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IMPROVEMENT OF THE SAFETY AND QUALITY OF REFRIGERATED READY-TO-EAT FOODS USING NOVEL MILD PRESERVATION TECHNIQUES

A SHARED-COST PROJECT

8th MEETING IN LE MUY (FRANCE) ORGANIZED BY THE INSTITUTE NATIONAL DES RECHERCHES AGRONOMIQUE (INRA), AVIGNON, FRANCE, 13 AND 14 JUNE 1996

RESEARCH AND MANAGEMENT PLANNING TO BE DISCUSSED DURING THE 8th MEETING OF THE PROJECT GROUP AT THE 8th MEETING, ORGANIZED BY THE INSTITUTE NATIONAL DES RECHERCHES AGRONOMIQUE (INRA), AVIGNON (FRANCE) AT LE MUY (FRANCE)

13 AND 14 JUNE 1996

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IMPROVEMENT OF THE SAFETY AND QUALITY OF REFRIGERATED READY-TO-EAT FOODS USING NOVEL MILD PRESERVATION TECHNIQUES

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Research progress and planning per participant

1: Agrotechnological Research Institute, Wageningen, the Netherlands

2: Department of Food Science, Agricult. Univ., Wageningen, the Netherlands

3: Institut National de la Recherche Agronomique, Montfavet, France with Les Crudettes, Fruidor S.A., Cavaillon, France

4: Ctr Coop. Intn. Rech. Agron. Dév., Syst. Agro-Alim. Rur. (CIRAD-SAR), and Ecole Natl. Sup. Agronomique (ENSAM), Montpellier, France

5: Inst. Techn. Agr. Products, Nation. Agr. Res. Found., Athens, Greece

6: Institute of Food Research, Norwich, United Kingdom

7: Dept. Life Sciences, Univ. Limerick, Limerick, Ireland

8: Nature's Best Ltd., Duleek, Ireland

Proposed schedule of meetings

Addresses and communication facilities

Agenda of the 8th Group Meeting at Montpellier, France

13 June 1996

09.00 Welcome and outline of the 8th meeting by the coordinator, Leon Gorris.
09.15 Presentation progress at ATO.DLO (Marjon Bennik & Leon Gorris)
10.15 Break for coffee/tea
10.45 Presentation of progress at WAU.DFS (Annette Verheul & Tjakko Abee)
11.45 Presentation of progress at INRA.AV & FRUID (Frédéric Carlin)
12.45 Lunch
14.00 Presentation of progress at CIRAD.SAR (Stéphane Guilbert & Natalie Gontard)
15.00 Presentation of progress at NARF (George Nychas)
16.00 Break for coffee/tea
16.15 Presentation of progress at IFRN (Mike Peck & Sandra Stringer)
17.15 Domestic planning
19.30 Diner

14 June 1996

09.00 Presentation progress at ULMK.DCLS (David O'Beirne)
10.00 Presentation progress at NBEST (Paddy Callaghan & Anne-Marie Kierans)
10.30 Break for coffee/tea
11.00 Summary of session results and project planning: tasks for work in last half year in tasks 9, 10 and 11. Critical assessment of research progress and achievements regarding set objectives and deliverables set forth in the Technical Annex. Collaboration and exchange of materials (coatings/packaging materials/software/data) between partners. Dissemination of research results. Miscellaneous. Closure of the meeting. Leon Gorris
12.30 Lunch

General objectives of the project

This project sets out to improve safety and quality of vegetable based ready-to-eat foods, *i.e.* fresh or minimally processed preparations and refrigerated, processed foods of extended durability (REPFEDs). Because of the difficulty of maintaining sufficiently low temperatures during refrigerated storage, one of the most commonly used mild preservation techniques, additional barriers to the growth of microorganisms are required. These barriers may be based on novel techniques such as biopreservation, modified atmosphere packaging (MAP) or coating (MAC), and coatings containing food-grade antimicrobial agents (active MAC). The present study will optimise these barriers in combination with refrigeration for use with fresh and processed vegetable foods. The optimised mild preservation techniques will be more environmentally friendly than those currently employed, but will effectively inhibit pathogenic bacteria and will reduce post-harvest losses due to microbial spoilage and physiological degradation of product quality.

