0.970 (±0.003)**	**0.965 (±0.003)**	**0.965 (±0.003)**

The pH of all ham samples decreased with increasing storage time at both 4 and 8°C. For ham samples at 4°C (Figure 1b), pH decreased from pH ~6.2 to ~5.5 with increasing incubation time for all controls and treatments. Variation in pH estimates was most pronounced for hams containing sodium lactate with differences in pH of up to 0.8 units observed between duplicate samples with or without added *L. monocytogenes*.

For ham samples at 8°C (Figure 1a), pH decreased from pH ~6.2 to ~5.1-5.7 with increasing incubation time for all controls and treatments. The pH of hams generally was highest for sodium lactate > lactate/diacetate blend > control samples.

The pH of most of the 4°C chicken samples remained unaltered for the duration of the trial, approximately pH 6.2, with the exception of the inoculated control chicken samples (Figure 2b). In those samples, i.e. untreated chicken inoculated with *L. monocytogenes*, the pH began to decrease after day 60.

For chicken samples incubated at 8°C the pH remained unchanged, pH ~6.2, up to day 40 (Figure 2a). Thereafter the pH of control samples inoculated with *L. monocytogenes* decreased with further incubation. In contrast, the uninoculated control samples mostly remained at a pH similar to that at the commencement of the trial. This was also observed for most of the inoculated and uninoculated 8°C samples treated with 3% (w/w) of PURASAL®P Opti.Form 4.

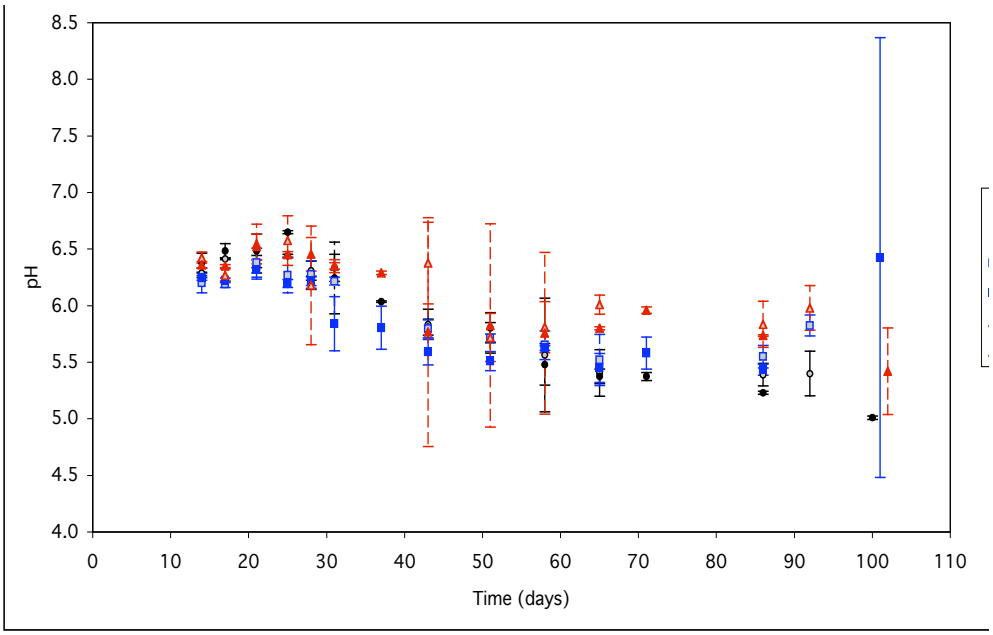


Figure 1(a) Temporal changes in pH of control ham (untreated-black symbols) and treated ham (lactate/diacetate blend, blue symbols; or sodium lactate, red symbols) with (closed symbols) or without (shaded symbols) the addition of *L. monocytogenes* at 4°C

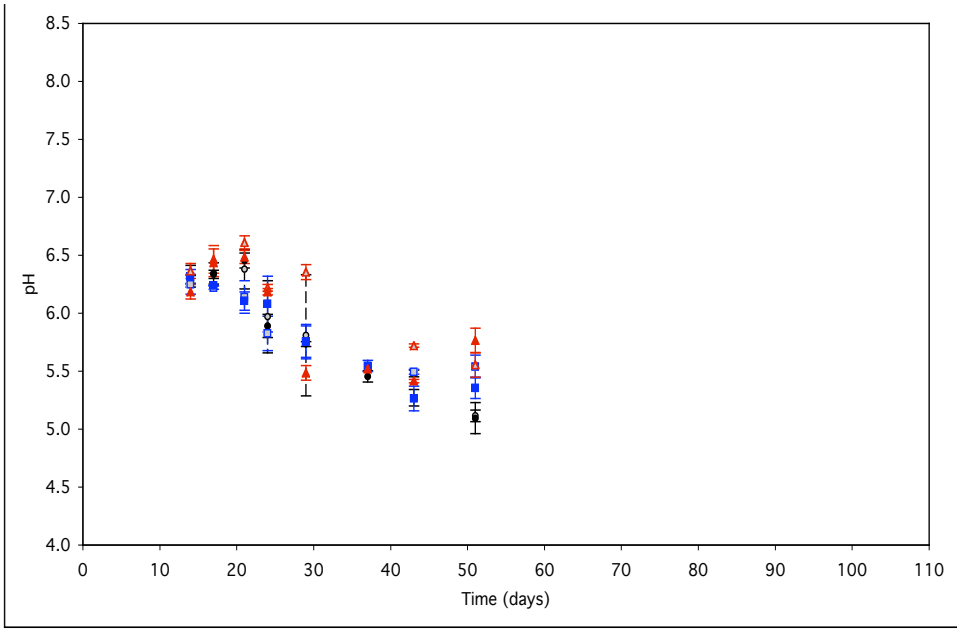


Figure 1(b) Temporal changes in pH of control ham (untreated-black symbols) and treated ham (lactate/diacetate blend, blue symbols; or sodium lactate, red symbols) with (closed symbols) or without (shaded symbols) the addition of *L. monocytogenes* at 8°C

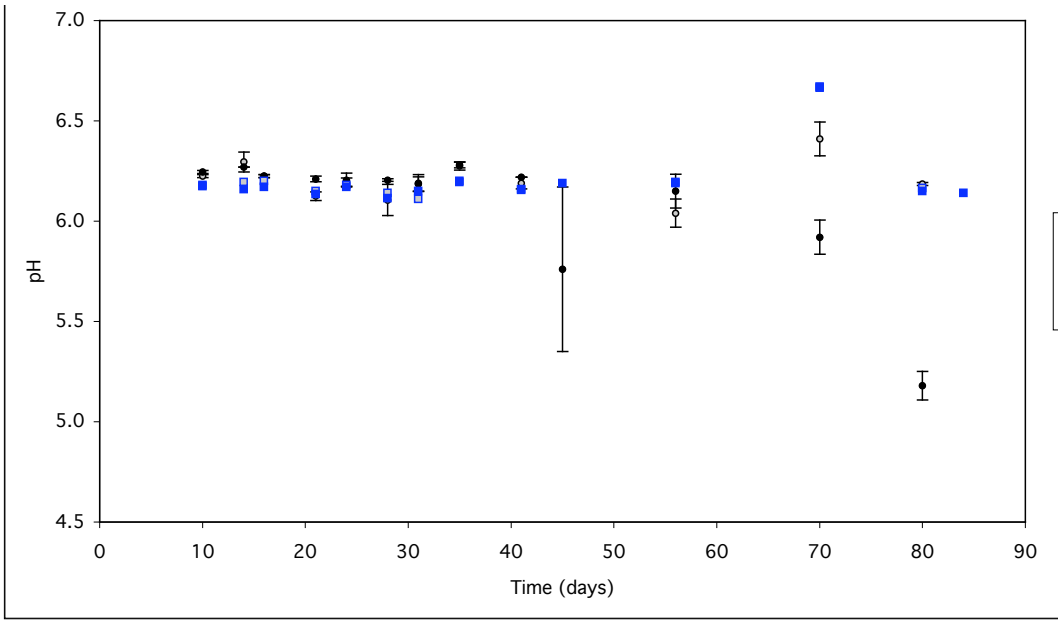


Figure 2(a) Temporal changes in pH of control chicken (untreated-black symbols) and treated chicken (lactate/diacetate blend, blue symbols) with (closed symbols) or without (shaded symbols) the addition of *L. monocytogenes* at 4°C

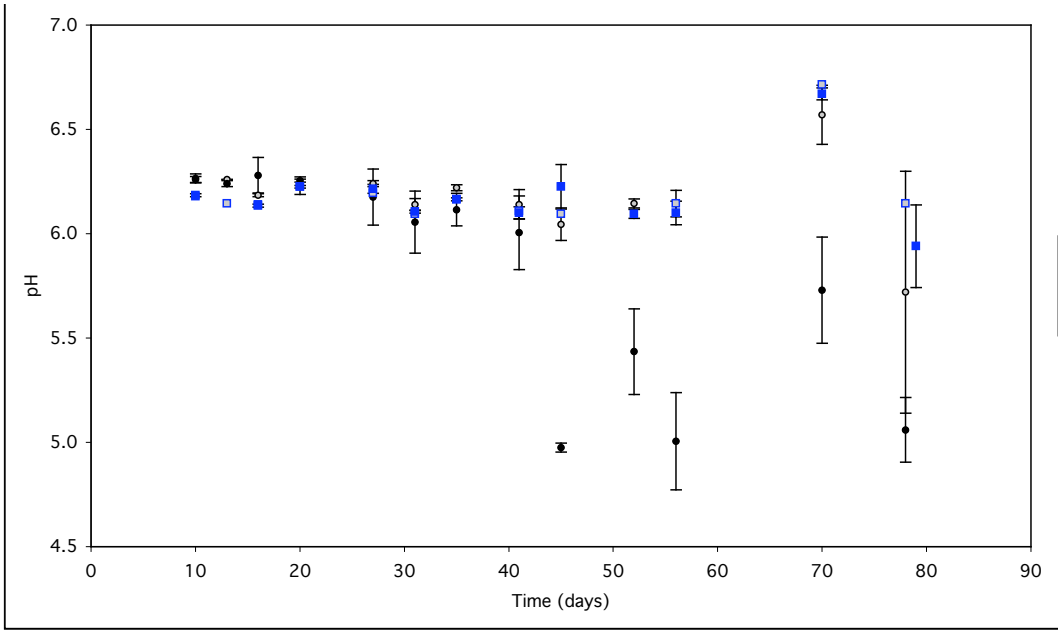


Figure 2(b) Temporal changes in pH of control chicken (untreated-black symbols) and treated chicken (lactate/diacetate blend, blue symbols) with (closed symbols) or without (shaded symbols) the addition of *L. monocytogenes* at 8°C

Microbiology-Ham

Untreated Controls

No *Listeria* spp. were detected in untreated, uninoculated control hams incubated at either 4 or 8°C (Figures 3 a and b). Thus, if present, levels of *L. monocytogenes* were below the limit of detection of the methods used (i.e. 2 log $CFU.g^{-1}$). Growth of total aerobic microflora was slower at 4°C than at 8°C and in both cases the TVC consisted predominantly of LAB.

When *L. monocytogenes* were inoculated into control samples and stored at 4°C a small amount, ~1 log $CFU.g^{-1}$ of growth was observed and a maximum population density (MPD) of ~ 4.5 log $CFU.g^{-1}$ was reached at day 37, equivalent to 26 days after inoculation (Figure 4a). Numbers then appeared to decrease after day 58 and then to return to a viable count similar to that at the commencement of the trial. Sampling of 10 replicates on completion of the trial, i.e. at day 110, revealed that *L. monocytogenes* count varied by up to 2 log $CFU.g^{-1}$ between individual samples.

The extent of growth in inoculated untreated control hams was greater at 8 than at 4°C (Figure 4b), at which temperature *L. monocytogenes* numbers increased by ~2 log $CFU.g^{-1}$, and the population appeared to reach MPD after day 23, equivalent to 12 days after inoculation. Variation in the number of *L. monocytogenes* recovered from samples at 8°C occurred after day 37 and in some cases was below the level of detection of the methods used. At both temperatures *L. monocytogenes* reached MPD when the total aerobic count reached ~8.7 log $CFU.g^{-1}$. Additional data collected on day 23 for the 8°C samples showed variation: ~<0.3 log $CFU.g^{-1}$, in TVC, LAB and *L. monocytogenes* numbers across 10 replicates.

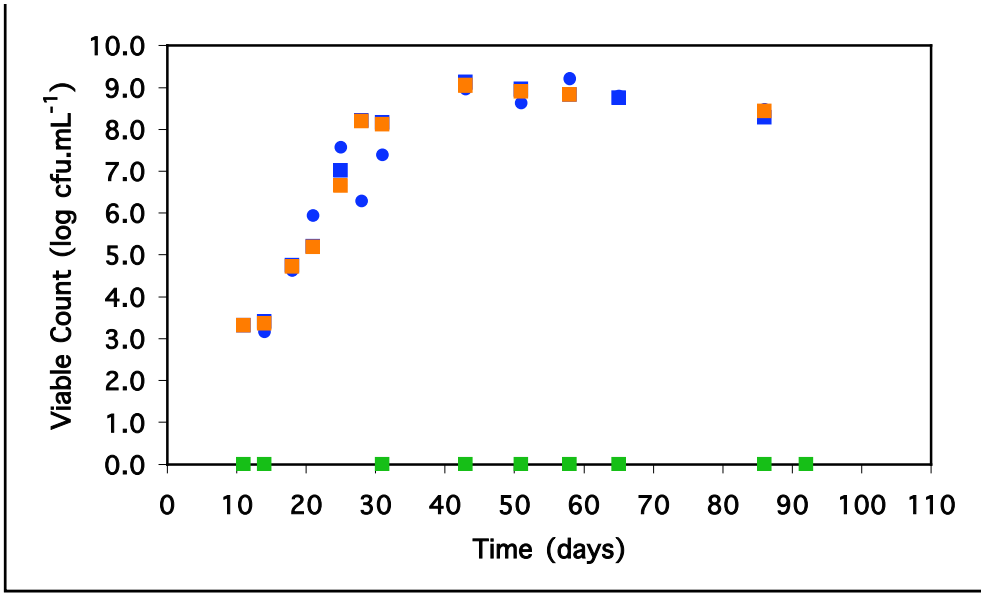


Figure 3(a) Temporal changes in microbial populations of untreated, uninoculated control hams during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts from duplicate samples are shown.

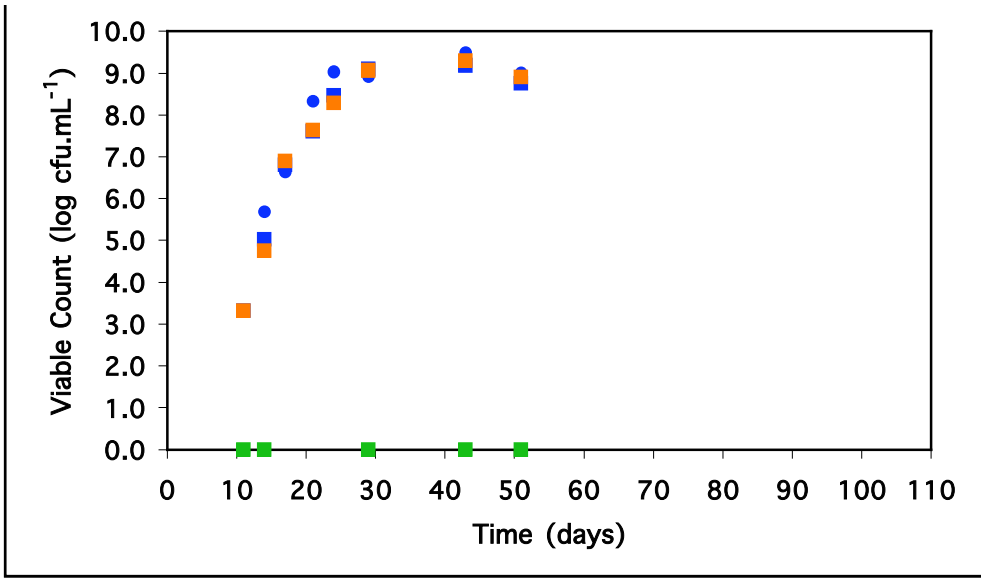


Figure 3(b) Temporal changes in microbial populations of untreated, uninoculated control hams during MAP storage at 8°C. Symbols as described for Figure 3a

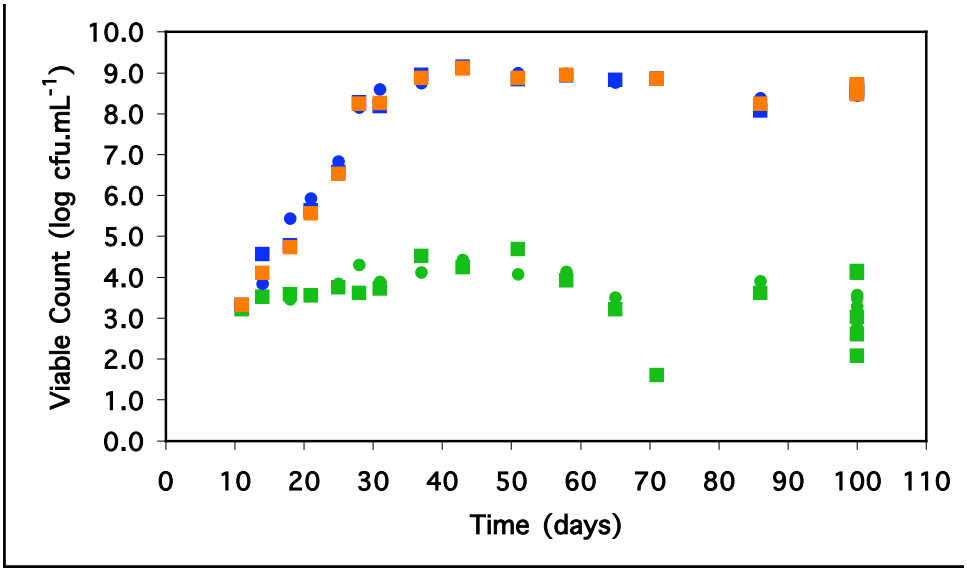


Figure 4a Temporal changes in microbial populations of untreated control hams inoculated with *L. monocytogenes* during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts are shown.

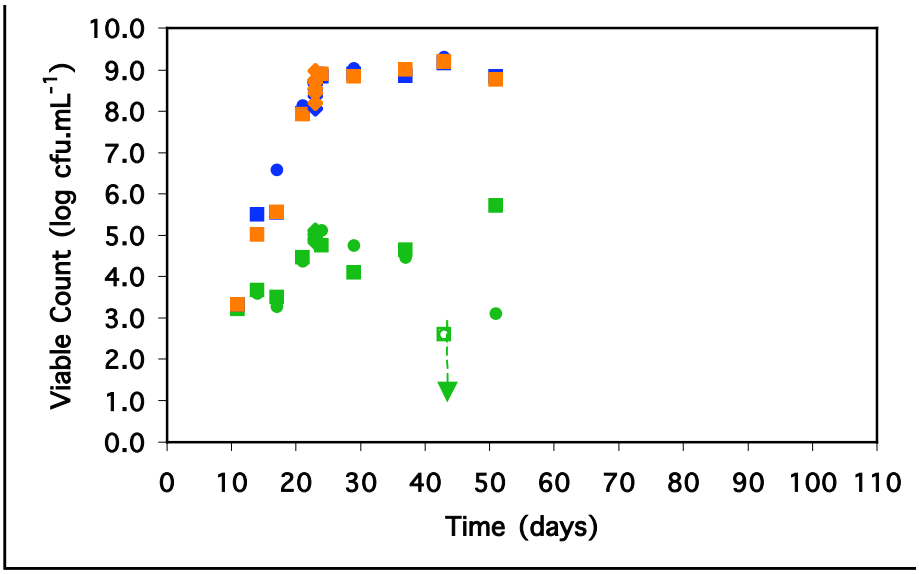


Figure 4b Temporal changes in microbial populations of untreated control hams inoculated with *L. monocytogenes* during MAP storage at 8°C. Symbols as described for Figure 4a. The open symbol denotes those cases where counts were below the level of detection; i.e. the arrow indicates that the count is below this point which represents the lower limit of detection for that sample.

Treated Controls-3% (w/w) of PURASAL®P Opti.Form 4

While overall growth was slower at 4 than at 8°C in ham treated with lactate/diacetate blend, at both temperatures the TVC was dominated by LAB (Figures 5 a and b). Variability in counts was observed between replicates samples at both temperatures. On day 25 at 4°C replicate samples differed by ~2.5 log CFU.g^{-1}. On day 17 at 8°C replicate samples differed by ~3.5 log CFU.g^{-1}.

In most cases no *Listeria* spp. were detected in uninoculated hams containing the lactate/diacetate blend incubated at either 4 or 8°C. Haemolytic *Listeria* spp were, however, detected on one occasion. 24 CFU.g^{-1} were present in one 8°C replicate sample at day 29 (Figure 5b). These isolates require further testing to determine whether they are *L. monocytogenes* or other haemolytic *Listeria* spp.

The addition of lactate and/or acetate salts appears to have had little effect on the time for the lactic acid bacteria (the numerically dominant organisms) to achieve stationary phase, nor the maximum population density that those organisms achieved. Similarly, the addition of *L. monocytogenes* did not appear to affect the time at which the lactic acid bacteria achieved stationary phase, nor their maximum population density When *L. monocytogenes* were inoculated into lactate/diacetate blend treated samples and stored at 4°C the increase in *L. monocytogenes* numbers was negligible, ≤0.4 log CFU.g^{-1}, up to day 65, equivalent to 54 days after inoculation (Figure 6a). Variability of ≥3 log CFU.g^{-1} was observed between replicate samples at days 71 and 101 respectively.