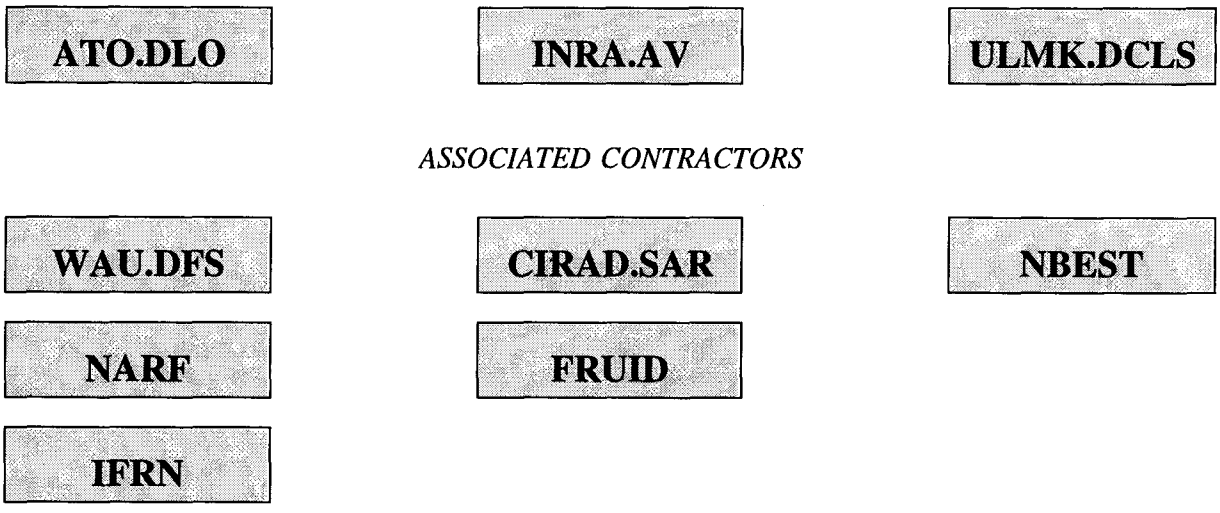
The range of foods which may be classified as ready-to-eat is quite extensive, including (mixtures of) raw vegetables, minimally processed (washed, trimmed, sliced) vegetables with or without dressings, and Sous Vide preparations (cooked vegetable and potato based dishes). A recent innovation has been the introduction of dishes composed of raw vegetables and cooked items. The physiological and microbiological characteristics of products within this range varies substantially. Fresh and minimally processed vegetables are metabolically active, but their activity depends strongly on the type of product and the type of processing applied. Due to their activity, these products create a modified atmosphere when they are packaged. Also, these products possess, part of, their natural epiphytic microbial flora. Pathogens may form part of this flora, posing a potential safety problem. The pathogens encountered under refrigerated storage conditions are mainly psychrotrophic (low-temperature) bacteria (*e.g. Listeria monocytogenes* and non-proteolytic *Clostridium botulinum*). Some mesophyllic pathogens are able to proliferate at abuse temperatures (*e.g. Salmonella typhimurium, Staphylococcus aureus, Bacillus cereus*). The number of epiphytes and pathogens present again will depend on the type of product and the type of processing applied. Different types of products need to be studied to appreciate the interactions between epiphytic flora and foodborne pathogens. With REPFEDs, the mild heat treatment included in the processing diminishes most of the microbial flora but not spores of sporeforming bacteria (*e.g. C. botulinum* and *B. cereus*). In the absence of any competitors, these organisms may proliferate under refrigeration conditions (*e.g.* non-proteolytic *C. botulinum*) or under conditions of temperature abuse.

The approach proposed combines the development of basic knowledge on microbiology, product physiology and preservation techniques with the practical evaluation of microbiological safety and product quality of the novel or optimised techniques. Computer modelling will be used to integrate the data (biochemical data on products as well as physico-chemical data on storage/packaging conditions) from the various activities into a predictive model. Because of the substantial variation in products, the proposed research will focus on a number of model food products from the categories: raw ingredients only, cooked and raw-ingredients, cooked ingredients only. The proposed research integrates studies on product factors (type, cultivar, initial quality, respiration rate), on microbiology (spoilage organisms, pathogens, their interactions and interaction with natural antimicrobial control systems) and on improved or novel mild preservation technologies (biopreservation agent, properties of MA packaging films or coating materials). It also integrates fundamental and applied studies. Fundamental studies are devoted to investigating the basic physiology of a number of foodborne pathogens in relation to key parameters (*e.g.* temperature, pH, a_w, O_2, CO_2, etc.), their interactions with non-pathogenic microorganisms present

on a product and to determination of optimal MA-gas compositions with respect to product quality. A further objective is to assess the effect of heat processing and subsequent mild preservation factors on the safety of REPFEDs in relation to non-proteolytic *C. botulinum*, a low temperature pathogen. The fundamental research yields information necessary for the applied studies on biopreservation, the optimization of MA packaging and the development of MA coatings which are fully biodegradable. The development of active, biodegradable MACs, incorporating food-grade preservatives, will be studied as well. The various studies will be designed to allow integration of the data on the three levels given above in a mathematical computer model. This model should be able to link information on product quality, microbiology and preservation technique and may be used to predict the effects of changes in a specific parameter on the shelf life of the product.

Organigram:

CONTRACTORS



ATO.DLO: Agrotechnological Research Institute (ATO-DLO), Wageningen, the Netherlands (participant 1).
WAU.DFS: Department of Food Science, Wageningen Agricultural University (WAU), Wageningen, the Netherlands (participant 2).
INRA.AV: Institut National de la Recherche Agronomique (INRA), Laboratoire de Technologie des Fruits et Legumes, Montfavet, France (participant 3).
CIRAD.SAR: Centre de Coopération Internationale en Recherche Agronomique, Departement des Systèmes Agro-Alimentaires et Ruraux, Montpellier, France (participant 4).
NARF: Institute of Technology of Agricultural Products (ITAP) of the National Agricultural Research Foundation (N.Ag.Re.F.), Athens, Greece (participant 5).

IFRN: Institute of Food Research (IFRN), Norwich, United Kingdom (participant 6).
ULMK.DCLS: Department of Chemical and Life Sciences (DCLS), University of Limerick, Limerick, Ireland (participant 7).
FRUID: Les Crudettes (LESC), Fruidor S.A., Cavaillon, France (participant 8).
NBEST: Nature's Best Ltd. (NBEST), Duleek, Ireland (participant 9).

Participant 1: Agrotechnological Research Institute (ATO.DLO)
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The Netherlands

A. ***1. (Sub-)Project title:***

(a) Microbial quality and safety of mildly preserved, fresh or freshly processed vegetables (biopreservation, gas-microbe interaction)
(b) Systematic investigation of the quality of fresh or freshly processed vegetables under modified atmospheres (gas-product interaction, packaging systems)
(c) Sensory analysis of mildly preserved, vegetable products *(year 4)*
(d) Compilation of an integrated computer model *(year 4)*

2. Project coordinator:
Dr. Leon G.M. Gorris

3. Project participants and 4. Resources:

Ir. Marjon H.J. Bennik	12 months/year
seconded researchers	16 "
Dr. Leon G.M. Gorris	5 "
Ir. Herman W. Peppelenbos	3 "

SUB-PROJECT (A): ***MICROBIAL QUALITY AND SAFETY OF MILDLY PRESERVED, FRESH OR FRESHLY PROCESSED VEGETABLES***

B. ***1. Description of the research topic or practical problem:***

Refrigerated storage in a Modified atmosphere packaging (MAP) is a rather new, mild preservation technique for minimally processed vegetable food products. Since it is a quite recent development, fundamental knowledge of determinative physiological, microbiological and physico-chemical parameters is limited. As for the microbiological determinants, focus has as yet been on a few pathogenic microorganisms. Little fundamental knowledge has been obtained on the microbial population dynamics associated with spoilage and on the role of spoilage microflora in competitive exclusion of pathogens. The natural micro flora of lactic acid bacteria is believed to be a natural, antimicrobial system in potential.

2. Goal of the research.
The goal of the project is to obtain more fundamental knowledge on the effects of the gas atmosphere composition on the growth and interactions (*viz.* the microbial population dynamics) of relevant pathogenic and spoilage microbes in refrigerated MAP systems. The feasibility of introducing an additional mild preservative hurdle in this system by using selective promotion of the growth of lactic acid bacteria, being a natural inhibitory system, will be investigated as well.

3. Short description of the approach

There will be two major lines of investigation:

1) *Fundamental studies*
   - To study the effects of the composition of the gas atmosphere, in relation with other relevant (bio)chemical and physical parameters, on key organisms (pathogens and spoilage organisms) in order to assess the effect of the gasses on the cell/molecular level (mechanistic research) and to obtain data to add to predictive models of bacterial growth being composed at WAU.DFS and elsewhere.
   - Attention will be given to the gas atmosphere conditions which specifically promote the growth of lactic acid bacteria (LABs), which may be used to suppress undesirable pathogenic or spoilage organisms. The mode of action of potentially useful LABs will be investigated.