The response at 8°C was more variable than at 4°C (Figure 6b). In general ~1.0 log CFU.g^{-1} of growth occurred in most samples by day 24, equivalent to 13 days after inoculation. However, on one occasion (21 days; equivalent to 10 days after inoculation) *L. monocytogenes* numbers had increased 3-fold to ~6.0 log CFU.g^{-1}. In some cases, including one sample at the end of the incubation period (day 51; equivalent to 40 days after inoculation), numbers remained similar to those at the commencement of the trial.

The extra data collected on day 23 for the 8°C samples showed little variation, ~0.5 log CFU.g^{-1}, for *L. monocytogenes* numbers across 10 replicates. The variation in TVC and LAB numbers was higher, up to ~2 log CFU.g^{-1}.

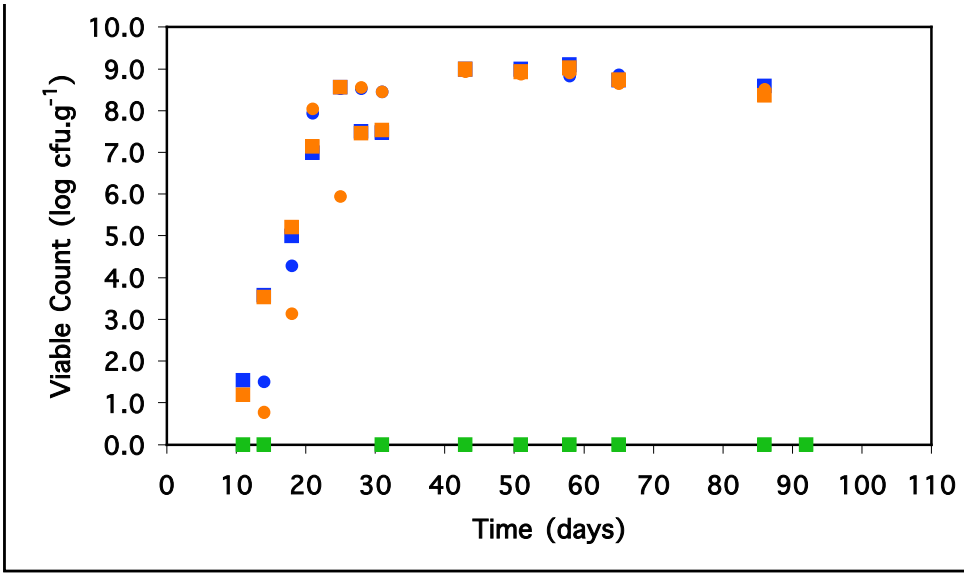


Figure 5(a) Temporal changes in microbial populations of uninoculated ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and presumptive *L. monocytogenes* (green symbols) counts from duplicate samples are shown.

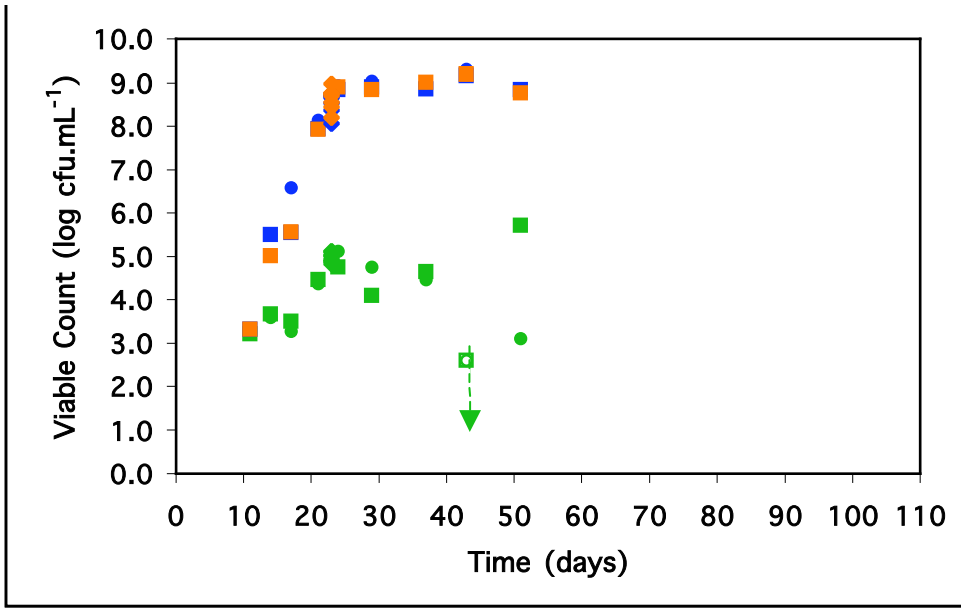


Figure 5(b) Temporal changes in microbial populations of uninoculated ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 during MAP storage at 8°C. Symbols as described for Figure 5a.

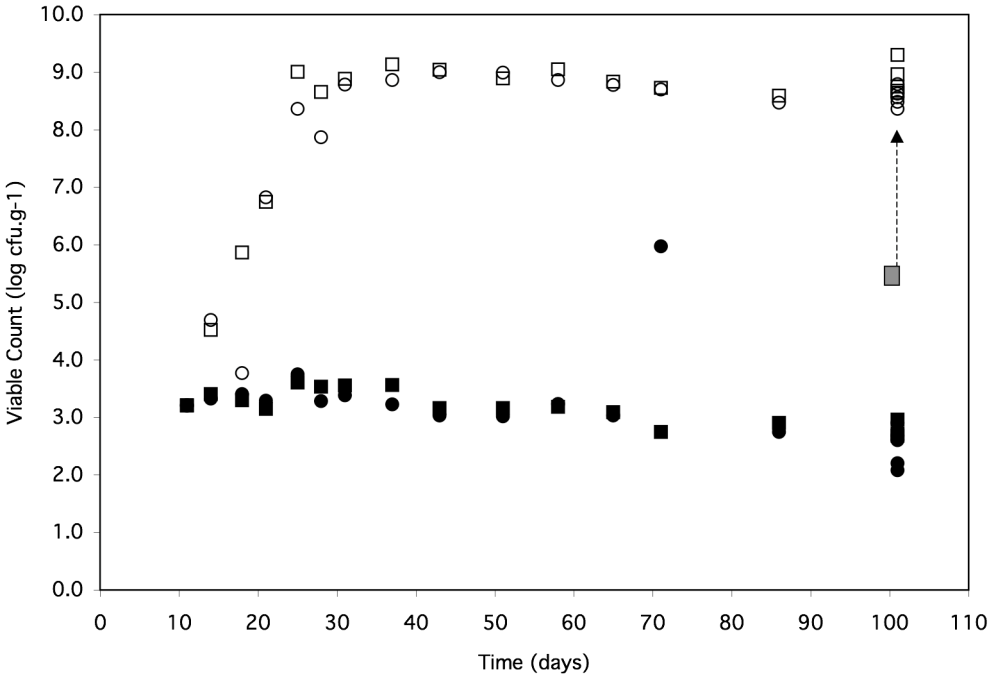


Figure 6(a) Temporal changes in microbial populations of hams treated with 3% (w/w) of PURASAL®P Opti.Form 4 inoculated with *L. monocytogenes* during MAP storage at 4°C. Total viable aerobic count (open squares), lactic acid bacteria (open circles) and *L. monocytogenes* (closed symbols) counts are shown. The open symbol denotes those cases where counts were above the level of detection; i.e. the arrow indicates that the count is anywhere above this point.

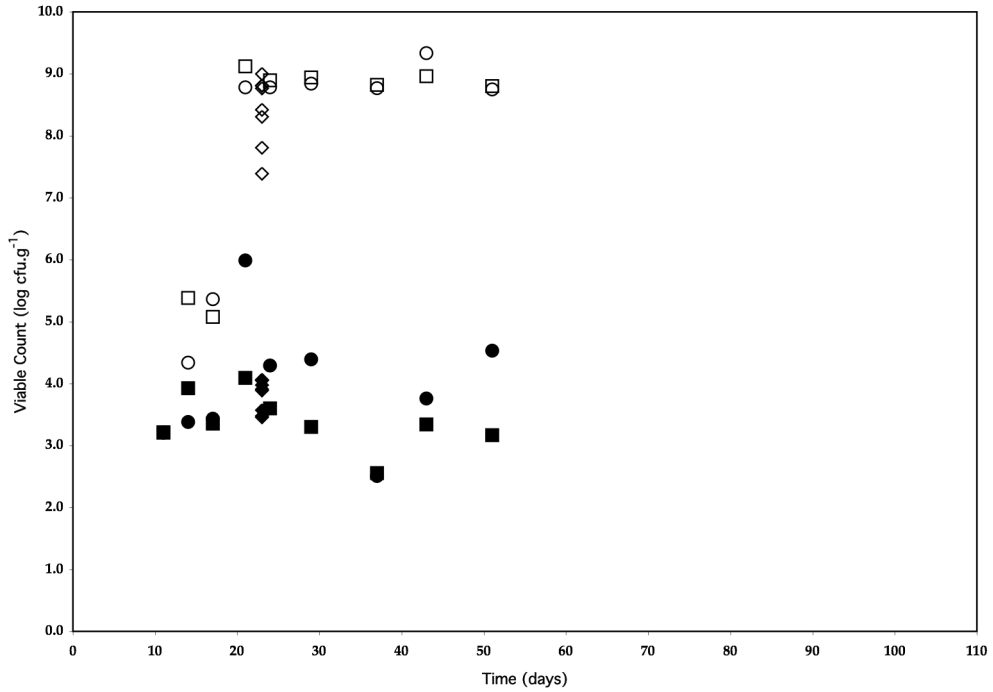


Figure 6(b) Temporal changes in microbial populations of hams treated with 3% (w/w) of PURASAL®P Opti.Form 4 and inoculated with *L. monocytogenes* during MAP storage at 8°C. Symbols as described for Figure 6(a).

Treated Controls-3% PURASAL®S/SP 60

In most cases no *Listeria* spp. were detected in uninoculated hams containing sodium lactate incubated at either 4 or 8°C (Figures 8 a and b). If present, however, levels of *L. monocytogenes* were below the limit of detection of the methods used (i.e. 2 log $CFU.g^{-1}$). Haemolytic *Listeria* spp were, however, detected on one occasion with 2 $CFU.g^{-1}$ present in a 4°C sample at day 31. These isolates require further testing to determine whether they are *L. monocytogenes* or other haemolytic *Listeria* species.

While overall growth was slower at 4 than at 8°C in ham treated with sodium lactate, the 4°C had slightly more variable TVC and LAB counts.

When *L. monocytogenes* were inoculated into ham samples containing sodium lactate and stored at 4°C, an increase in *L. monocytogenes* numbers occurred in some samples after a lag period up to day 21, equivalent to 10 days after inoculation (Figure 8a). An ~1 log $CFU.g^{-1}$ increase was observed for one sample at day 25. The *L. monocytogenes* numbers in remaining samples either showed a negligible increase, ≤0.4 log $CFU.g^{-1}$, or remained unchanged up to day 70, equivalent to 59 days after inoculation. Thereafter the variability in *L. monocytogenes* numbers increased. On the final sampling occasion a total of 10 replicates were assessed and counts in *L. monocytogenes* varied by up to ≥3 log $CFU.g^{-1}$.

At 8°C growth occurred in most samples (Figure 8b). In many cases this was without a lag period and MPD stabilised at around 4.5 log $CFU.g^{-1}$. In other samples log linear growth occurred, with numbers increasing to ~7.0 log $CFU.g^{-1}$ for one sample. For some samples no growth of *L. monocytogenes* occurred.

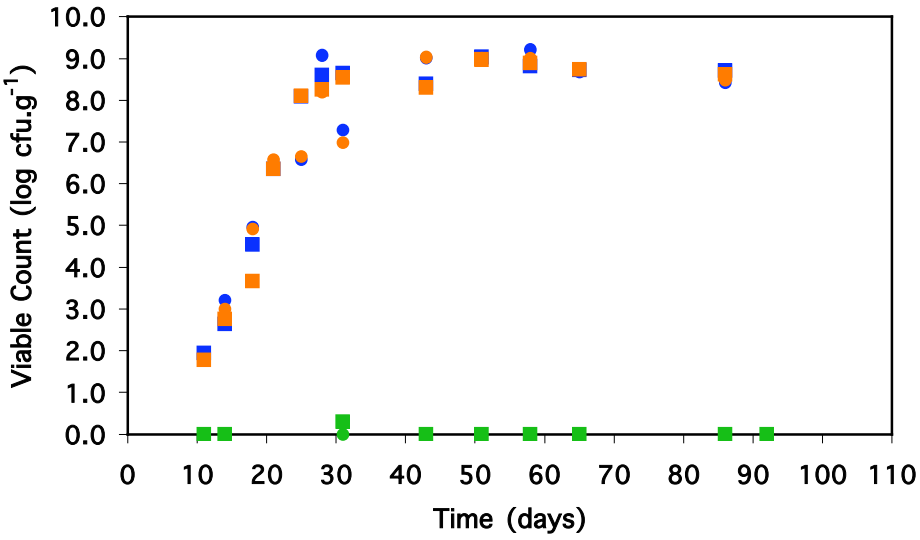


Figure 7(b) Temporal changes in microbial populations of uninoculated ham treated with 3% PURASAL®S/SP 60 during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts from duplicate samples are shown.

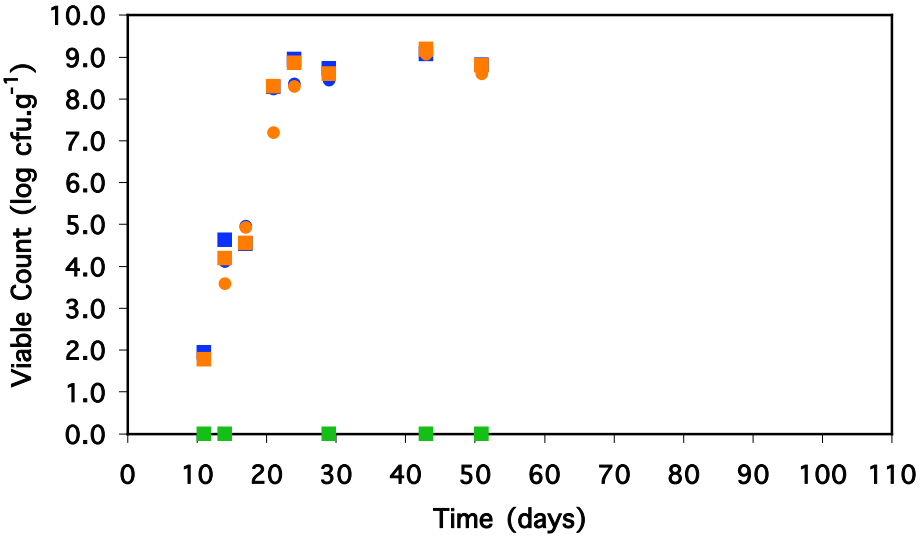


Figure 7(b) Temporal changes in microbial populations of uninoculated ham treated with 3% PURASAL®S/SP 60 during MAP storage at 8°C. Symbols as described for Figure 7(a).

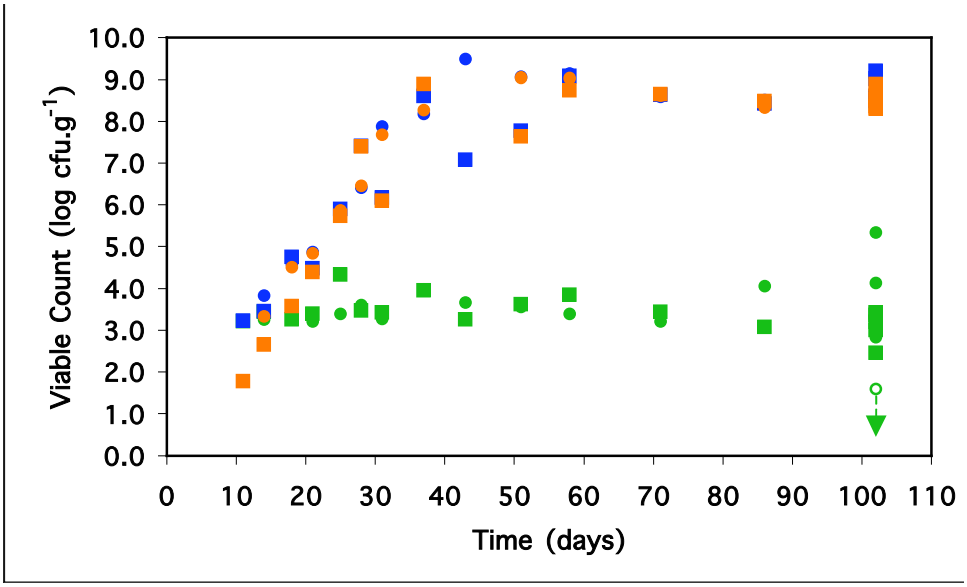


Figure 8(a) Temporal changes in microbial populations of hams treated with 3% PURASAL®S/SP 60 and inoculated with *L. monocytogenes* during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts are shown. The open symbol denotes those cases where counts were below the level of detection; i.e. the arrow indicates that the count is anywhere below this point.

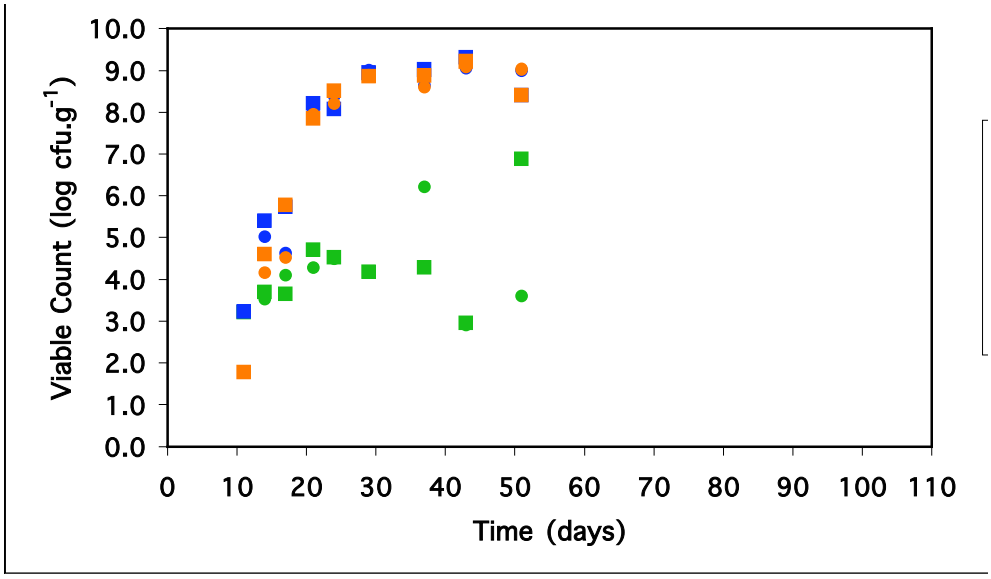


Figure 8(b) Temporal changes in microbial populations of hams treated with 3% PURASAL®S/SP 60 and inoculated with *L. monocytogenes* during MAP storage at 8°C. Symbols as described for Figure 9(a).

Despite the TVC numbers in hams with added weak acid salts observed at the commencement of trials in comparison to control untreated hams, ham shelf life was not improved at either 4 or 8°C (Figures 9 a and b; note the data presented are for duplicate samples only and do not include those occasions where a greater number of replicates were analysed). At the two test temperatures the growth response of the total aerobic microflora in treated hams was similar to that of untreated control hams.

Addition of *L. monocytogenes* to the hams did not affect the growth response of the total aerobic microflora in most cases (Table 5). At 8°C the generation time estimates were similar for uninoculated and inoculated hams, or slightly faster for inoculated hams, regardless of treatment. Similarly at 4°C the control hams showed little systematic effect of addition of *L. monocytogenes*. However, for treated hams at 4°C the data are more variable. Notably, the addition of Opti.Form 4 reduced the growth of TVC compared to either untreated or lactate-treated hams.

Table 5 Generation time (days) of total aerobic microflora in untreated and weak acid salt treated hams at 4 or 8°C. Average and standard deviation of estimates at each temperature are marked in bold.

Temp. (°C)	Untreated Control		3% (w/w) PURASAL®P Opti.Form 4		3% PURASAL®S/SP 60	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
4	1.18	1.16	0.51	0.81	0.74	1.48
	1.17 (±0.02)		**0.66 (±0.21)**		**1.11 (±0.53)**	
			All 4°C = 0.96 (±0.36)			
8	0.65	0.75	0.46	0.55	0.57	0.76
	0.70 (±0.07)		**0.51 (±0.06)**		**0.67 (±0.13)**	
			All 8°C = 0.63 (±0.12)			

In practically all cases addition of weak acid salts to ham samples suppressed the growth of *L. monocytogenes* in comparison to control untreated hams at 4°C (Figure 9a). PURASALS/SP®60 (3% w/w) was generally more effective than PURASAL®P Opti.Form 4 3% (w/w), with no significant increase in *L. monocytogenes* numbers observed for most of the trial. A small amount of growth (~0.6 log CFU.g^{-1}) was observed in 3% (w/w) of PURASAL®P Opti.Form 4 treated hams. Growth of *L. monocytogenes* was observed on both types of treated hams but only well after the product had been stored longer that its nominal shelf life. It should be noted that for ease of interpretation a shortened time scale has been used in Figure 9a as it was not possible to calculate

the average for the 10 replicate samples from day 101 due to the estimates exceeding the upper counting limit, ≥ 5.2 log CFU.g^{-1}, on 2 occasions.

At 8°C the results were more variable, although in general 3% (w/w) of PURASAL®P Opti.Form 4 was the most effective treatment (Figure 9b). Growth of *L. monocytogenes* was restricted to ~ 1 log CFU.g^{-1} in most samples on most occasions; the exceptions being a 3 log CFU.g^{-1} increase in one replicate of the day 21 samples, equivalent to 10 days after inoculation (see Figure 6b).

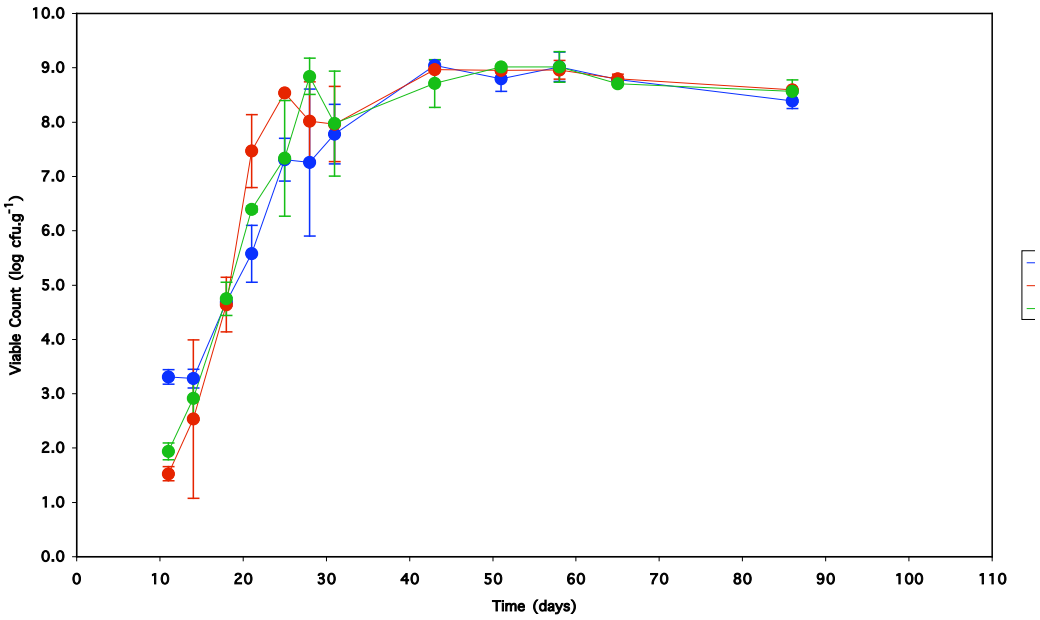


Figure 9(a) Temporal changes in total viable aerobic count of either untreated ham (blue symbols), ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) or ham treated with 3% PURASAL®S/SP 60 (green symbols) at 4°C. Error bars denote standard deviations based on duplicate samples.

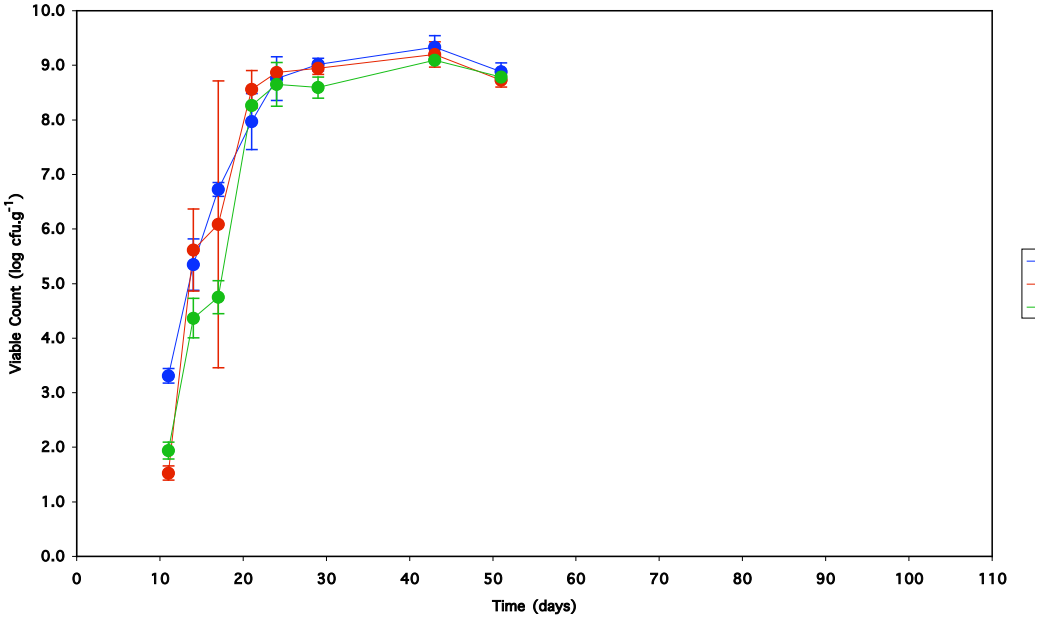


Figure 9(b) Temporal changes in total viable aerobic count of either untreated ham (blue symbols), ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) or ham treated with 3% PURASAL®S/SP 60 (green symbols) at 8°C. Error bars denote standard deviations based on duplicate samples.

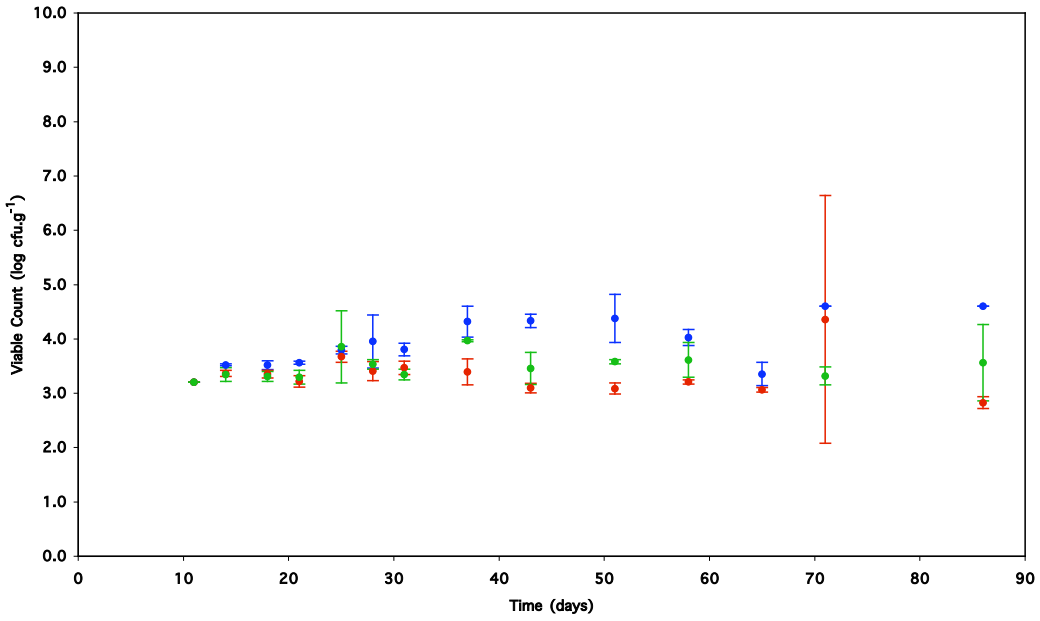


Figure 10(a) Temporal changes in *L. monocytogenes* numbers after 86 days for either untreated ham (blue symbols), ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) or ham treated with 3% PURASAL®S/SP 60 (green symbols) at 4°C. Error bars denote standard deviations based on duplicate samples.

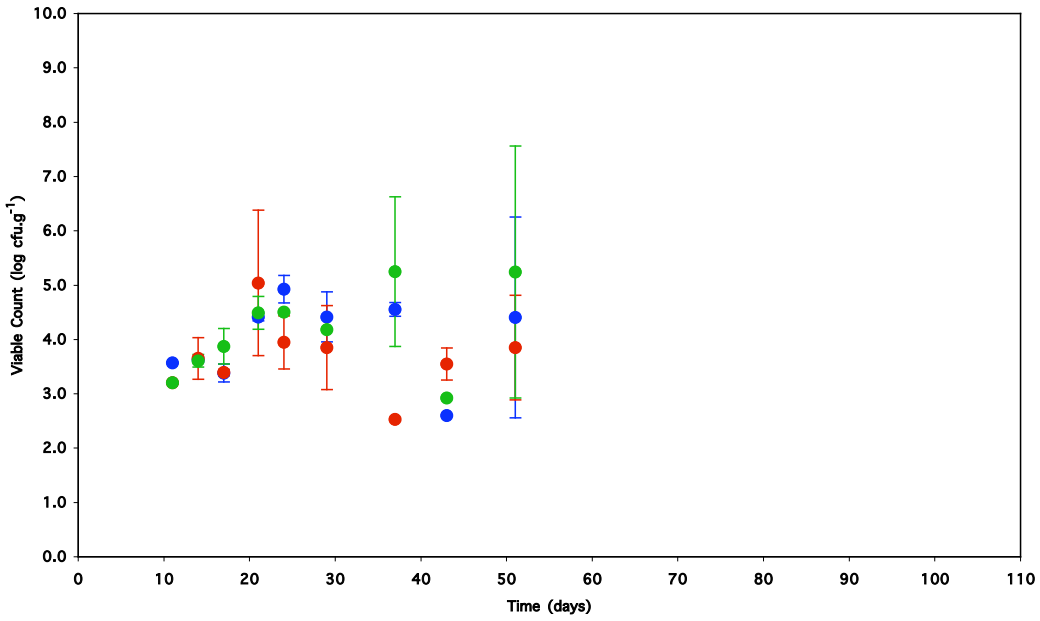


Figure 10(b) Temporal changes in *L. monocytogenes* numbers for either untreated ham (blue symbols), ham treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) or ham treated with 3% PURASAL®S/SP 60 (green symbols) at 4 and 8°C. Error bars denote standard deviations based on duplicate samples.

Microbiology - Chicken

Untreated Controls

No *Listeria* spp. were detected in untreated, uninoculated control chicken samples incubated at either 4 or 8°C (Figures 11 a and b). Thus, if present, levels of *L. monocytogenes* were below the limit of detection of the methods used (i.e. 2 log CFU.g^{-1}). Growth of total aerobic microflora and LAB was highly variable at both temperatures, but more so at 4°C. In some cases duplicate samples for either temperature varied by as much as 4-5 log CFU.g^{-1}. In some cases the TVC consisted predominantly of LAB. On other occasions fewer LAB were recovered.

When *L. monocytogenes* were inoculated into control chicken samples and stored at 4°C growth was observed after an apparent lag period up to day 28, equivalent to 19 days after inoculation (Figure 11a). Thereafter *L. monocytogenes* appeared the dominant microflora in most samples, i.e. it comprised most of the total aerobic viable count. Up to ~ 2 log CFU.g^{-1} variability in *L. monocytogenes* was observed between replicates samples.

A similar response was observed at 8°C, with *L. monocytogenes* growth observed after an apparent lag period up to day 16, equivalent to day 7 after inoculation (Figure 15). As for 4°C samples, *L. monocytogenes* appeared the dominant microflora in most samples. The variability in replicate samples was, however, less than observed at 4°C.

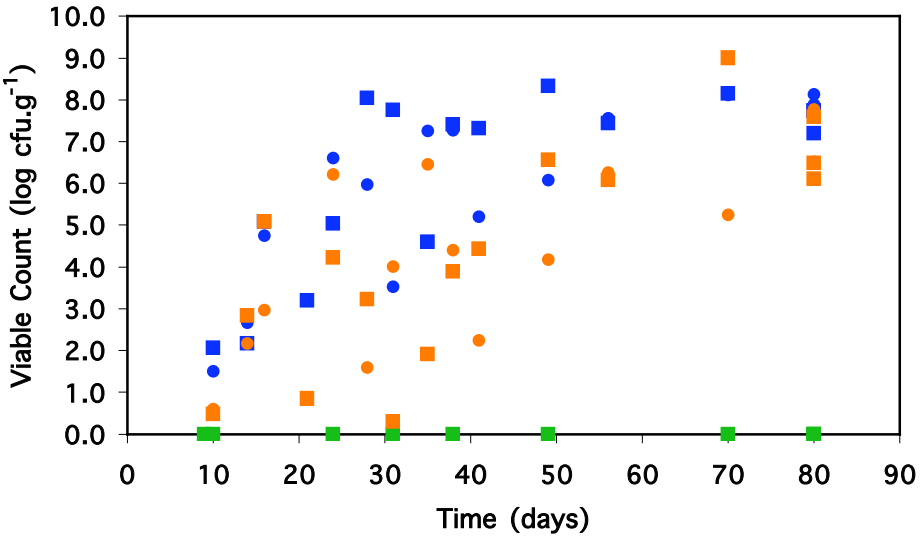


Figure 11(a) Temporal changes in microbial populations of untreated, uninoculated control chicken during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts are shown.

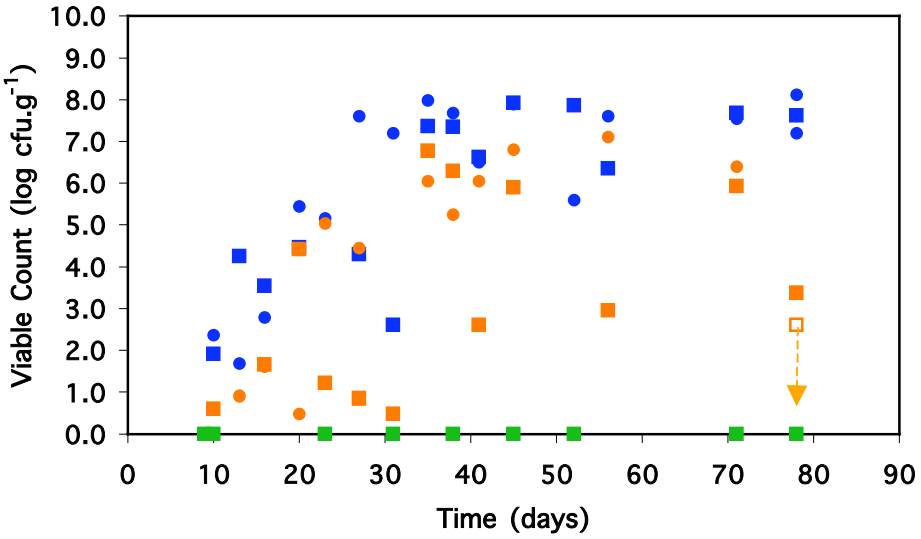


Figure 11(b) Temporal changes in microbial populations of untreated, uninoculated control chicken during MAP storage at 8°C. Symbols as described in Figure 11a. The open symbol denotes those cases where counts were below the level of detection; i.e. the arrow indicates that the count is anywhere below this point.

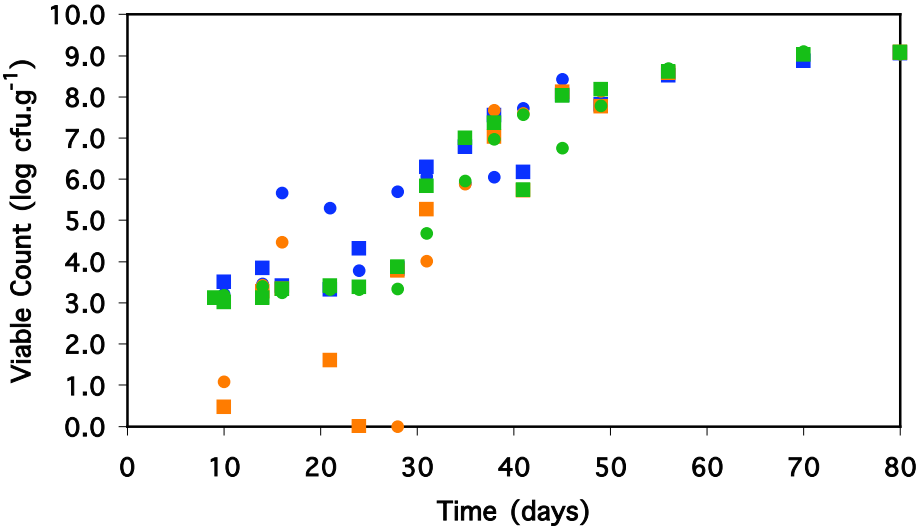


Figure 12(a) Temporal changes in microbial populations of untreated control chicken samples inoculated with *L. monocytogenes* during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts from duplicate samples are shown.

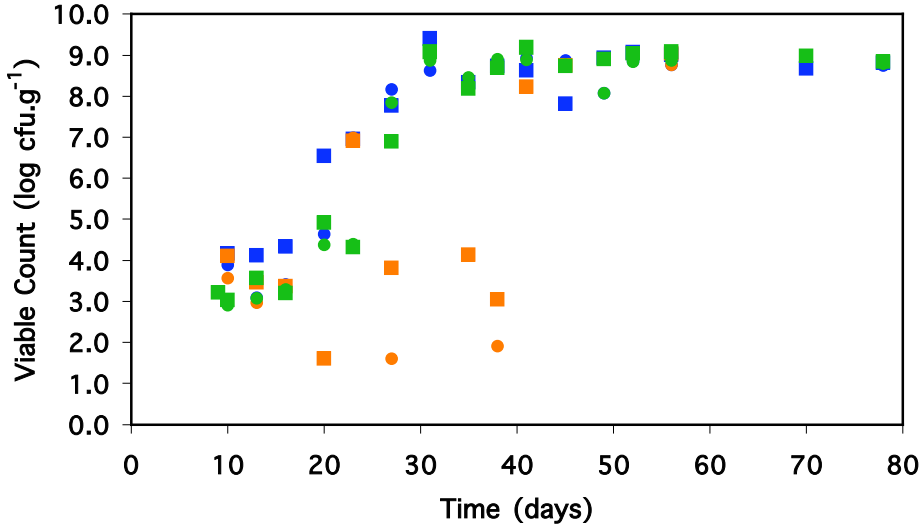


Figure 12(b) Temporal changes in microbial populations of untreated control chicken samples inoculated with *L. monocytogenes* during MAP storage at 8°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts from duplicate samples are shown.

Treated Controls-3% (w/w) of PURASAL®P Opti.Form 4

No *Listeria* spp. were detected in most uninoculated chicken containing the lactate/diacetate blend incubated at either 4 or 8°C (Figures 13 a and b). If present, however, levels of *L. monocytogenes* were below the limit of detection of the methods used (i.e. 2 log $CFU.g^{-1}$). Haemolytic *Listeria* spp were, however, detected on one occasion with 114 $CFU.g^{-1}$ present in a 4°C sample at day 50. These isolates require further testing to determine whether they are *L. monocytogenes* or other haemolytic *Listeria* species. No significant increase in total aerobic viable count occurred in 4°C samples until after day 40. After day 80 the total aerobic counts remained below 6.3 log $CFU.g^{-1}$. At 8°C the growth response was more variable with estimates from replicates samples varying by as much as 6.3 log $CFU.g^{-1}$. While growth occurred as early as day 16 in some samples, other samples showed no increase after day 45.

When *L. monocytogenes* were inoculated into samples treated with lactate/diacetate blend and stored at 4°C no significant increase in *L. monocytogenes* numbers occurred until after day 50, equivalent to 41 days after inoculation (Figure 14 a). Thereafter *L. monocytogenes* numbers generally increased but with that increase characterised by high variability between samples.. Sampling of 5 and 6 replicates near completion of the trial, i.e. on day 80 and 84, revealed that *L. monocytogenes* count varied by up to 5.4 log $CFU.g^{-1}$ between individual samples. In most cases throughout the trial *L. monocytogenes* appeared to be the dominant microflora, i.e. it comprised most of the total aerobic viable count.

At 8°C the increase in *L. monocytogenes* numbers began earlier than at 4°C, at day 38. *L. monocytogenes* appeared the dominant microflora in almost all cases, i.e. it comprised most of the total aerobic viable count.

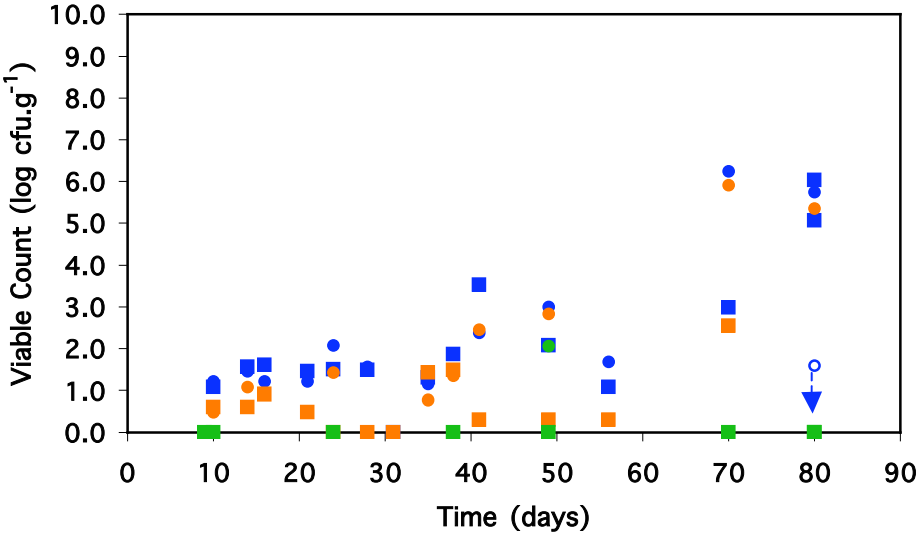


Figure 13(a) Temporal changes in microbial populations of uninoculated chicken samples treated with 3% (w/w) of PURASAL®P Opti.Form 4 during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts are shown. The open symbol denotes those cases where counts were below the level of detection; i.e. the arrow indicates that the count is anywhere below this point.