2) *Applied studies*
   - To investigate the influence of the above parameters on interactions between spoilage organisms and harmless epiphytes on the one side and pathogenic microorganisms on the other *in vitro* (in a petri-dish system) and *in situ* (in a MAP system with produce).
   - Investigation of the possibility to bring natural antimicrobial systems to expression. One such system resides within the population of LABs, which occur naturally on most vegetables, the expression of which may be manipulated through the composition of the gas atmosphere in a MAP system.

4. State-of-the-art

The use of Modified Atmosphere Packaging (MAP) and Vacuum Packaging (VP) to extend the shelf-life of perishable food products has experienced a strong upsurge lately. Although applied mostly with non-respiring products, both techniques offer considerable prospects for respiring products as well. Respiring products, like fresh and freshly processed vegetables and fruits, stay metabolically active after harvest and this activity is essential for keeping their quality.
With Modified Atmosphere Packaging (MAP), the atmosphere inside a package generally is low in oxygen (O_2) and high in carbon dioxide (CO_2), with typical values at equilibrium of 2-3% and 5% respectively. The composition of the atmosphere is the net result of the initial gas composition, the gas exchange through the packaging material and the respiratory activity of the produce. Recently, a so called Moderate Vacuum Packaging (MVP) system has become available in the Netherlands. In this system, a respiring produce is packaged in a rigid, airtight container under 400 mB atmospheric pressure which is stored at a refrigeration temperature. The initial gas composition is that of normal air (21% O_2, 0.04% CO_2 and 78% N_2) but at a reduced partial gas pressure. Thus, the available amount of O_2 is about one third of the normal. As with MAP, the lower O_2 content stabilises the post-harvest product quality by slowing down the metabolism of the produce and the growth of spoilage microorganisms. The mechanism of action and the safety of the MVP system were studied in the first part of the project. Mungo bean sprouts and chicory endive were used as the model products.

C. 1. Overview of the work performed from 1-1-1996 to 30-6-1996

I) *MODELING AND PREDICTING OF BACTERIAL GROWTH*

Growth of pathogens and bacteria relevant to spoilage of vegetables under the influence of different gas phase conditions was monitored on agar surfaces (see previous reports). Growth data obtained were analysed by fitting with the modified Gompertz equation (Bennik *et al.* 1995).
These data were now analysed with a equation described by Jószef Baranyi (Baranyi *et al.* 1993) who kindly provided the inhouse modeling program of the IFR Reading Laboratory, UK, called DModel. This model allows for bacterial curve fitting, but moreover for predicting bacterial growth on the basis of interpolation of obtained results. This is not possible using the modified Gompertz equation.

Materials and methods:

Bacterial growth data of pure cultures grown on agar surfaces were obtained in year 2 and 3 of the project. These data were now fitted with the model of Jozsef Baranyi, IFR, Reading and compared to curve fitting that was done previously using the modified Gompertz equation.

Results and discussion

Curve fitting of L. monocytogenes *data*
Figure 1 shows the curve fits with the DModel and the Modified Gompertz equation (upper and lower part, respectively) for the growth of *Listeria monocytogenes* under a gasphase atmosphere of 1.5% O_2 and 0, 5, 20 or 50% CO_2. Both programs generate the calculated maximum specific growth rate (μ_m), the maximum population density and the lag phase. A lag phase was not observed, and the maximum population densities were not effected by a CO_2 concentration up to 50%. However, maximum specific growth rates were reduced under elevated CO_2 concentrations. Figure 2 shows the μ_m of *L. monocytogenes* as a function of the CO_2 concentration. This relation is described by the function: $\mu_m = 0.081724 - 0.00093 * [CO_2]$ (linear regression analysis) and is presented in figure 1.