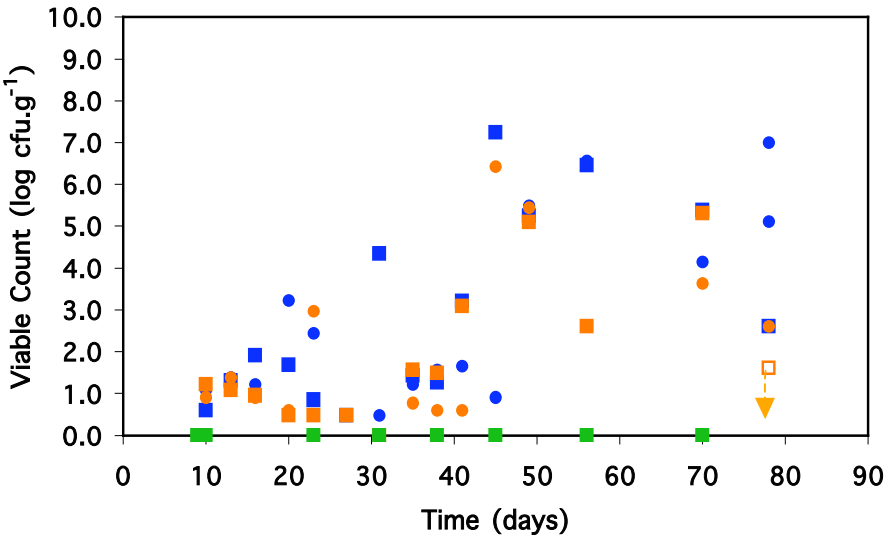


Figure 13(b) Temporal changes in microbial populations of uninoculated chicken samples treated with 3% (w/w) of PURASAL®P Opti.Form 4 during MAP storage at 8°C. Symbols are as described for Figure 13a.

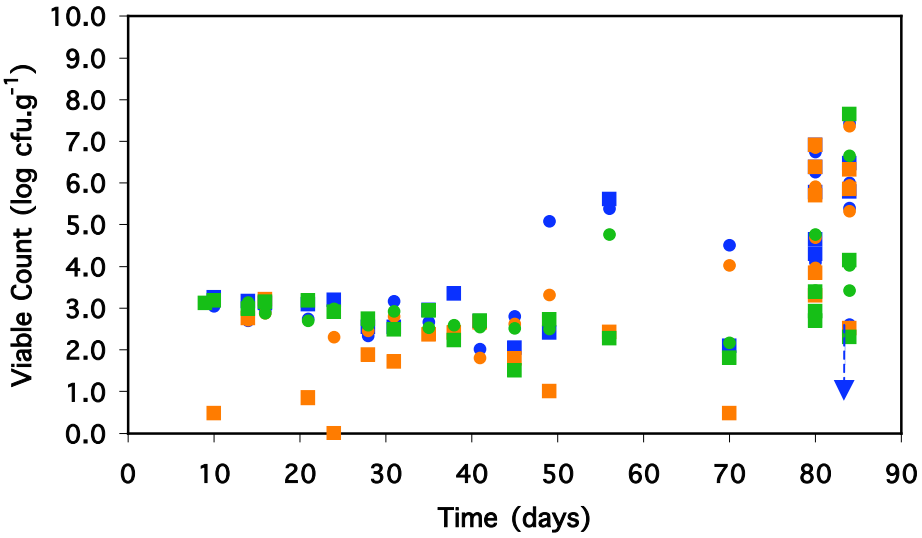


Figure 14(a) Temporal changes in microbial populations of chicken samples treated with 3% (w/w) of PURASAL®P Opti.Form 4 and inoculated with *L. monocytogenes* during MAP storage at 4°C. Total viable aerobic count (blue symbols), lactic acid bacteria (orange symbols) and *L. monocytogenes* (green symbols) counts are shown. The open symbol denotes those cases where counts were below the level of detection; i.e. the arrow indicates that the count is anywhere below this point.

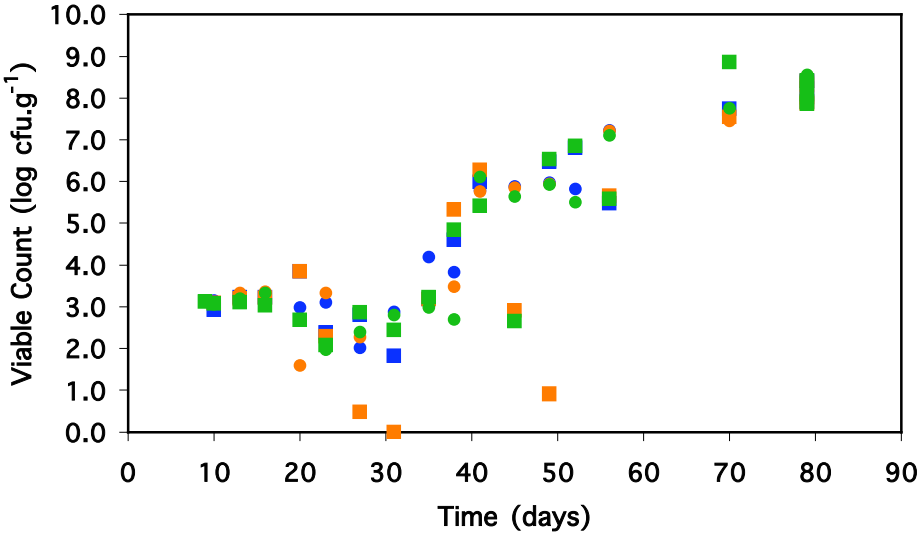


Figure 14(b) Temporal changes in microbial populations of chicken samples treated with 3% (w/w) of PURASAL®P Opti.Form 4 and inoculated with *L. monocytogenes* during MAP storage at 4°C. Symbols are as described for Figure 14a.

Despite the variability of the TVC and LAB counts in the lactate/diacetate blend chicken samples there are some trends (Figures 15 a and b). In general, addition of 3% (w/w) of PURASAL®P Opti.Form 4 to the chicken samples resulted in lower total aerobic and LAB counts at both 4 and 8°C in comparison to untreated control chicken samples. The effect is more pronounced at the lower temperature.

Addition of 3% (w/w) of PURASAL®P Opti.Form 4 to chicken samples did suppress the growth of *L. monocytogenes* at both 4 and 8°C up to days 41 and 22 respectively (Figure 16). Conversely, extensive growth of *L. monocytogenes* occurred in the untreated samples. In untreated samples stored at 4°C, *L. monocytogenes* displayed a lag time of 12 days after inoculation, while at 8°C a lag time of 2 – 3 days was apparent. In both treatments *L. monocytogenes* eventually grew to levels of ~10^9 cfu.g^{-1}.

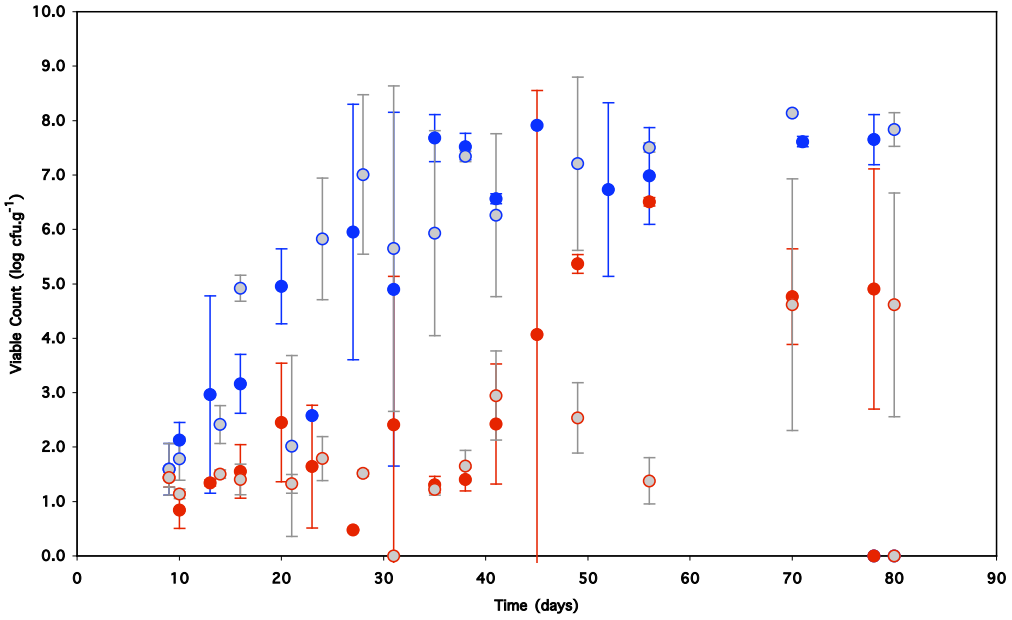
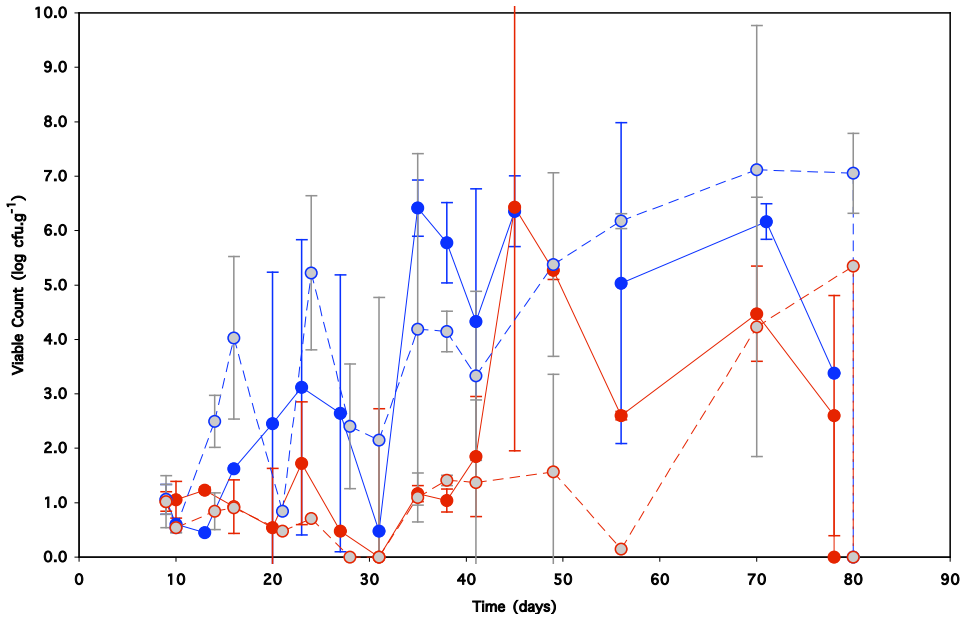


Figure 15(a) Temporal changes in total aerobic viable count for either untreated chicken (blue symbols) or chicken treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) at 4°C (shaded symbols) and 8°C (solid symbols). Error bars denote standard deviations based on duplicate samples.



Figures 15(b) Temporal changes in lactic acid bacteria count for either untreated chicken (blue symbols) or chicken treated with 3% (w/w) of PURASAL®P Opti.Form 4 (red symbols) at 4°C (shaded symbols) and 8°C (solid symbols). Error bars denote standard deviations based on duplicate samples.

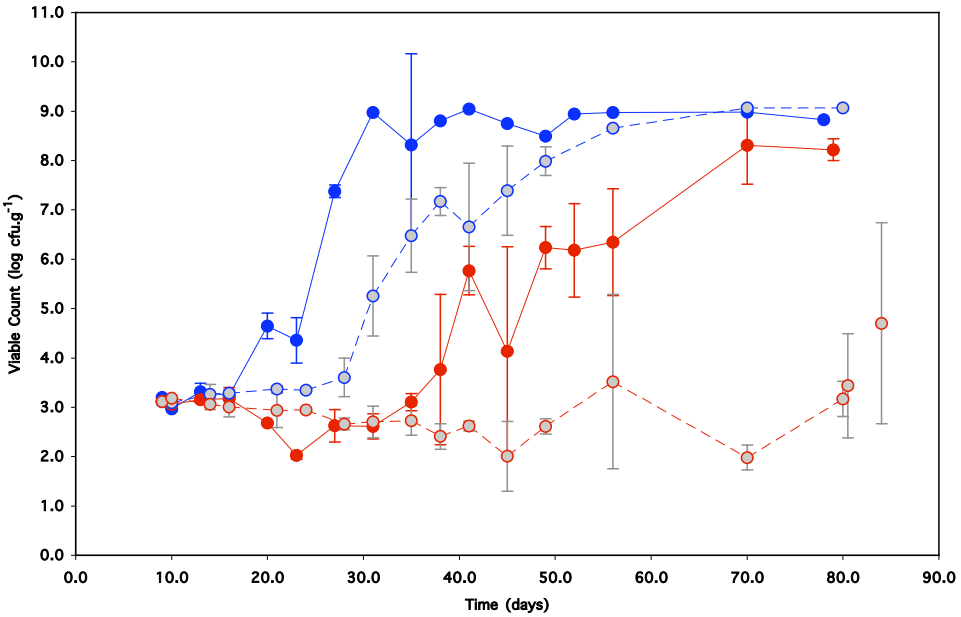


Figure 16 Temporal changes in *L. monocytogenes* numbers for either untreated chicken (solid symbols) or chicken treated with 3% (w/w) of PURASAL® P Opti.Form 4 (shaded symbols) at 4°C (red symbols) and 8°C (blue symbols). Error bars denote standard deviations based on duplicate samples except for those data at day 80 and 84 where a greater number of replicate samples were analysed.

Conclusions

Collectively, the results indicate that the addition of lactate or lactate and diacetate salts can reduce the risk of listeriosis from smallgoods by reducing the potential for growth of *L. monocytogenes*. It must be stressed, however, that while significant inhibition of growth of *L. monocytogenes* is observed in either chicken or ham samples stored at 4°C, the inhibition achieved at 8°C was more modest. Importantly, in almost all product and treatments growth of *L. monocytogenes* was observed within the nominal shelf life typical of MAP, sliced, processed cooked meat products, *viz*. 6- 8 weeks. The exception was Opti.Form 4-treated ham stored at 4°C for which the suppression of *L. monocytogenes* growth was complete.

The effect of other microorganisms on the potential growth of *L. monocytogenes* was also highlighted. Despite the similarity in the physico-chemical composition of the two products tested (i.e. chicken, ham), *L. monocytogenes* grew to much higher levels in the chicken samples. This is probably because the microbial load on the chicken at the time of inoculation of the product with *L. monocytogenes* was much less than in the ham challenge trials. As discussed and exemplified in various reports (PRMS.019, 2003; Ross, 2004; Stiles, 1996), when the total level of any microorganism present in a batch of foods reaches stationary phase, other populations of microoganisms present stop growing as well. This has been termed the Jameson Effect. In the case of MAP or VP smallgoods, the organisms that are dominant and reach stationary phase first are usually lactic acid bacteria. The difference in the results of the ham trials with lactate and the chicken trials with lactate is an excellent example of the role of lactic acid bacteria, and the Jameson Effect in general, on the potential risk from listeriosis in processed meats. In short, in products that have low levels of 'background' microbiota, the potential growth of *L. monocytogenes* - and hence the risk from the product - are greater, irrespective of other treatments. It must be noted, however, that unrealistically high levels of *L. monocytogenes* were used in these trials compared to 'natural' levels of contamination. This was necessary to be able to reliably measure changes in *L. monocytogenes* numbers over the life of the trial. Nonetheless, the principle that background microbiota can play an important role in controlling the risk of *L. monocytogenes* has been clearly demonstrated.

It was also observed that lactate and diacetate salts in combination can reduce the growth rate of other bacteria present on processed meat products and, as such, can extend their shelf life. This was observed most clearly in treated chicken samples. The results also suggest that a mixture of lactate and diacetate salts in ham inhibits the growth of indigenous microbiota more than lactate

salts alone, but an increase in shelf life (adjudged by the time taken for the TVC to reach 10^9cfu.g$^{-1)}$ was not observed. The reason for the difference is not known but, while the initial microbiota of the ham products was dominated by lactic acid bacteria, this was not the case for the chicken product. The identities of the non-lactic acid bacteria on the processed chicken were not determined, but it is possible that these organisms were more inhibited by lactate than are lactic acid bacteria.

PART 2 - MODEL SYSTEM DEVELOPMENT

OVERVIEW AND OUTCOMES

The second objective of this project was to develop a model system that would enable the systematic study of interactions of anti-listerial components in processed meats. The ultimate aim was to develop reliable predictive models for smallgoods manufacturers so that they might be able to develop product formulations and packaging systems that prevent listerial growth. As discussed, the ability to demonstrate that *L. monocytogenes* could not grow in the product would potentially provide considerable regulatory relief. This aspect of the project was also proposed to be used as a vehicle for Honours student training.

The project was offered to completing 3rd year BSc and BBiotech students, but none took up the project during 2006. A Summer Studentship was offered and taken up by Mr. Adam Teo in late 2006/early 2007. Mr. Teo clearly demonstrated (results not shown) that the presence of nitrite reduced *L. monocytogenes* growth rate, and moved the growth/no growth boundary to milder levels of other factors. Those effects were consistent with expectations from published studies showing he inhibitory effect of nitrite on *L. monocytogenes* growth (Duffy *et al*., 1994; Augustin *et al*., 2005). Unfortunately, for family reasons, Mr. Teo was unable to continue with the project as an Honours student. This project was then included in the Honours studies of Mr. Pragesh Devendran in mid-2007, but has met with limited success due to technical reasons. The work is, however, continuing but has taken a different direction, as discussed below.

A model that does include all abiotic factors that are considered to affect *L. monocytogenes* growth rate in ready-to-eat processed meats was published in early 2007. The model also includes terms for the prediction of interactions of factors that might, in combination, *preclude* growth. The model has been evaluated using 71 sets of data for growth of *L. monocytogenes* in smoked and/or marinated fish products, and found to accurately predict the growth, or growth potential, in 68 cases. The model development and evaluation is described in Mejholm and Dalgaaard (2007), a copy of which is included as Appendix 2 to this document.

The Mejholm and Dalgaaard model is based on a model developed earlier at University of Tasmania for Meat and Livestock Australia in project PRMS.012. The PRMS.012 model incorporated elements from other published studies of Devlieghere and colleagues in Belgium (Devlieghere *et al*., 1998; 1999; 2000; 2001), also active in predictive microbiology of *Listeria monocytogenes* in processed meats. Elements of the Belgian group's work are also included in the Mejholm and Dalgaaard model. The new model also uses modelling techniques developed by Le Marc *et al*. (2002) to predict combinations of factors that preclude growth.

While the model was validated against fish products, many of the antilisterial factors used in those products are the same as those used or proposed for use with processed meats. Thus, factors in the model include:

- temperature; (range: 2 to 15°C)
- water activity (or aqueous phase salt); (range: 2 to 9% aqueous phase NaCl; i.e. a_w ~0.985 to 0.943)
- pH; (range: 5.8 to 7.5)
- lactate concentration (including both added sodium or potassium lactate or lactate present due to muscle metabolism); (range: added lactate 0 to 3% wt/vol)
- diacetate concentration; (range: 0 to 0.5% wt/vol)
- phenol concentration (essentially a measure of total smoke components); (range: 0 to 20 ppm)
- CO_2 level for MAP-packed products; (range: 0 to 3100 ppm in aqueous phase) and
- nitrite concentration; (range: 0 to 350 ppm).

While nitrite is not used with lightly preserved fish products, the term was included in the original PRMS.071 model and was retained by Mejholm and Dalgaard (2007) to make the model relevant to more types of product, including processed meats.

The reasons for inclusion of these factors are that they have all been demonstrated to inhibit *L. monocytogenes* growth to a greater, or lesser, extent and would all be expected to contribute to the Hurdle Effect. Mejholm and Dalgaard (2007) provide a concise review of relevant studies demonstrating the inhibitory effects of these factors. The full model is described in detail in Appendix 2.

In the current project, the model has been incorporated into spreadsheet software that converts parameter values relevant to product formulation and storage conditions into predictions of growth rate, or whether grow is expected to occur. The spreadsheet has been developed so that more usual units of measurement can be used, rather than the values used in the model itself, with the software undertaking the necessary conversions. A "screen capture" of the model is shown in Figure 17, overleaf, and the software is included on a CD-ROM with this report.

The model predictions from the software can be used in one of two ways. In the first, product formulations are entered and, if the combined effect is predicted to prevent *L. monocytogenes* growth, the software notifies the user that no growth is predicted. For some combinations of factors, however, growth prevention is not predicted. Nonetheless, the predicted growth rate may

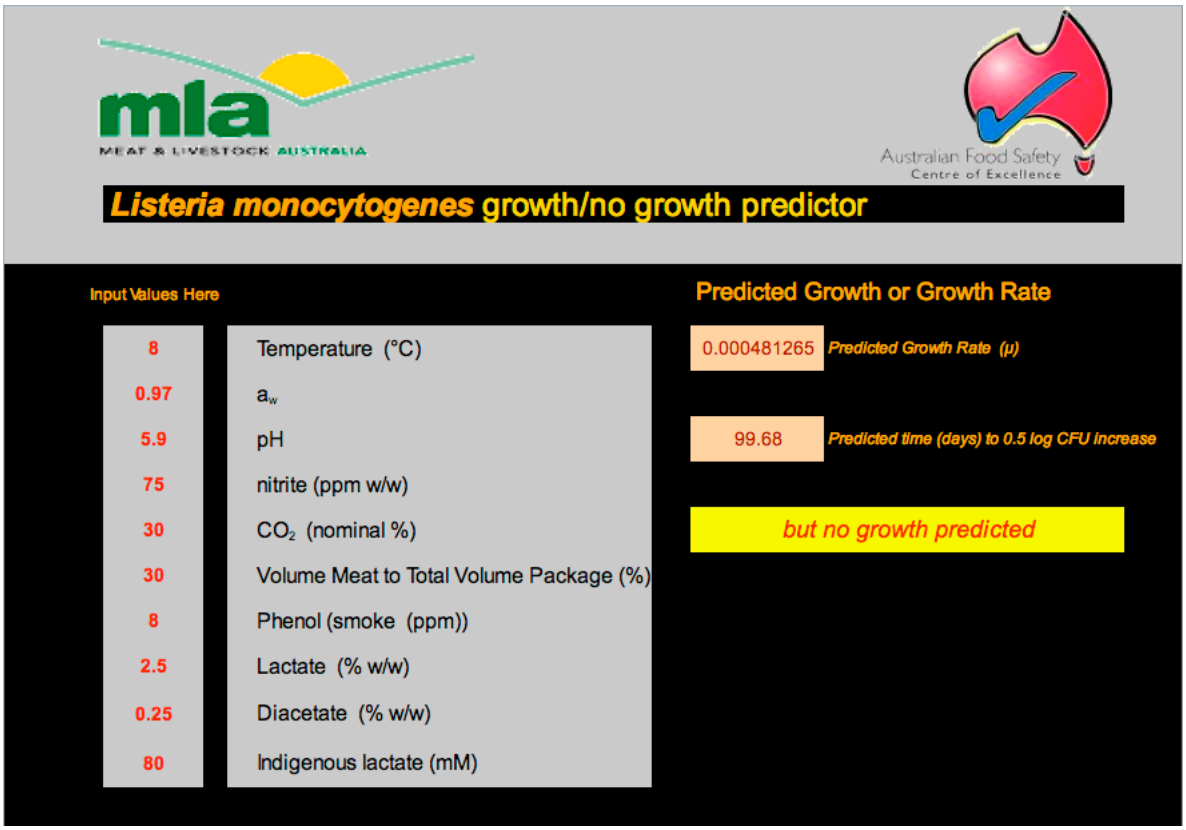


Figure 17. Screen-capture of software encompassing the predictive model of Mejholm and Dalgaard (2007), for prediction of growth rate and growth potential of *L. monocytogenes* in ready-to-eat meat and fish products.

be very slow, and unlikely to lead to measurable increases in *L. monocytogenes* levels. For this reason a second output of the model is the predicted time to a 0.5 logCFU increase in levels of *L. monocytogenes*. If this time exceeds the shelf life of the product, the product essentially will be shelf stable, particularly because the model does not consider the effects of lag time of *L. monocytogenes* contaminating the product, nor the effect of other microbiota

A conclusion of PRMS.071 was that the model produced in that project over-predicts the potential for growth when compared to published studies for *L. monocytogenes* growth in food Initial experiments with the Mejholm and Dalgaard (2007) model have demonstrated that it is *less* conservative than the model produced in PRMS.071, the precursor to the current project. In other words, for any given combination of lactate, water activity and temperature, the new model will predict 'no growth' at more moderate levels than the model from PRMS.071. When the effects of nitrite and phenolics are also included, the model predicts that yet more moderate levels of lactate and diacetate may be adequate to prevent *L. monocytogenes* growth in smallgoods products.

The maximum permissible level of nitrite in processed meat is 125 ppm. At this level, nitrite is predicted by the model to extend the time to detectable growth of *L. monocytogenes* by

approximately 50%. At 50 ppm nitrite the reduction in growth rate is less, in the range 10 - 15%. Typical levels of phenolics in processed meats range from 2 – 18 ppm. Assuming a moderate level of smoke compounds (8 ppm) extends the time to detectable growth of *L. monocytogenes* by approximately a further 50%. Thus, inclusion of realistic values for nitrite and smoke compounds in the model, has a very large effect on the predicted rate of growth. In turn, these effects on predicted growth rate result in lower levels of other factors being predicted to be required to prevent *L. monocytogenes* growth in processed meat product. Carbon dioxide levels are predicted to have relatively less effect, e.g. doubling CO_2 levels from 20 to 40 % is predicted to delay time-to-detectable-growth by ~10% only. Unfortunately, when realistic values for smallgoods products are included in the model (*viz*. 4°C, a_w 0.975; pH 6.2; 75 ppm nitrite, 30% CO_2, 8 ppm phenol, and 3% added lactate and 80mM indigenous lactate), the model predicts growth, albeit slow. However, if the pH were reduced to 6.08, no growth would be expected to occur. Under these conditions it is, apparently, the level of undissociated lactic acid that leads to predicted cessation of growth. For example, eliminating the CO_2, phenol and nitrite hurdles leads to a predicted pH limit for growth of 6.05, i.e. there is a relatively minor contribution of these factors at this pH/lactate combination. For this reason, the estimate of the minimum concentration of undissociated lactic acid that leads to complete inhibition of growth becomes a fundamentally important parameter and it is suggested that this parameter be carefully verified. For example, while the Mejholm and Dalgaard model assumes a level of 3.79mM undissociated lactic acid as preventing growth, derived from earlier studies at University of Tasmania, more recent studies in our laboratories have resulted in estimates of 4.55 mM undissociated lactic acid as being required to prevent growth. At levels of diacetate that are sensorially acceptable, the analogous estimate of the minimum concentration of undissociated acetic acid is not as important to the predicted probability of growth. (In contrast, pH levels of 5.8 or less are predicted by the PRMS.071 model to be required to prevent *L. monocytogenes* growth in typical processed meats such as hams). From initial studies with the model, it appears that 'no growth' is usually predicted if the time predicted for a 0.5logCFU increase in *L. monocytogenes* exceeds ~ 20 days.

With the presentation of the Mejholm and Dalgaard model, and the model validation studies already undertaken, the original objectives of this part of the project have been superseded and are now largely redundant. Rather, the impetus should now be on further studies to validate the model, in particular at factor combinations predicted to be at, or near, the limits of growth of *L. monocytogenes* in the multi-factor space relevant to processed meat products. At the time of reporting this work has commenced.

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Appendix 1. Lactate and Acetate Analyses

Figures A1 and A2, below, present the results of analyses of lactate and acetate achieved in the final product.

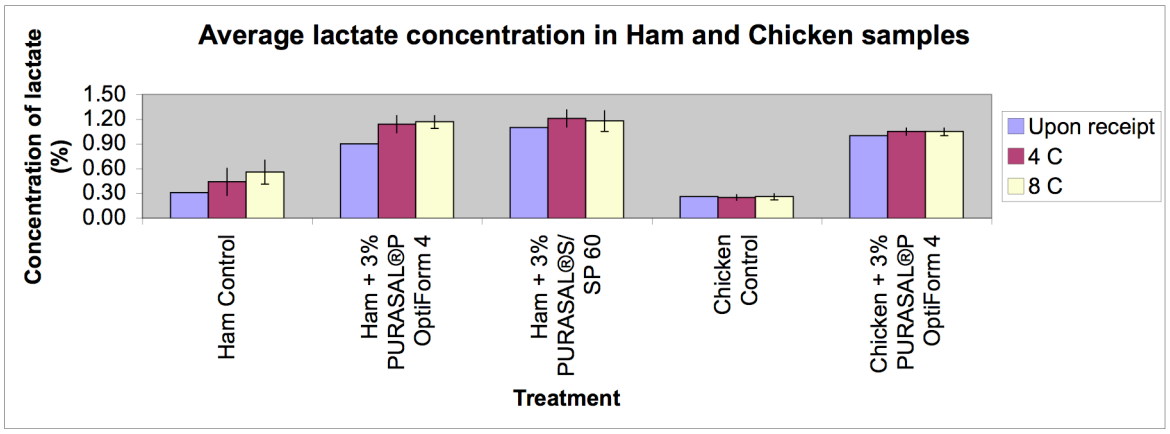

Figure A1. Average lactate concentrations determined in hams and chicken samples according to treatment and storage temperature.

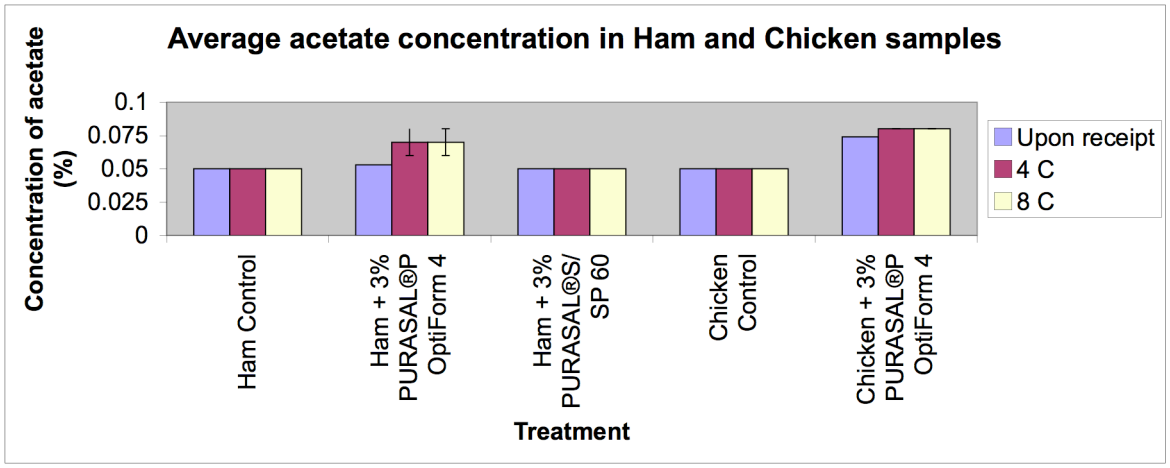

Figure A2. Average acetate concentrations in hams and chicken samples according to treatment and storage temperature.

The change in acetate and lactate levels as a function of storage time in various treatments are shown in Figures A3 and A4, overleaf.

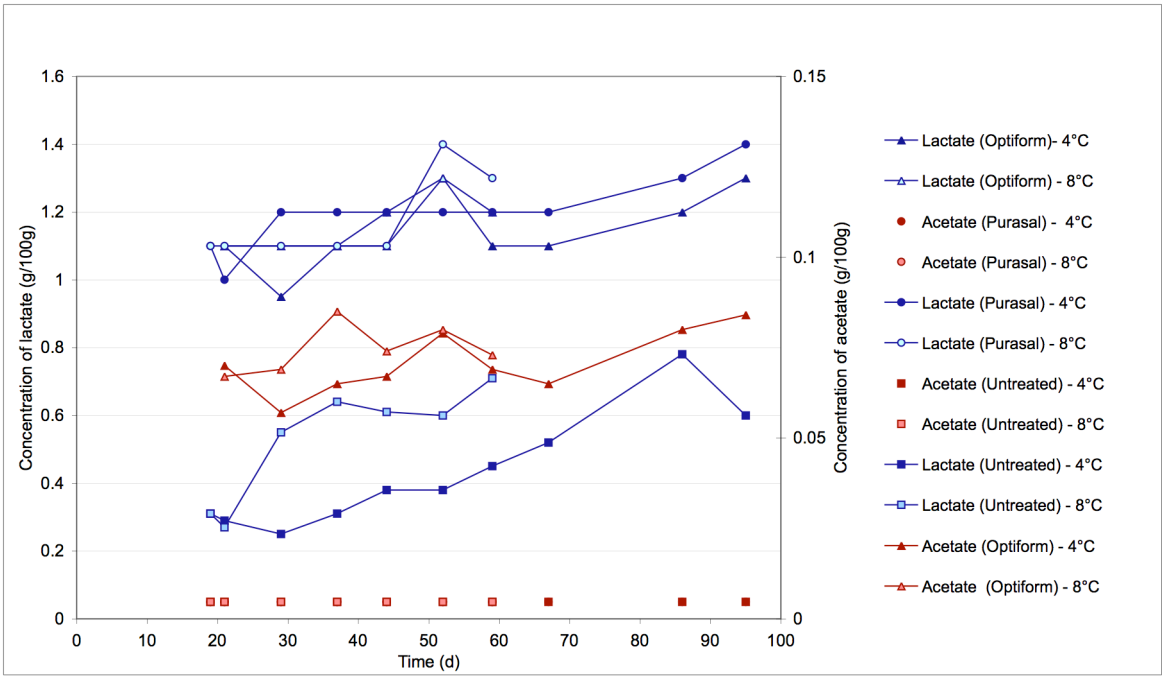

Figure A3. Changes in lactate and acetate levels in control and treated HAM samples stored at 4 or 8°C.

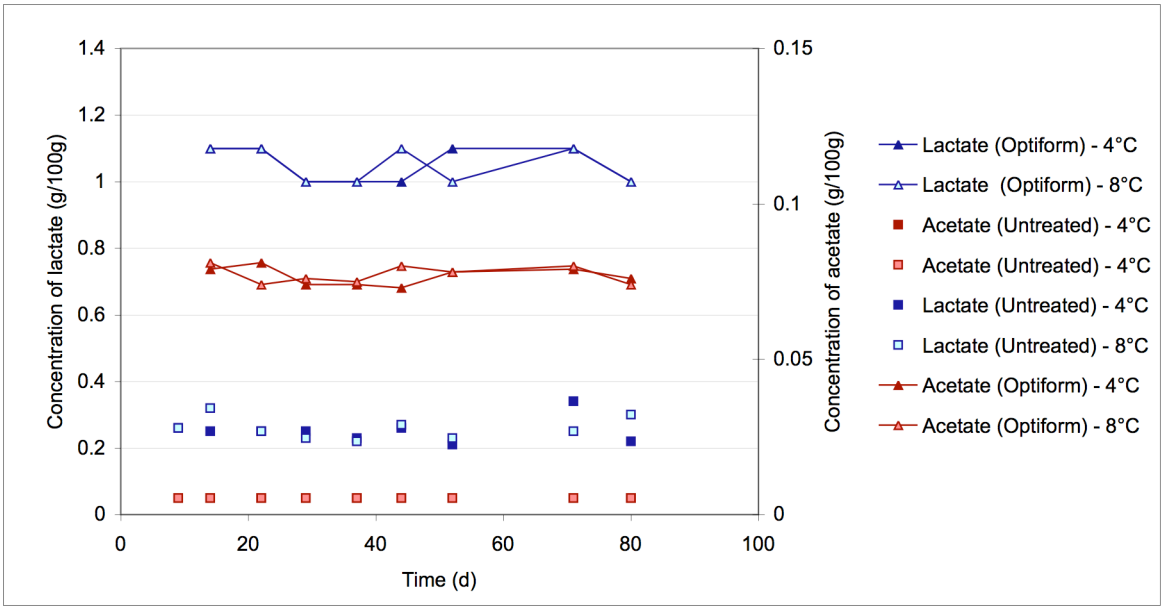

Figure A4. Changes in lactate and acetate levels in control and treated CHICKEN samples stored at 4 or 8°C.

Appendix 2. Mejholm and Dalgaard (2007)

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Modeling and Predicting the Growth Boundary of *Listeria monocytogenes* in Lightly Preserved Seafood

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ABSTRACT

The antimicrobial effect of diacetate and lactate against *Listeria monocytogenes* was evaluated in challenge tests with vacuum-packaged or modified atmosphere packaged (MAP) cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon. MAP cold-smoked salmon with the addition of 0.15% (wt/wt) diacetate prevented the growth of *L. monocytogenes* for more than 40 days at 8°C, whereas the addition of 0.15% (wt/wt) diacetate reduced the growth rate of the pathogen in MAP cold-smoked Greenland halibut. This difference between the two types of products was explained by a higher content of naturally occurring lactate in cold-smoked salmon (0.77 to 0.98%, wt/wt) than in cold-smoked Greenland halibut (0.10 to 0.15%, wt/wt). In fact, the addition of 0.15% (wt/wt) diacetate and 0.75% (wt/wt) lactate to MAP cold-smoked Greenland halibut prevented the growth of *L. monocytogenes* for more than 45 days at 8°C. A mathematical model that included the effect of diacetate, lactate, CO_2, smoke components, nitrite, pH, NaCl, temperature, and interactions between all these parameters was developed to predict the growth boundary of *L. monocytogenes* in lightly preserved seafood. The developed growth boundary model accurately predicted growth and no-growth responses in 68 of 71 examined experiments from the present study as well as from literature data. Growth was predicted for three batches of naturally contaminated cold-smoked salmon when a no-growth response was actually observed, indicating that the model is fail-safe. The developed model predicts both the growth boundary and growth rate of *L. monocytogenes* and seems useful for the risk management of lightly preserved seafood. Particularly, the model facilitates the identification of product characteristics required to prevent the growth of *L. monocytogenes*, thereby making it possible to identify critical control points, and is useful for compliance with the new European Union regulation on ready-to-eat foods (EC 2073/2005).

Despite considerable efforts to improve process hygiene and sanitation procedures, it has not been possible to successfully prevent the contamination of several types of ready-to-eat (RTE) seafood with *Listeria monocytogenes*. This is critical, as typical product characteristics, including pH, salt, and smoke components, are insufficient to prevent the growth of *L. monocytogenes*, e.g., in chilled and vacuum-packaged (VP) smoked and gravad products. Especially products with a long, chilled shelf life constitute a risk with respect to the growth of *L. monocytogenes* to critical concentrations *(17, 46, 47, 49)*.

Cold-smoked and gravad seafoods are lightly preserved RTE products of considerable economic importance. Control of *L. monocytogenes* in these products represents a substantial challenge to reduce the risk of listeriosis and to prevent recalls due to a lack of compliance with national and international regulations. For RTE seafood, European Union (EU) regulations differentiate between products that are able or unable to support the growth of *L. monocytogenes* and allow 100 CFU g^{-1} in the latter (EC 2073/2005). In the United States, the U.S. Food and Drug Administration demands the absence of *L. monocytogenes* in 25-g samples of lightly preserved seafood *(47)*. Preventing the growth of *L. monocytogenes* to high concentrations is the strategy expected to have the greatest impact on reducing cases of listeriosis *(49)*. Consequently, preservation procedures that prevent the growth of *L. monocytogenes* in lightly preserved seafood are of major interest, particularly procedures that can be used without altering the sensory characteristics of classical lightly preserved seafood.

Traditionally, cold-smoked and gravad seafoods have been distributed as VP products, but recently, the use of modified atmosphere packaging (MAP) with CO_2 has been increasing *(1, 33, 43)*. Carbon dioxide in MAP delays the growth of *L. monocytogenes* in several lightly preserved foods *(15, 33)*, and 100% CO_2 prevents growth in cold-smoked salmon at 4°C *(43)*. More frequently, organic acids and especially combinations of diacetate and lactate have been reported to prevent the growth of *L. monocytogenes* in various meat products *(6, 18, 40, 41)* as well as in chilled VP cold-smoked salmon *(50)*. Depending on concentrations, the addition of diacetate and lactate may alter the sensory characteristics of seafood, and further studies are needed to identify the combinations of these compounds that prevent the growth of *L. monocytogenes* without having a negative effect on product properties. Storage conditions (temperature and atmosphere) and product characteristics (NaCl, pH, smoke components, and naturally occurring lactate) in lightly preserved seafood most likely influence the antimicrobial effect of added diacetate and lactate.

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Thus, a mathematical model to predict the combined effect of all these parameters on the growth boundary of *L. monocytogenes* would be of considerable practical importance to assist in the formulation of safe products in compliance with the EU regulation on RTE seafood (EC 2073/2005).

Mathematical models for the growth limits of *L. monocytogenes* as a function of temperature, pH, NaCl, CO_2, smoke components (phenol), and organic acids are available *(3–5, 23, 26, 27, 45),* and the combined effect of diacetate, lactate, salt, and moisture in cooked meat at 4°C has also been modeled *(25, 40).* Clearly, it is interesting to use the combined effect of product characteristics and storage conditions as a means of identifying critical control points for *L. monocytogenes* in lightly preserved seafood. However, to our knowledge, mathematical models that include the effect of diacetate and lactate in combination with the environmental parameters that most likely influence the growth of *L. monocytogenes* in lightly preserved seafood are not available.

The objectives of the present study were to evaluate and mathematically model the antimicrobial effect of diacetate and lactate against *L. monocytogenes* in chilled, lightly preserved seafood. Cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon were studied to evaluate the importance of different product characteristics on the growth of *L. monocytogenes.* A new growth boundary model was developed to predict the effect of diacetate, lactate, CO_2, smoke components, nitrite, pH, NaCl, temperature, and interactions between the parameters on the growth boundary of *L. monocytogenes* in lightly preserved seafood.

MATERIALS AND METHODS

Challenge tests. A series of challenge tests were carried out with cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon. Two different batches of cold-smoked salmon and cold-smoked Greenland halibut as well as one batch of marinated salmon, marinated Greenland halibut, and gravad salmon were studied in a total of 24 challenge tests. Products were VP or MAP and stored at 8 or 15°C, as shown in Table 1. Salmon (*Salmo salar*) from aquaculture in Norway and Greenland halibut (*Reinhardtius hippoglossoides*) caught in the North Atlantic Ocean were used as raw materials and processed into sliced and packed fillets by a company in Denmark. After processing, the products were frozen and transported to the Danish Institute for Fisheries Research in their frozen state. The frozen, processed fillets were thawed overnight at 5°C and divided into slices. For each batch of each product, slices were randomly divided into subbatches prior to the addition of organic acids. Selected subbatches of the five products were added from 0.0 to 0.15% (wt/wt) diacetate and from 0.0 to 1.5% (wt/wt) lactate as shown in Table 1. A total of 0.183% (wt/wt) sodium diacetate (98%; Spectrum S1266, Spectrum, New Brunswick, N.J.) and 2.36% (vol/wt) sodium lactate (60% [wt/wt] syrup, Sigma L-1375, Sigma, St. Louis, Mo.) were added to the samples, corresponding to concentrations of 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate, respectively. Proportional concentrations of sodium diacetate and sodium lactate were added for other levels of diacetate and lactate. Solutions containing sodium diacetate and sodium lactate or only sodium diacetate were prepared by dissolving sodium diacetate in deionized water corresponding to 1% (vol/wt) of the weight of the product in question, and subsequently, when required, sodium lactate was added. To all subbatches was added the same amount of liquid, with sterile water being added to products with less than 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate (Table 1). For each subbatch, the liquid was added as four portions, and after each addition, the product was manually tumbled to ensure an even distribution of liquid and preservatives. Following this treatment, all subbatches were stored at 1 to 2°C for 24 h to allow distribution of preservatives in the samples.

Inoculation and packaging of samples. All subbatches were inoculated with a mixture of four *L. monocytogenes* isolates (94-203D, 95-54A, 95-442A, and 94-167B) previously obtained from seafood *(17, 21).* Initially, each isolate was grown (25°C, 1 day) in brain heart infusion broth (Oxoid CM225, Oxoid, Basingstoke, UK) and subsequently precultured (5°C, 4 to 5 days) in brain heart infusion broth with 4% NaCl. Preculturing at 5°C in brain heart infusion broth with 4% NaCl was chosen in order to adapt the isolates to the experimental conditions of the challenge tests resembling the actual salt concentrations and storage conditions of lightly preserved seafood. Precultures were harvested in late exponential growth phase, defined as a relative change in absorbance of 0.05 to 0.2 at 540 nm (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The inoculum was prepared by mixing the four precultures and diluting them in 0.85% NaCl to a cell density of approximately 10^4 *L. monocytogenes* ml^{-1} (MIX-Lm). To all subbatches was added 1% (vol/wt) MIX-Lm as four portions of 0.25% (vol/wt), and after each addition, the products were manually tumbled to distribute *L. monocytogenes* on the samples.

After inoculation, 100-g portions of the differently treated products (Table 1) were either VP or packaged in a modified atmosphere initially containing 40% CO_2 and 60% N_2 (AGA Ltd., Copenhagen, Denmark). A Multivac A 300/16 packaging machine (Multivac Ltd., Vejle, Denmark) and a packaging film (NEN 40 HOB/LLPDE 75, Amcore Flexibles, Horsens, Denmark) with low gas permeability (0.45 ± 0.15 cm^3 m^{-2} atm^{-1} for O_2 and 1.8 ± 0.6 cm^3 m^{-2} atm^{-1} for CO_2) were used. For MAP samples, the gas/fish ratio was approximately 2:1. Following packaging, subbatches were stored at 8 or 15°C, and data loggers (TinytagPlus, Gemini Data Loggers Ltd., Chichester, UK) recorded the storage temperatures continuously throughout the storage period.

Microbiological and chemical analyses. At regular intervals during the storage of each subbatch, three samples were analyzed by microbiological methods. Product samples of 20 g were diluted 10-fold in chilled (5°C) physiological saline (0.85% NaCl) with 0.1% peptone and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made in chilled physiological saline (0.85% NaCl) with 0.1% peptone. *L. monocytogenes* was determined by spread plating (37°C, 2 days) on PALCAM agar (Oxoid CM0877) with PALCAM Selective Supplement (Oxoid SR0150). Lactic acid bacteria (LAB) were enumerated by pour plating (25°C, 3 days) in nitrite actidione polymyxin agar with pH 6.2 *(14),* and aerobic plate counts were determined by spread plating (15°C, 7 days) on Long and Hammer agar with 1% NaCl *(48).*

Characteristics of the seven different batches of the five types of products (cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon) were determined by an analysis of three samples from each batch at the start of the challenge tests (day 0). Dry matter and pH were measured as previously described *(10).* Salt content was determined by an automated potentiometric titration method *(2).* Diacetate and lactate were determined by a previously described

TABLE 1. *Product characteristic of cold-smoked salmon (CSS), marinated salmon (MS), cold-smoked Greenland halibut (CSGH), marinated Greenland halibut (MGH), and gravad salmon (GS)*

Experimental design					Measured product characteristics and storage conditions						
			Added (%, wt/wt)								
Products and sub-batches (code)	Atmosphere	Temp (°C)	Diacetate	Lactate	pH	Water phase lactate (%)	Water phase diacetate (%)	Water phase salt (%)	Phenol (ppm)	Temp (°C)	% CO_2[a]
CSS-1 (a)	VP	8	—	—	6.0	0.98 ± 0.36	—[b]				—
CSS-1 (b)	MAP	8	—	—	6.1	0.90 ± 0.08	—[b]	5.26 ± 0.18	8.9 ± 0.9	7.8 ± 0.5	30.0 ± 1.7
CSS-1 (c)	MAP	8	0.15	—	5.9	0.77 ± 0.07	0.14 ± 0.04				
CSS-1 (d)	MAP	8	0.15	1.5	5.8	2.84 ± 0.29	0.19 ± 0.04				
CSS-2 (a)	MAP	15	—	—	6.0	0.67 ± 0.02	0.05 ± 0.00	3.58 ± 0.50	13.5 ± 1.5	14.3 ± 0.2	25.3 ± 0.8
CSS-2 (b)	MAP	15	0.15	—	5.9	0.60 ± 0.00	0.17 ± 0.01				
MS-1 (a)	MAP	8	—	—	6.2	0.35 ± 0.07	—[b]				
MS-1 (b)	MAP	8	0.015	—	6.2	0.33 ± 0.02	—[b]				
MS-1 (c)	MAP	8	0.03	—	6.2	0.40 ± 0.04	0.04 ± 0.01	3.67 ± 0.28	—[b]	7.8 ± 0.2	21.5 ± 1.7
MS-1 (d)	MAP	8	0.045	—	6.2	0.33 ± 0.05	0.03 ± 0.01				
MS-1 (e)	MAP	8	0.06	—	6.2	0.42 ± 0.06	0.05 ± 0.01				
MS-1 (f)	MAP	8	0.12	—	6.1	0.40 ± 0.02	0.10 ± 0.01				
CSGH-1 (a)	VP	8	—	—	6.4	0.15 ± 0.01	—[b]				—
CSGH-1 (b)	MAP	8	—	—	6.5	0.13 ± 0.00	—[b]	3.74 ± 0.05	16.2 ± 1.3	7.7 ± 0.5	26.0 ± 1.8
CSGH-1 (c)	MAP	8	0.15	1.5	6.2	2.26 ± 0.15	0.16 ± 0.08				
CSGH-2 (a)	MAP	8	0.15	—	6.2	0.10 ± 0.00	0.14 ± 0.01				
CSGH-2 (b)	MAP	8	0.15	0.25	6.3	0.33 ± 0.01	0.14 ± 0.01				
CSGH-2 (c)	MAP	8	0.15	0.5	6.2	0.57 ± 0.01	0.14 ± 0.01	4.64 ± 0.10	20.1 ± 1.7	7.6 ± 0.4	22.9 ± 1.8
CSGH-2 (d)	MAP	8	0.15	0.75	6.2	0.79 ± 0.03	0.16 ± 0.00				
CSGH-2 (e)	MAP	8	0.15	1.0	6.2	1.03 ± 0.03	0.15 ± 0.00				
MGH-1 (a)	MAP	8	—	—	6.8	0.17 ± 0.00	—[b]	3.40 ± 0.13	—[b]	7.8 ± 0.2	16.2 ± 0.4
MGH-1 (b)	MAP	8	0.15	0.9	6.7	0.62 ± 0.01	0.11 ± 0.00				
GS-1 (a)	MAP	15	—	—	6.3	0.64 ± 0.01	—[b]	3.03 ± 0.11	4.9 ± 0.8	14.3 ± 0.2	24.1 ± 1.1
GS-1 (b)	MAP	15	0.15	—	6.1	0.62 ± 0.02	0.12 ± 0.01				

[a] Equilibrium concentrations in headspace gas.
[b] Measured but not detected (ND).

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high-pressure liquid chromatography method *(12).* External standards were used for the identification and quantification of the compounds. Smoke components were estimated as phenols by a spectrophotometric method *(7).* Finally, the equilibrium composition of gas in MAP samples was measured with a Combi Check 9800-1 gas analyzer (PBI, Dansensor, Ringsted, Denmark). The sensorial effect of adding diacetate and lactate to the products was examined prior to the challenge test. Different concentrations of diacetate (0.0 to 0.20%, wt/wt), lactate (0.0 to 2.0%, wt/wt), or both were added to samples of fish as previously described. Subsequently, the sensorial properties (appearance, taste and smell, and texture) of the products were evaluated by an internal panel (five to six panelists), which judged the samples as either acceptable or not acceptable.

Statistical analyses and curve fitting. A one-way analysis of variance was used to determine whether differences between the mean values of repeated measurements were statistically significant. Calculations were carried out by Statgraphics Plus version 5.1 (Manugistics Inc., Rockville, Md.). To determine the lag time (expressed as hours) and maximum specific growth rate (μ_{max}; per hour), the four-parameter logistic model *(9)* was fitted to growth data determined on PALCAM and nitrite actidione polymyxin agar in a challenge test. The software package Fig.P (version 2.98, Biosoft, Cambridge, UK) was used for curve fitting. An *F*-test to compare fits of the three- and four-parameter logistic models was used to evaluate whether lag phases of the microbial growth curves were significant *(9).*

Modeling and predicting the growth of *L. monocytogenes* in lightly preserved seafood. Sixty μ_{max} values were obtained from the growth of *L. monocytogenes* in lightly preserved seafood as a function of storage conditions (temperature and CO_2) and product characteristics (water phase salt concentration or water activity [a_w], pH, lactic acid, nitrite, CO_2, smoke components [phenol], and diacetate). Eighteen growth curves were generated in the present study, and 42 μ_{max} values were obtained from the literature, together with relevant storage conditions and product characteristics *(17, 24, 35, 36, 43, 44, 50).* A relatively limited number of μ_{max} values were obtained from the literature, as many previous studies unfortunately did not report or measure important antimicrobial parameters, such as the concentration of smoke components (phenol) or even, in some cases, the a_w, pH, or lactic acid concentrations. Growth data were compared with predictions from existing secondary models and expanded versions of these models by calculation of bias and accuracy factor values from observed and predicted μ_{max} values *(11, 38).* The effect of temperature, water phase salt or a_w, and pH as well as concentrations of lactic acid, nitrite, and smoke components (phenol) was predicted by the secondary model previously studied by Giménez and Dalgaard *(17).* This model was then expanded by the addition of a term for the growth-inhibiting effect of CO_2 and a term for the effect of diacetate as indicated in equation 1. The CO_2 term was adapted from the model of Devlieghere et al. *(15)* and transformed into a model component $(CO_{2\ max} - CO_{2\ equilibrium})/(CO_{2\ max} - CO_{2\ opt})$ with a value between 0 and 1. The optimal concentration of carbon dioxide ($CO_{2\ opt}$) for the growth of *L. monocytogenes* was assumed to be zero. A $CO_{2\ max}$ value of 3,140 ppm was used with a 95% confidence interval of 2,917 to 3,365 ppm of CO_2 *(15).* The diacetate term $(1 - \sqrt{[DAC_U]/[MIC_{U\ DAC}]})$ was obtained from equations previously used to express the antimicrobial effect of organic acids *(3, 26).* In addition, the temperature and a_w terms of Giménez and Dalgaard *(17)* were reformulated to obtain terms with values between 0 and 1 in resemblance with the other model components. For the temperature term $((T - T_{min})/(T_{ref} - T_{min}))^2$, this was achieved by introducing a reference temperature (T_{ref}) of 25°C. The original a_w term ($a_w - a_{w\ min}$) was transformed to $(a_w - a_{w\ min})/(a_{w\ opt} - a_{w\ min})$, where the optimal a_w ($a_{w\ opt}$) for the growth was assumed to be 1.0.

$$\mu_{max} = b \cdot \left[\frac{(T - T_{min})}{(T_{ref} - T_{min})}\right]^2 \cdot \frac{(a_w - 0.923)}{(0.077)} \cdot [1 - 10^{(4.97 - pH)}] \cdot \left(1 - \frac{[LAC_U]}{[3.79]}\right) \cdot \left[\frac{(350 - NIT)}{350}\right]^2 \cdot \frac{(28.1 - P)}{(28.1)} \cdot \frac{(3{,}140 - CO_{2\ equilibrium})}{(3{,}140)} \cdot \left(1 - \sqrt{\frac{[DAC_U]}{[MIC_{U DAC}]}}\right) \cdot \xi \quad (1)$$

where $[LAC_U]$ is the concentration (expressed as millimoles) of undissociated lactic acid, NIT is the concentration of nitrite (expressed as parts per million), *P* is the concentration of phenols (expressed as parts per million), and $CO_{2\ equilibrium}$ is the concentration of dissolved CO_2 (expressed as parts per million) at equilibrium. The concentration of dissolved CO_2 (expressed as parts per million) was calculated by the measured percentage of CO_2 in the headspace gas at equilibrium and Henry's constant at the appropriate storage temperature *(39).* $[DAC_U]$ is the concentration (expressed as millimoles) of undissociated diacetate, and $[MIC_{U\ DAC}]$ is the theoretical concentration of undissociated diacetate preventing the growth of *L. monocytogenes.* The MIC of diacetate $[MIC_{U\ DAC}]$ was established in brain heart infusion broth (Oxoid CM225) (pH 6.0) at 8°C. The antimicrobial effect of 10 different concentrations of diacetate (0.0, 0.025, 0.05, 0.075, 0.10, 0.125, 0.15, 0.20, 0.35, and 0.50%, wt/vol) was examined against each of the four *L. monocytogenes* isolates (94-203D, 95-54A, 95-442A, and 94-167B) previously described. Isolates of *L. monocytogenes* were cultured as previously described, and an inoculum of 10^3 *L. monocytogenes* ml^{-1} was used. For each treatment, the growth of the four *L. monocytogenes* isolates was determined in triplicate by automated absorbance measurements at 540 nm (Bioscreen C, Labsystems, Helsinki, Finland). For each absorbance growth curve, the maximum specific growth rate (μ_{max} per hour) was determined by the logistic model *(13).* Square root–transformed μ_{max} values were plotted against concentrations of undissociated diacetate, and the MIC_U was estimated by a simple square root model *(3).* The concentration (expressed as millimoles) of undissociated organic acid was calculated as $[HA]/1 + 10^{pH - pK_a})$. [HA] was the total concentration (expressed as millimoles) of organic acid, and the pK_a values of 3.86 and 4.76 were used for lactic acid and diacetate, respectively. A P_{max} value of 28.1 ppm of phenol with a standard error of 2.8 ppm was used *(17).*

By using the approach of Le Marc et al. *(26),* a term was added to the expanded model of Giménez and Dalgaard *(17)* for the interaction between the environmental parameters and in this way was used to predict the effect of temperature, water phase salt or a_w, pH, lactate, nitrite, CO_2, smoke components (phenol), and diacetate on both the μ_{max} and the growth boundary of *L. monocytogenes* (equation 1). In brief, this approach expands the growth rate models by adding a term for the interactive inhibiting effect of environmental parameters. The hypothesis is that the contribution of each environmental factor to the interaction can be derived from its separated effect on the μ_{max} values *(26).* A term for the effect of interaction between environmental parameters (ξ) was included in equation 1, as indicated in equation 2, with contributions from the different environmental parameters calculated as indicated in equations 3 and 4. In equation 4, e_i represents environmental factors. It is assumed that (i) if ψ is lower than a predefined threshold value (θ), then no interactive effect between

environmental parameters occurs ($\xi = 1$); (ii) if ψ is higher than 1, then a no-growth response occurs ($\xi = 0$); and (iii) if ψ is lower than 1 and higher than θ, then the growth rate (μ_{max} per hour) is reduced, depending on the value of ψ. A threshold value (θ) of ½ was used, as suggested by Le Marc et al. *(26).*

The expanded model with interactions between environmental parameters (equation 1) was fitted to 39 of the 60 obtained μ_{max} values (previously described), and the values of the parameters b and T_{min} were estimated. This expanded model (equation 1) was fitted using nonlinear regression and the software SigmaStat (Systat Software GmbH, Erkrath, Germany). The remaining 21 μ_{max} values were used to evaluate the performance of the fitted model. An *F*-test was used to evaluate if terms for the different environmental parameters in the model had a significant effect on the μ_{max} values of *L. monocytogenes.*

$$\xi\{\varphi(T, a_w, \text{pH}, [\text{LAC}], \text{NIT}, P, CO_2, [\text{DAC}])\} = \begin{cases} 1, & \psi \le \theta \\ 2(1 - \psi), & \theta < \psi < 1 \\ 0, & \psi \ge 1 \end{cases} \quad (2)$$

where $\xi(\varphi(T, a_w, \text{pH}, [\text{LAC}], \text{NIT}, P, CO_2, [\text{DAC}]))$ is the term describing the effect of interactions between environmental parameters on μ_{max}.

$$\varphi_T = \{1 - [(T - T_{min})/(T_{ref} - T_{min})]\}^2$$

$$\varphi_{a_w} = (1 - \sqrt{(a_w - 0.923)/0.077})^2$$

$$\varphi_{pH} = [1 - \sqrt{1 - 10^{(4.97 - pH)}}]^2$$

$$\varphi_{NIT} = [1 - (350 - \text{NIT}/350)]^2$$

$$\varphi_{[LAC];[DAC]} = \{1 - [(1 - \sqrt{[LAC_U]/3.79}) \cdot (1 - \sqrt{[DAC_U]/[MIC_{UDAC}]})]\}^2$$

$$\varphi_P = [1 - \sqrt{(28.1 - P)/28.1}]^2$$

$$\varphi_{CO_2} = [1 - \sqrt{(3{,}140 - CO_{2\,\text{equilibrium}})/3{,}140}]^2 \quad (3)$$

$$\psi = \sum_i \frac{\varphi_{e_i}}{2 \prod_{j \ne i} (1 - \varphi_{e_j})} \quad (4)$$

The contribution of lactate and diacetate to the interaction term ($\varphi_{[LAC];[DAC]}$ in equation 3) was modeled by the multiplication of their effects as previously described *(8).*

The model recently suggested by Augustin et al. *(5)* was used to predict the effect of temperature, water phase salt or a_w, pH, nitrite, smoke components (phenol), CO_2, and interactions between these environmental parameters on the growth rate (μ_{max} value) of *L. monocytogenes.* In addition, this model was expanded by lactate and diacetate terms from equation 1 and the contribution of these organic acids to the effect of interaction between environmental parameters (equations 3 and 4).

Growth and no-growth responses of *L. monocytogenes* in lightly preserved seafood were obtained, together with product characteristics from the present study ($n = 24$), from challenge tests reported in the literature ($n = 34$) and from 13 lots of naturally contaminated cold-smoked salmon *(11, 35, 43, 50).* A no-growth response was defined as an increase in *L. monocytogenes* concentration that was less than 0.5 log CFU g^{-1} for the duration of the experiment. These growth and no-growth data were compared with predictions from two growth boundary models: (i) the expanded and calibrated model from the present study (equation 1) and (ii) model #5 from Augustin et al. *(5).* In addition, the growth and no-growth data ($n = 71$) were compared with the growth probability models, including the effect of (i) temperature, a_w, and pH *(22, 23, 27)*; (ii) temperature, a_w, pH, and lactic acid *(45)*; and (iii) salt, moisture, lactate, and diacetate *(25).* Growth and no-growth responses were defined as probability values above 0.5 and below 0.5, respectively. The model of Legan et al. *(25),* developed for the prediction of the growth and no-growth response at 4°C and expressed as the time to a 1-log (CFU per gram) increase of *L. monocytogenes,* was compared only with experiments carried out at this specific temperature ($n = 6$). Predicted and observed growth and no-growth responses were compared by calculating the correct prediction percentage that corresponded to the percentage of all samples that were correctly predicted *(19).* Furthermore, the positive predictive value, representing the probability that growth is observed when growth is predicted, and the negative predictive value, representing the probability that the no-growth response is observed when a no-growth response is predicted, were estimated *(5).*

RESULTS

Product characteristics and storage conditions. Table 1 shows the characteristics and storage conditions of the products and subbatches studied. The concentration of naturally occurring water phase lactate was substantially higher in cold-smoked salmon, marinated salmon, and gravad salmon than in cold-smoked Greenland halibut and marinated Greenland halibut ($P < 0.01$), and the content of smoke components (phenol) was significantly lower ($P < 0.01$) in marinated salmon, marinated Greenland halibut, and gravad salmon than in cold-smoked salmon and cold-smoked Greenland halibut (Table 1). The addition of 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate had no adverse effects on the sensory characteristics of the samples, whereas the addition of 0.2% (wt/wt) diacetate and 2.0% (wt/wt) lactate seemed to affect the texture of the products negatively (data not shown).

Microbiological changes. No lag phases of *L. monocytogenes* were observed in VP and MAP products without added diacetate (Fig. 1 and Table 2). MAP, when compared with VP, significantly reduced both μ_{max} and the maximum population density (expressed as log CFU per gram) of *L. monocytogenes* in cold-smoked salmon ($P < 0.01$), whereas this was not observed for cold-smoked Greenland halibut ($P = 0.15$ to 0.69). *L. monocytogenes* grew significantly ($P < 0.01$) faster in cold-smoked Greenland halibut than in cold-smoked salmon, and MAP extended the time to a 100-fold increase in the concentration of *L. monocytogenes* by approximately 70 to 75% in cold-smoked salmon but by only 23% in cold-smoked Greenland halibut (Table 2).

Importantly, the addition of 0.15% (wt/wt) diacetate prevented the growth of *L. monocytogenes* for more than 40 days at 8°C in MAP cold-smoked salmon with 0.77 to 0.98% water phase lactate (Fig. 1 and Table 2). The pathogen grew without a significant lag phase in MAP cold-smoked Greenland halibut with added 0.15% (wt/wt) diacetate (Table 2), but the naturally occurring water phase lactate concentration of this product was only 0.13 to 0.15% (Table 1). In cold-smoked Greenland halibut with 0.79 to 1.03% water phase lactate, the addition of 0.15% (wt/wt) diacetate was sufficient to prevent the growth of *L. mono-*

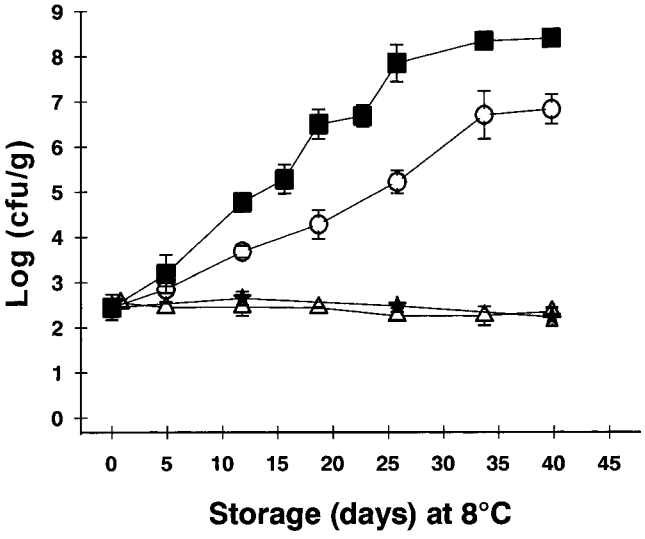

FIGURE 1. *Evolution of* Listeria monocytogenes *in cold-smoked salmon stored at 8°C.* ■, *VP;* ○, *MAP;* △, *MAP + 0.15% (wt/wt) diacetate; and* ★ *MAP + 0.15% (wt/wt) diacetate + 1.5% (wt/wt) lactate. Error bars represent the standard deviation (*n = *3).*

cytogenes for more than 45 days at 8°C (Table 2). The addition of 0.15% (wt/wt) diacetate strongly inhibited the growth of *L. monocytogenes* in MAP cold-smoked salmon (0.60% water phase lactate) stored at 15°C, and its concentration increased only by 1.3 log (CFU per gram) during 14 days of storage. In MAP marinated Greenland halibut, the growth of *L. monocytogenes* was not affected by the addition of 0.15% (wt/wt) diacetate and 0.9% (wt/wt) lactate (Table 2). In MAP gravad salmon with 0.62% water phase lactate, the addition of 0.15% (wt/wt) diacetate had only a limited inhibitory effect on the growth of *L. monocytogenes* at 15°C (Table 2).

The initial concentrations of LAB varied between 1.5 ± 0.1 and 2.6 ± 1.4 log (CFU per gram) in cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, and marinated Greenland halibut. In gravad salmon, the initial concentration of LAB was 3.4 ± 0.1 log (CFU per gram). MAP, when compared with VP, had no significant effect ($P = 0.06$ to 0.29) on the μ_{max} values of LAB, but it significantly reduced ($P < 0.01$) the maximum population density (expressed as log CFU per gram) in cold-smoked salmon and cold-smoked Greenland halibut. The addition of 0.15% (wt/wt) diacetate to MAP cold-smoked salmon prevented the growth of LAB for more than 25 days at 8°C, and only a small increase in the concentration of LAB and no increase in aerobic plate counts were observed after 40 days (Table 2). The inhibitory effect of 0.15% (wt/wt) diacetate against LAB was reduced in MAP cold-smoked salmon at 15°C when compared with storage at 8°C (Table 2). In MAP cold-smoked Greenland halibut, the growth of LAB and aerobic plate counts was observed in subbatches added 0.15% (wt/wt) diacetate and as much as 1.5% (wt/wt) lactate (Table 2).

Modeling and predicting the growth of *L. monocytogenes* in lightly preserved seafood. The average MIC of diacetate ($MIC_{diacetate}$) against *L. monocytogenes* in liquid medium was established in the present study as 4.8 ± 0.3 mM undissociated diacetate at 8°C. No significant differences ($P > 0.05$) in the MICs of diacetate were found between the four examined isolates of *L. monocytogenes.*

The addition of terms for the antilisterial effect of CO_2 and diacetate to the model of Giménez and Dalgaard *(17)* slightly improved the model and changed the bias and accuracy factor values from 1.4 and 1.8 to 1.3 and 1.7 (Table 3). However, the bias factor values of both the original *(17)* and the expanded model increased as a function of the storage temperature (Table 3). This effect of storage temperature on the bias factor was eliminated by the fitting of equation 1 to 39 of the 60 obtained μ_{max} values and a constant (b value) of 0.6802, and a T_{min} value of −2.3°C was estimated with a standard error of 1.0°C (Table 3). The fitting of equation 1 showed a significant effect ($P < 0.01$) of temperature, smoke components (phenol), diacetate, and water phase salt or a_w on the growth rate (μ_{max}) of *L. monocytogenes.* Average bias and accuracy factor values for the fitted model (equation 1) were 1.0 and 1.5 (Table 3) for the data used to determine T_{min} and b and 1.1 and 1.6 for the 21 μ_{max} values used to validate the growth rate model (Table 3). The model of Augustin et al. *(5),* including the effect of temperature, pH, a_w, nitrite, phenol, CO_2, and interactions between the parameters, resulted in bias and accuracy factor values of 0.7 and 1.9. However, bias and accuracy factor values of 0.4 and 2.6 were obtained when the model of Augustin et al. *(5)* was expanded by terms that modeled the effect of lactate and diacetate, as in equation 1 (data not shown).

The growth and no-growth model (equation 1) developed in the present study, including the effect of temperature, water phase salt or a_w, pH, lactate, nitrite, CO_2, smoke components (phenol), diacetate, and the interactions between all these parameters (ξ), clearly performed better than previously developed models that predicted the growth boundary of *L. monocytogenes* (Table 4). The proposed model accurately predicted whether the growth of *L. monocytogenes* was observed or not in 96% of the tested experiments ($n = 71$), which represented different types of lightly preserved seafood (Fig. 2 and Table 4). In comparison, the models of Tienungoon et al. *(45),* Koutsoumanis and Sofos *(22, 23),* Le Marc et al. *(27),* and Augustin et al. *(5)* resulted in correct prediction percentages of 68, 68, 68, 51, and 70, respectively. Predictions by the model of Legan et al. *(25)* resulted in a correct prediction percentage of 83 when applied to experiments carried out at 4°C. Importantly, when no growth was predicted by the model of the present study, no growth was actually observed, resulting in a negative predictive value of 100%. In contrast, the models of Legan et al. *(25),* Le Marc et al. *(27),* and Augustin et al. *(5)* in some cases predicted a no-growth response when growth was actually observed in cold-smoked salmon and cold-smoked Greenland halibut. The developed model (equation 1) predicted the growth of *L. monocytogenes* in the 13 naturally contaminated batches of cold-smoked salmon, although a no-growth response was actually observed in three of the lots (Table 4).

TABLE 2. *Kinetic parameters describing the growth of* Listeria monocytogenes *and lactic acid bacteria (LAB) in cold-smoked salmon (CSS), marinated salmon (MS), cold-smoked Greenland halibut (CSGH), marinated Greenland halibut (MGH), and gravad salmon (GS) at 8°C, 15°C, or both*

Products and subbatches (code)	*Listeria monocytogenes*				Lactic acid bacteria (LAB)		
	Lag phase (days)	μ_{max} (h^{-1})	MPD[a] (log CFU g^{-1})	Time to 100-fold increase (days)	Lag phase (days)	μ_{max} (h^{-1})	MPD (log CFU g^{-1})
CSS-1 (a)	NS[b]	0.021 ± 0.002	8.4	10.8	NS	0.025 ± 0.005	8.3
CSS-1 (b)	NS	0.013 ± 0.002	7.1	18.7	NS	0.024 ± 0.003	7.5
CSS-1 (c)	>40	0	—[c]	>40	38	ND[d]	2.7
CSS-1 (d)	>40	0	—	>40	27	ND	2.8
CSS-2 (a)	NS	0.038 ± 0.003	6.6	3.2	NS	0124 ± 0.026	7.5
CSS-2 (b)	NS	0.012 ± 0.000	3.6	>14	NS	0.051 ± 0.004	6.0
MS-1 (a)	NS	0.032 ± 0.002	7.3	5.7	NS	0.053 ± 0.006	8.7
MS-1 (b)	NS	0.027 ± 0.002	6.7	6.9	NS	0.051 ± 0.006	8.7
MS-1 (c)	NS	0.021 ± 0.006	5.8	9.0	NS	0.049 ± 0.001	8.8
MS-1 (d)	NS	0.018 ± 0.002	5.4	10.7	NS	0.048 ± 0.001	8.8
MS-1 (e)	NS	0.014 ± 0.003	4.8	13.9	NS	0.045 ± 0.001	8.8
MS-1 (f)	NS	0.005 ± 0.002	3.9	37.6	NS	0.047 ± 0.001	8.8
CSGH-1 (a)	NS	0.029 ± 0.003	7.1	6.0	NS	0.036 ± 0.007	8.1
CSGH-1 (b)	NS	0.026 ± 0.001	7.0	7.4	NS	0.033 ± 0.007	7.6
CSGH-1 (c)	>40	0	—	>40	NS	0.026 ± 0.004	7.1
CSGH-2 (a)	NS	0.006 ± 0.001	6.1	33.8	NS	0.020 ± 0.002	7.6
CSGH-2 (b)	12	0.006 ± 0.002	6.0	23.1	NS	0.017 ± 0.002	7.6
CSGH-2 (c)	28	0.003 ± 0.002	4.3	>45	10	0.021 ± 0.004	6.9
CSGH-2 (d)	>45	0	—	>45	6	0.017 ± 0.003	7.1
CSGH-2 (e)	>45	0	—	>45	14	0.017 ± 0.003	6.8
MGH-1 (a)	NS	0.044 ± 0.006	6.5	4.4	NS	0.088 ± 0.007	7.4
MGH-1 (b)	NS	0.039 ± 0.004	6.1	4.9	NS	0.086 ± 0.016	7.3
GS-1 (a)	NS	0.105 ± 0.005	6.6	0.9	NS	0.138 ± 0.015	8.6
GS-1 (b)	NS	0.034 ± 0.006	5.8	2.3	NS	0.100 ± 0.013	8.9

[a] MPD, maximum population density within the storage period.
[b] Lag phase was not significant (NS) ($P > 0.05$).
[c] —, not significantly different from the inoculation level.
[d] ND, not determined as no growth was observed.

TABLE 3. *Observed and predicted maximum specific growth rates for* Listeria monocytogenes *in VP and MAP cold-smoked salmon (CSS), marinated salmon (MS), cold-smoked Greenland halibut, marinated Greenland halibut and gravad salmon as evaluated by bias and accuracy factors*

											Bias/accuracy factor values based on comparison of observed and predicted μ_{max}			
Reference	*n*	Temp (°C)	Water phase salt (%)[a]	pH	a_w[a]	Phenol (ppm)	Nitrite (ppm)[b]	% CO_2[c]	Water phase lactate (%)	Water phase diacetate (%)	Giménez and Dalgaard *(17)* (b = 0.1478, T_{min} = 0.88°C)	Expanded Giménez and Dalgaard *(17)* model[d] (b = 0.1478, T_{min} = 0.88°C)	Equation 1 (b = 0.6802, T_{min} = −2.3°C)	Augustin et al. *(5)* model #5
Data used for development of model														
This study	6	7.6–7.8	3.74–5.26	6.0–6.5	0.969–0.978	8.9–20.1	ND	0–30	0.10–0.98	0.0–0.14	1.4/1.6	1.1/1.3	0.6/1.7	0.1/9.3
This study	4	14.3	2.95–5.26	5.9–6.5	0.969–0.983	4.8–20.1	ND	0–30	0.10–0.98	0.0–0.17	3.4/3.4	2.2/2.2	1.2/1.4	1.2/1.7
36	3	5	3–6	6.2	0.964–0.983	0	—	0	0.70[e]	—	1.0/1.6	1.0/1.6	1.1/1.6	1.0/1.6
36	3	10	3–6	6.2	0.964–0.984	0	—	0	0.70[e]	—	1.5/1.5	1.5/1.5	1.0/1.3	0.9/1.3
44	6	4	3.7–4.27	6.1[e]	0.975–0.979	10.7–11.2	—	0	0.70[e]	—	0.9/2.2	0.9/2.2	1.2/2.0	—[f]
44	6	8	3.43–4.47	6.1[e]	0.974–0.980	8.8–10.6	—	0	0.70[e]	—	1.4/1.4	1.4/1.4	1.1/1.2	0.6/1.6
44	5	12	5.5–5.6	6.1[e]	0.967	9.7–11	—	0	0.70[e]	—	1.9/2.7	1.9/2.7	1.1/2.1	1.0/1.1
24	1	5.5	4.92	6.1	0.971	14.6	ND	0	0.59	—	0.6/1.6	0.6/1.6	0.5/1.9	—[f]
24	1	9.9	4.92	6.1	0.971	14.6	ND	0	0.59	—	1.2/1.2	1.2/1.2	0.8/1.3	0.2/5.6
17	1	2	4.9	6.03	0.971	12.6	ND	0	0.37	—	0.4/2.4	0.4/2.4	1.1/1.1	—[f]
17	1	5	4.9	6.03	0.971	12.6	ND	0	0.37	—	2.3/2.3	2.3/2.3	2.4/2.4	—[f]
17	1	10	4.9	6.03	0.971	12.6	ND	0	0.37	—	2.3/2.3	2.2/2.2	1.5/1.5	0.6/1.8
17	1	17.5	4.9	6.03	0.971	12.6	ND	0	0.37	—	1.9/1.9	1.9/1.9	1.0/1.0	1.0/1.1
								Average bias/accuracy factor values			1.4/1.8	1.3/1.7	1.0/1.5	0.7/1.9
Data used for model validation														
This study	8	7.8	3.40–3.67	6.1–6.8	0.979–0.980	0	ND	16.2–21.5	0.17–0.62	0.0–0.11	2.4/2.4	1.7/1.7	1.3/1.4	1.4/1.5
35	6	5–10	2–3	6.1	0.983–0.989	0	0–145	0	0–2.01[g]	—	2.4/2.4	2.4/2.4	0.7/1.9	1.9/1.9
43	3	4–10	3.5[e]	6.3	0.980[e]	6.0[e]	—	0–100[h]	0.70[e]	—	1.6/2.7	1.0/1.7	0.9/1.3	0.7/2.0
50	4	4–10	3.5[e]	6.1	0.980[e]	6.0[e]	—	0	0–0.84[g]	0–0.06[g]	1.3/3.4	1.2/2.4	1.4/1.7	0.6/2.9
								Average bias/accuracy factor values			2.0/2.5	1.6/2.0	1.1/1.6	1.4/2.0

[a] Relation between percentage of water phase salt (% WPS) and water activity (a_w) calculated as $a_w = 1 - 0.0052471 \times \%\ WPS - 0.00012206 \times \%\ WPS^2$ *(37)*.
[b] Measured but not detected (ND).
[c] Equilibrium concentrations in headspace gas.
[d] Expanded with the CO_2 term of Devlieghere et al. *(15)* and the acetate term of Le Marc et al. *(26)*. See Equation 1.
[e] If not reported in the literature, the following product characteristics were used for prediction of μ_{max} values in CSS: 3.5% water phase salt (a_w = 0.980), pH 6.1, 6 ppm of phenol, and 0.70% water phase lactate (natural content of lactate in CSS).
[f] No growth predicted by the model.
[g] Added concentrations (%, wt/wt) of diacetate, lactate, or both to the products.
[h] Initial concentrations of CO_2 (equilibrium concentrations not reported in the study).

TABLE 4. *Growth and no growth responses of* Listeria monocytogenes *in lightly preserved seafood as predicted by growth boundary models and probability models*

Reference	Storage conditions and products characteristics: Products	Packaging	Temp (°C)	Water phase salt (%)	pH	Phenol (ppm)	Sodium nitrite (ppm)	Added (%, wt/wt): Diacetate	Added (%, wt/wt): Lactate[a]	Observed (growth or no growth)	Growth boundary models: No. of expts correctly predicted: Equation 1	Augustin et al. *(5)* model #5[b]	Tienungoon et al. *(45)*[c, d]	Koutsoumanis and Sofos *(23)*[c, e]	Le Marc et al. *(27)*[c, e]
Inoculated															
This study															
Growth	CSS, MS, CSGH, MGH, GS	VP/MAP	8–15	3.03–5.26	5.9–6.8	0.0–20.1	ND[f]	0–0.15	0–0.9	19	19	15	19	19	11
No growth	CSS, CSGH	MAP	8	3.74–5.25	5.8–6.2	8.9–20.1	ND	0.15	0–1.5	5	5	5	0	0	2
35															
Growth	CPS[g]	VP	5–10	2–3	6.1	—	0–145	—	0–2.01	11	11	11	11	11	11
No growth	CPS	VP	5–10	2–3	6.1	—	0–145	—	1.61–2.41	11	11	0	0	0	0
43															
Growth	CSS	VP/MAP	4–10	—[h]	6.3	—[h]	—[h]	—	—	3	3	3	3	3	2
No growth	CSS	MAP	4	—[h]	6.3	—[h]	—[h]		—	1	1	1	0	0	1
50															
Growth	CSS	VP	4–10	—[h]	6.1	—[h]	—[h]	0–0.06[i]	0–0.84	4	4	4	4	4	3
No growth	CSS	VP	4–10	—[h]	6.1	—[h]	—[h]	0.05–0.20	0.59–2.80	4	4	0	0	0	3
Naturally contaminated															
11															
Growth	CSS	VP	5	3.5–5.8	6.1–6.3	—[h]	ND[f]	—	0.67–1.04	10	10	10	10	10	0
No growth	CSS	VP	5	3.7–8.9	6.2–6.3	—	0–60	—	0.88–1.11	3	0	1	1	1	3
									Correct prediction percentage (CPP)[j]		96	70	68	68	51
									Positive predictive value (PPV)[j]		94	72	67	67	64
									Negative predictive values (NPV)[j]		100	64	100	100	31

[a] If not reported, measured samples were assumed to contain 0.70% water phase lactate (natural content of lactate in CSS) in addition to the added amount of lactate.
[b] Includes the effect of temperature, pH, a_w, nitrite, phenol, CO_2, and interactions between parameters.
[c] Predicts the probability of growth expressed as values between 0 and 1 (0–100% probability of growth). Growth or no-growth were predicted when the growth probabilities were above 0.5 or below 0.5, respectively.
[d] Includes the effect of temperature, pH, a_w, and lactic acid.
[e] Includes the effect of temperature, pH, a_w, and interactions between the parameters.
[f] Not detected in the product.
[g] Cold-processed salmon (CPS). Produced as cold-smoked salmon without the smoking procedure.
[h] If not reported, measured products were assumed to contain 3.5% water phase salt (a_w = 0.980), 6 ppm of phenol, and 0 ppm of sodium nitrite.
[i] Recalculated from the added concentrations of PURASAL P Opti.Form 4 containing 4% sodium diacetate and 56% potassium lactate (60% [vol/vol] lactate syrup).
[j] CPP, percentage of all samples that were correctly predicted; PPV, representing the probability that growth is observed when growth is predicted; NPV, representing the probability that no growth is observed when no growth is predicted.

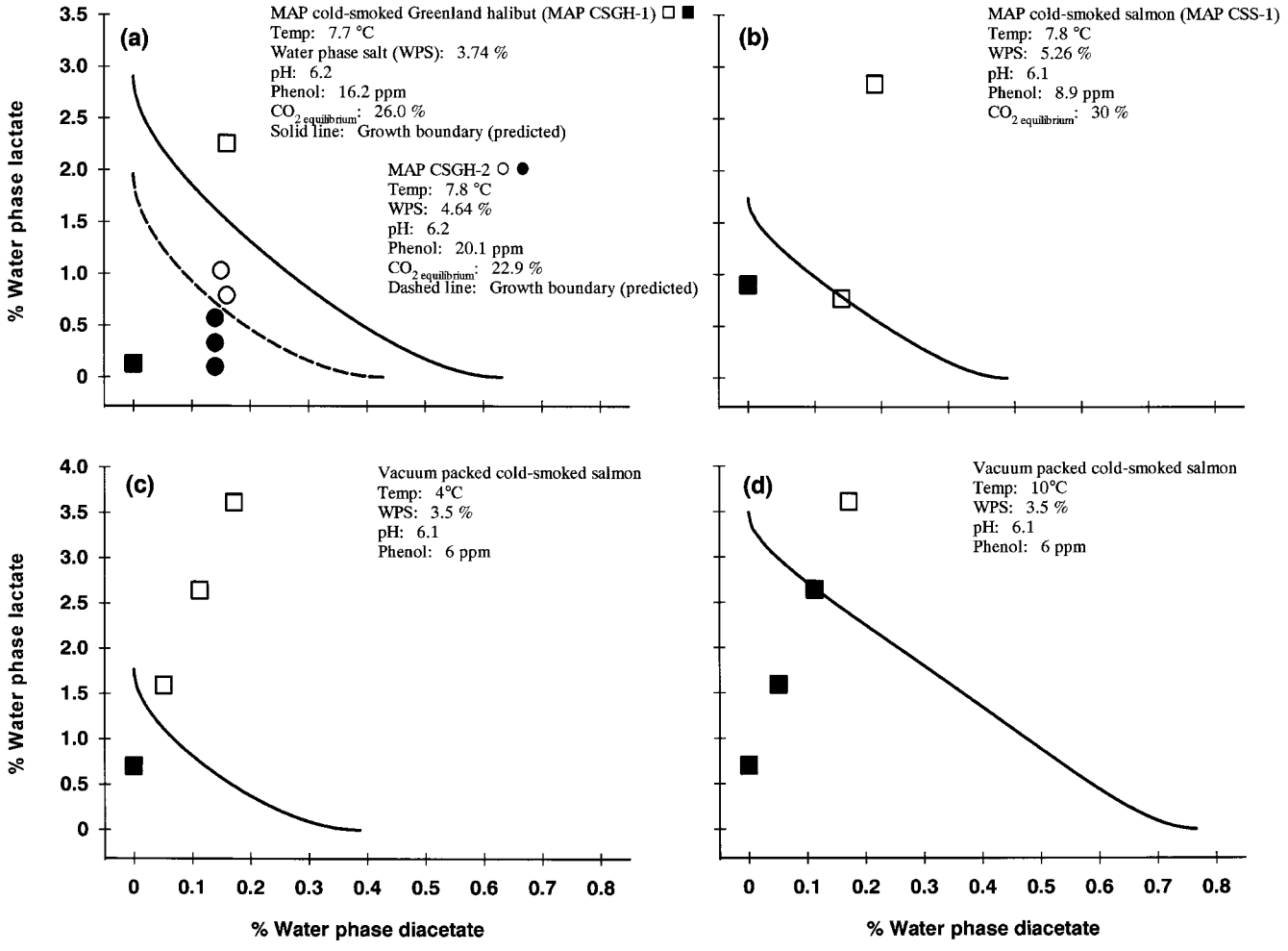

FIGURE 2. *Comparison of predicted growth boundaries and observed growth and no-growth responses of* Listeria monocytogenes *in MAP cold-smoked Greenland halibut (a), MAP cold-smoked salmon (b), and vacuum-packaged (VP) cold-smoked salmon stored at 4 and 10°C (c and d). Growth data of VP cold-smoked salmon stored at 4 and 10°C were reported by Yoon et al. (50). Open and solid symbols represent no-growth and growth of* L. monocytogenes, *respectively.*

DISCUSSION

The growth boundary model developed in the present study correctly predicted 68 of 71 growth and no-growth responses in lightly preserved seafood, but growth was predicted for three lots of naturally contaminated cold-smoked salmon where no-growth was observed (Table 4). The model performed substantially better than the existing *L. monocytogenes* growth boundary models *(5, 25, 27, 45)* (Table 4). The model of Legan et al. *(25)* also seemed promising, but its use is restricted to a storage temperature of 4°C only. The model developed in the present study included the effect of temperature, water phase salt or a_w, pH, lactate, nitrite, phenol, CO_2, and diacetate as well as interactions between these parameters (equation 1), and its superior performance when compared with the existing and less complex models is most likely due to an important inhibiting effect of all these parameters with respect to the growth boundary of *L. monocytogenes* in lightly preserved seafood. In contrast to the present study, Augustin et al. *(5)* found the growth of *L. monocytogenes* in lightly preserved seafood to be better predicted when the effect of phenol was excluded from their model. We observed a pronounced effect of smoke components on the growth boundary of *L. monocytogenes,* particularly in products with more than 10 ppm of phenol (Fig. 3d). Phenol concentrations above 10 ppm are common in smoked seafood *(16, 17, 24, 29).* The phenol term in equation 1 therefore is important to predict both the growth ($P < 0.01$) and the growth boundary of *L. monocytogenes* in smoked seafood, and in the present study, this was shown specifically for cold-smoked Greenland halibut with 16 to 20 ppm of phenol (Tables 1 and 4). In fact, smoke components (phenol) can be used together with sensorially acceptable concentrations of diacetate in an attempt to control the growth of *L. monocytogenes* in lightly preserved seafood (Table 5, line 5).

Within predictive food microbiology, growth boundary models have most often been developed from large sets of growth and no-growth data typically obtained from broth cultures in microwell plates and by polynomial models, logistic regression, or artificial neural networks *(39).* The present study used an alternative approach suggested by Le Marc et al. *(26).* The idea behind this approach is to divide the space of environmental parameters into three regions where the effect of interaction between the environmental

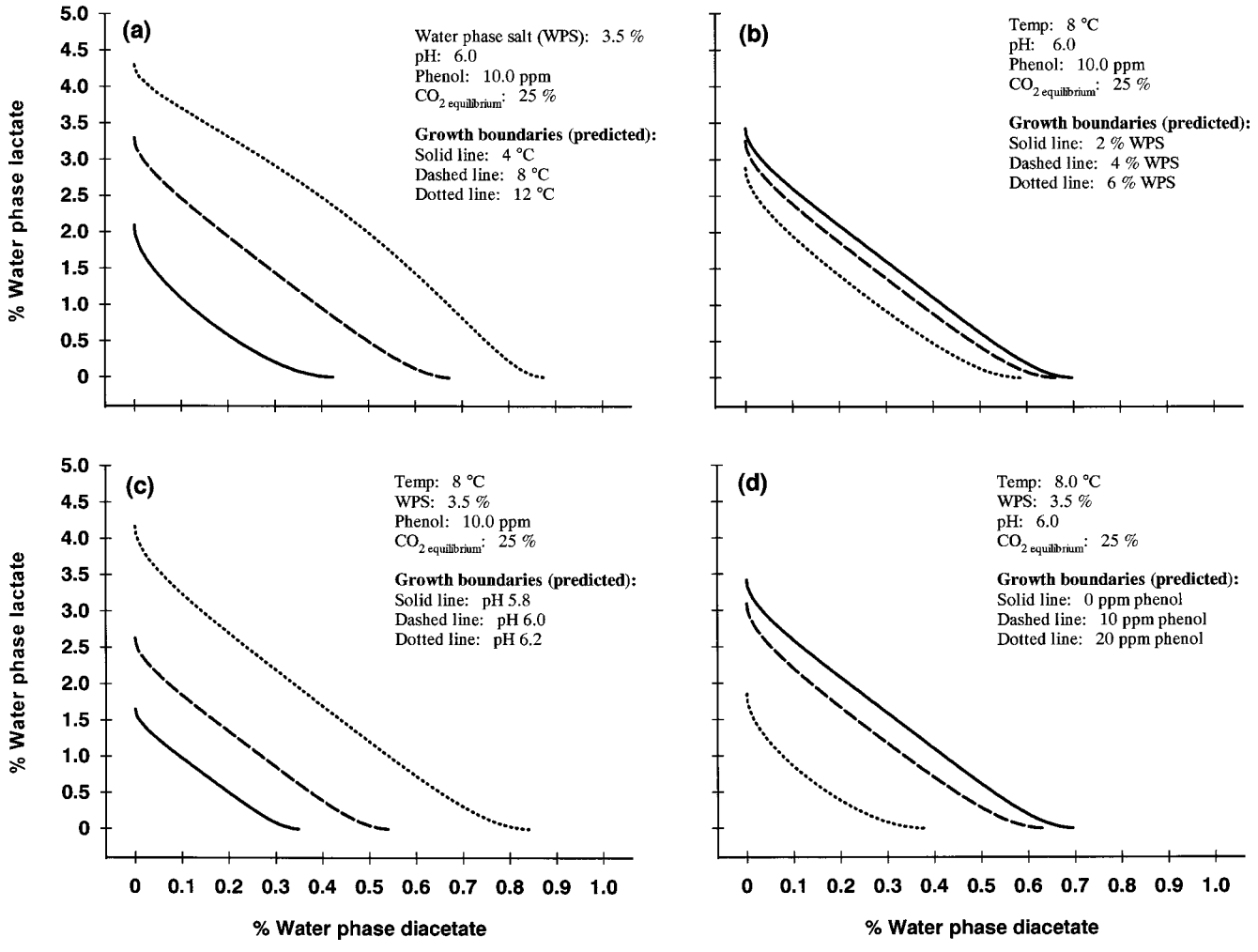

FIGURE 3. *Effect of temperature (a), water phase salt (WPS) (b), pH (c), and phenol (d) on the growth and no-growth boundary of* Listeria monocytogenes *at different concentrations of diacetate and lactate. Default parameters used were 8°C, pH 6.0, 3.5% water phase salt, 10 ppm of phenol, 25% CO_2 (equilibrium), and 0 ppm of nitrite.*

parameters is expressed by the parameter ξ; this interaction term can prevent growth (ξ = 0), reduce growth rate (0 < ξ < 1), or have no effect on growth rate (ξ = 1) (equation 2). The Le Marc approach to the development of growth boundary models thus relies on growth rate data. For *L. monocytogenes,* very large amounts of such data and existing growth rate models are available, and this facilitated the development of a growth boundary model for *L. monocytogenes* in the present study. We used μ_{max} values for *L. monocytogenes* from challenge tests with lightly preserved seafood and in this way developed a product-specific growth boundary model: (i) an existing model for the growth of *L. monocytogenes* was expanded to include the effect of diacetate, CO_2, phenol, nitrite, lactate, pH, water phase salt or a_w, and temperature; (ii) a term to take into account the inhibitory effect of the interaction between the environmental parameters was added (equation 1); and (iii) the model was calibrated to growth data (μ_{max} values) ob-

TABLE 5. *Combinations of environmental parameters preventing growth of* Listeria monocytogenes *as predicted by the growth boundary model*

Temp (°C)	Water phase salt (%)	pH	Phenol (ppm)	Nitrite (ppm)	CO_2 (%)[a]	Water phase lactate (%)	Water phase diacetate (%)
5.0	4.5	6.0	13	0	25	0.70	0.07
8.0	4.5	6.0	13	0	25	0.70	0.22
8.0	6.0	6.0	13	0	25	0.70	0.16
8.0	4.5	5.9	13	0	25	0.70	0.14
8.0	4.5	6.0	18	0	25	0.70	0.05
8.0	4.5	6.0	13	100	25	0.70	0.11
8.0	4.5	6.0	13	0	98	0.70	0

[a] Equilibrium concentrations in headspace gas.

tained for *L. monocytogenes* in challenge tests with lightly preserved seafood with well-characterized product characteristics and storage conditions (Table 3). Subsequently, the developed model was used to predict the growth and no-growth of *L. monocytogenes* in lightly preserved seafood in order to validate the growth boundary model.

For RTE foods, EU regulations (EC 2073/2005) include a critical limit (m = M) of 100 CFU g^{-1} for *L. monocytogenes* if a product is unable to support the growth of this pathogen. To determine if lightly preserved seafood, depending on product characteristics and storage conditions, is able or unable to support the growth of *L. monocytogenes,* the model developed in the present study seems most useful, particularly because critical control points for *L. monocytogenes* have not been identified during the processing and chilled distribution of VP cold-smoked salmon and similar lightly preserved seafood *(49).* The present study has documented that the combined effect of several product characteristics is predictable and that it can be used to prevent the growth of *L. monocytogenes* in lightly preserved seafood. Thus, the developed growth boundary model facilitates a selection of relevant product characteristics and storage conditions useful as critical control points for different seafood (Table 5). The developed growth boundary model can be used actively by the seafood industry in connection with product development and quality control of existing products as a means of ensuring that products comply with the EU regulation on RTE foods. Furthermore, if documentation of growth and no-growth responses is needed by the authorities or retailers, the model can be used to predict if *L. monocytogenes* is able or unable to grow in a given product. The more general use of the developed model was shown when previously estimated growth rates ($n = 6$) of *L. monocytogenes* in cooked and peeled MAP shrimps *(32)* were compared with predictions of equation 1, resulting in bias and accuracy factor values of 0.9 and 1.1. Product characteristics of cooked and peeled MAP shrimps (<2% water phase salt, pH 7.5, and 0.05% water phase lactate) differ substantially from the characteristics of the products examined in the present study (Table 1). On the basis of our product validation studies, the range of applicability for equation 1 seems to include temperature (2 to 15°C), water phase salt (2 to 9%), pH (5.8 to 7.5), water phase lactate (<3.0%), smoke components (phenol) (<20 ppm), CO_2 equilibrium (0 to 100%), and water phase diacetate (<0.2%). The number of studies that examine the effect of nitrite was not sufficient to establish a reliable range of applicability for this preservative.

The suggested model is complex, and to improve its usefulness and value to the seafood sector, it should be incorporated in application software such as the Seafood Spoilage and Safety Predictor (available at: http://www.difres.dk/micro/sssp/).

The developed growth boundary model is fail-safe and has a relatively wide range of applicability, as validation studies have included seafood with varied product characteristics and inoculations with different cocktails of *L. monocytogenes* with a total of 13 strains (Table 4). The growth boundary of *L. monocytogenes* as a function of environmental parameters depends on the initial concentration of the pathogen *(23).* The low initial concentrations of *L. monocytogenes* in naturally contaminated cold-smoked salmon *(11)* may therefore explain why growth was predicted in three batches of the product when a no-growth response was actually observed (Table 4). Furthermore, differences in the physiological state between strains of *L. monocytogenes* on naturally contaminated and inoculated products might explain these results. Additional product validation of a predictive model is always desirable, and evaluation of different *L. monocytogenes* isolates, particularly evaluation of naturally contaminated seafood with new combinations of environmental parameters, will benefit the growth boundary model developed in the present study. To improve the suggested model, the calibration carried out in the present study to obtain a T_{min} value of −2.3°C can be performed for other environmental parameters. This, however, requires a significant amount of new growth data with corresponding and carefully determined product characteristics and storage conditions. Furthermore, the inhibiting effect of the dominating spoilage microbiota, i.e., the Jameson effect, should be taken into account when the growth of *L. monocytogenes* in lightly preserved seafood is predicted *(17).* To do so, models for the growth of LAB and possibly other spoilage microorganisms must be expanded with terms for relevant environmental parameters, including diacetate and smoke components (phenol), and validated in well-characterized, lightly preserved seafood.

Treatment of MAP cold-smoked salmon with diacetate provided an effective method to prevent the growth of *L. monocytogenes* at 8°C (Fig. 1 and Table 2). The growth of *L. monocytogenes* was reduced only in MAP cold-smoked Greenland halibut treated with diacetate, whereas a combination of diacetate and lactate prevented growth (Table 2). The higher content of naturally occurring lactate in cold-smoked salmon (0.77 to 0.98% water phase lactate) when compared with cold-smoked Greenland halibut (0.13 to 0.15% water phase lactate) explained this difference between the products. The capability of equation 1 to predict the combined effect of diacetate and lactate on the growth boundary of *L. monocytogenes* in lightly preserved seafood and the importance of a thorough product characterization were obvious when the results of cold-smoked Greenland halibut were compared (Fig. 2a). In cold-smoked Greenland halibut with 0.15% (wt/wt) diacetate and 0.5% (wt/wt) lactate, growth of *L. monocytogenes* was observed, whereas a no-growth response was observed in samples with 0.15% (wt/wt) diacetate and 0.75% (wt/wt) lactate. In spite of these small differences in product characteristics, the model of the present study correctly predicted whether growth was observed or not (Fig. 2a).

The antimicrobial effect of diacetate and lactate against *L. monocytogenes* has previously been documented for different types of meat products *(18, 31, 40, 41).* Glass et al. *(18)* showed that 0.1% sodium diacetate and 1.0% sodium lactate prevented the growth of *L. monocytogenes* in smoked wieners for 60 days at 4.5°C. In beef bologna, the growth of *L. monocytogenes* was prevented for 45 days at 4°C by the addition of 0.2% sodium diacetate and 2.5%

sodium lactate *(31),* and in wieners and smoked-cooked ham treated with 0.15% sodium diacetate and 1.5% potassium lactate, comparable antimicrobial effects were observed for 18 weeks at 4°C *(40).* Stekelenburg *(41)* showed that the growth of *L. monocytogenes* was inhibited at 4°C in frankfurter sausage treated with 0.1% sodium diacetate. The natural content of lactate in frankfurter sausage was 0.68% potassium lactate corresponding to 0.47% lactate. However, treatment of frankfurter sausage with 0.1% sodium diacetate and 1.4% potassium lactate prevented the growth of *L. monocytogenes* for >28 days at 4°C, once again confirming that a certain amount of lactate has to be present to prevent the growth of *L. monocytogenes* in products treated with sensorially acceptable concentrations of diacetate. The model of the present study was evaluated on data from meat products ($n = 23$), including the growth and no-growth responses of *L. monocytogenes* and product characteristics *(18, 41).* The correct prediction percentage of the newly developed model was 83 (data not shown). In comparison, the model of Augustin et al. *(5)* had a correct prediction percentage of 53.

Only recently have studies been conducted that examine the antimicrobial effect of diacetate and lactate in lightly preserved seafood. Yoon et al. *(50)* showed that treatment of VP cold-smoked salmon with 0.06% sodium diacetate and 0.84% potassium lactate prevented the growth of *L. monocytogenes* for 32 days at 4°C, whereas 0.2% sodium diacetate and 2.8% potassium lactate were needed to prevent the growth of *L. monocytogenes* at 10°C. Previous studies have established a similar relationship between the antimicrobial effect of diacetate-lactate and the applied storage temperature *(6, 30, 31).* Results of the present study showed a pronounced antimicrobial effect of diacetate in MAP cold-smoked salmon stored at 15°C. In MAP cold-smoked salmon treated with 0.15% (wt/wt) diacetate, the growth of *L. monocytogenes* was prevented for 7 days at 15°C, and after 14 days, the concentration of *L. monocytogenes* had increased by only 1.3 log (CFU per gram) (Table 2). The effect of storage temperature on the antimicrobial effect of diacetate and lactate was obvious when the growth boundaries of *L. monocytogenes* were predicted (Fig. 3a).

Although MAP did not prevent the growth of *L. monocytogenes,* a substantial extension of the time to a 100-fold increase in the concentration of *L. monocytogenes* was observed in MAP cold-smoked salmon when compared with VP samples. The growth boundary model developed in the present study predicted that equilibrium concentrations of approximately 98% CO_2 were needed to prevent the growth of *L. monocytogenes* in MAP cold-smoked salmon with typical storage and product characteristics (Table 5, line 7). Szabo and Cahill *(43)* showed that the packaging of cold-smoked salmon in 100% CO_2 prevented the growth of *L. monocytogenes* at 4°C and reduced the growth at 10°C. The model of the present study (Table 4) accurately predicted the results obtained by Szabo and Cahill *(43).*

Model simulation showed a pronounced effect of pH on the growth boundary of *L. monocytogenes.* Smaller concentrations of diacetate, lactate, or both were needed to prevent the growth of *L. monocytogenes* at pH 5.8 when compared with pH values of 6.0 and 6.2 (Fig. 3c). This effect of pH is most likely explained by the higher concentration of undissociated lactic acid and diacetate molecules at pH 5.8. This hypothesis was confirmed by the results of the present study, showing no antimicrobial effect of 0.15% (wt/wt) diacetate and 0.9% (wt/wt) lactate against *L. monocytogenes* in MAP marinated Greenland halibut with pH 6.7 to 6.8 (Table 2). The impact of water phase salt or a_w on the growth limit of *L. monocytogenes* was not as pronounced as noticed for the temperature, pH, and phenol (Fig. 3b).

In subbatches of cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon supporting the growth of LAB, these bacteria constituted the dominating natural microflora. This is in agreement with previous studies of both VP and MAP cold-smoked salmon *(20, 28, 34).* The growth of LAB was strongly inhibited at 8°C in MAP cold-smoked salmon treated with 0.15% (wt/wt) diacetate, resulting in only a negligible increase in the concentration of LAB throughout the challenge test (Table 2). In contrast, treatment of MAP cold-smoked Greenland halibut with 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate inhibited the growth of LAB to a much lesser extent (Table 2). Stekelenburg and Kant-Muermans *(42)* showed that treatment of VP, cooked, cured ham with 0.1% sodium diacetate had no antimicrobial effects against *Lactobacillus curvatus* at 4°C, whereas 0.2% sodium diacetate or 1.98% sodium lactate inhibited the growth of this microorganism. In VP frankfurter sausage, no antimicrobial effect of 0.1% sodium diacetate against *Lactobacillus sake* was observed, whereas 1.68% potassium lactate and 0.12% sodium diacetate or 1.8% potassium lactate inhibited growth at 4°C *(41).* In addition to a pronounced antimicrobial effect against *L. monocytogenes,* the results of the present study indicate that diacetate and lactate can be used to prolong the sensory shelf life, especially of MAP cold-smoked salmon.

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