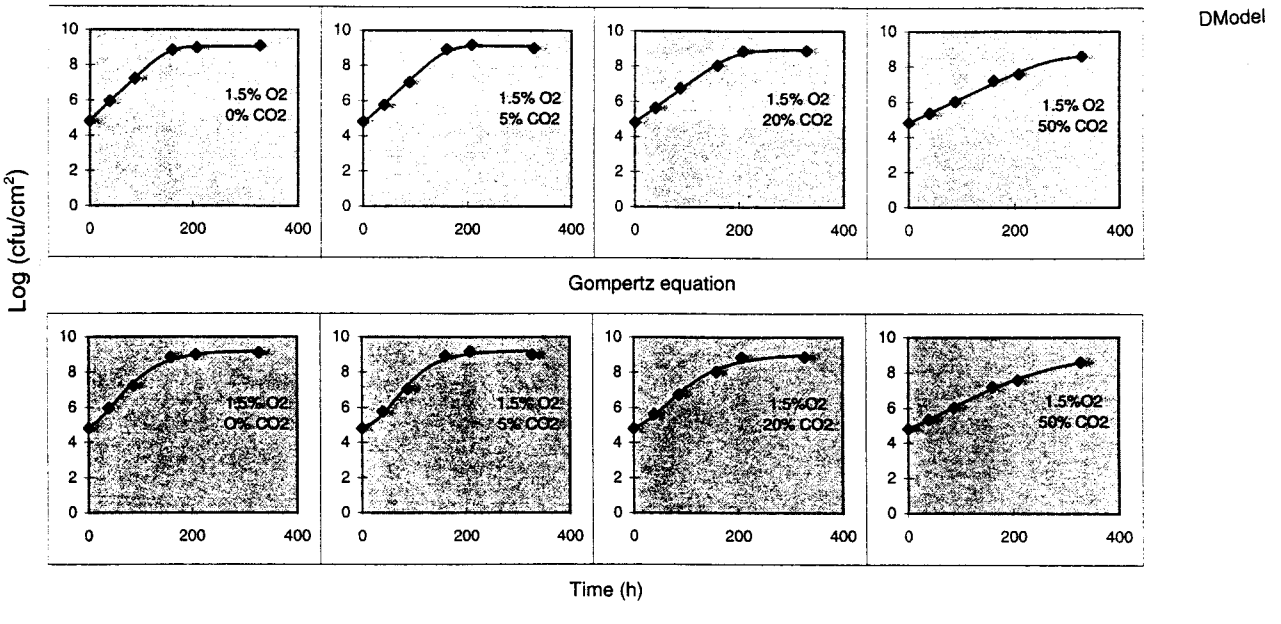

Figure 1. Growth of *Listeria monocytogenes* under gasphase atmospheres of 1.5% O_2 and 0, 5, 20 or 50% CO_2 (from left to right). Curve fits with the DModel (upper 4 graphs) and the Modified Gompertz equation (lower 4 graphs) are presented.

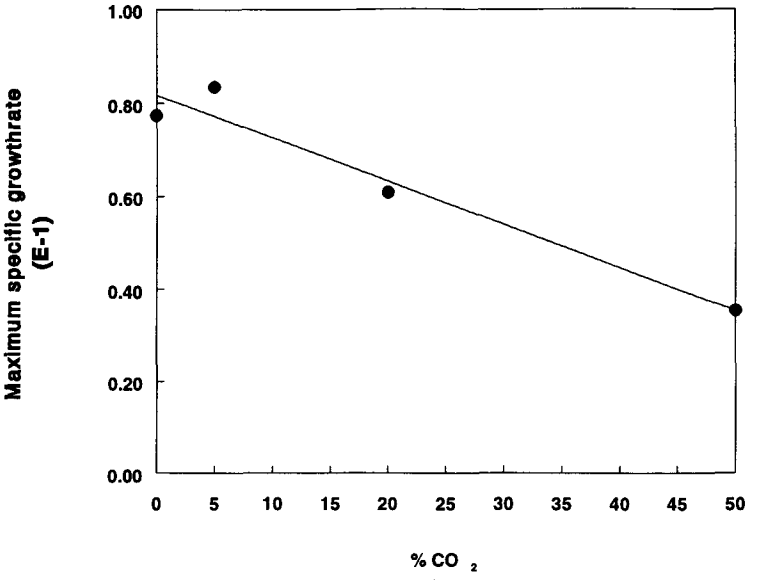

Figure 2. The maximum specific growth rate (μ_m) of *L. monocytogenes* as a function of the CO_2 concentration.

Growth prediction of L. monocytogenes

Figure 3 shows the predicted growth curve for the growth of *Listeria monocytogenes* under a gasphase atmosphere of 1.5% O_2 and 0, 5, 20 or 50% CO_2 (line y1, y2, y3 and y4 respectively), using the DModel. Additionally, the predicted growth under 10 and 35% CO_2 is indicated by the dotted lines. These predictions are based on the calculated relationship between μ_m and CO_2 and are only valid for interpolation of data (e.g. if the original data were obtained in the range of 0-50% CO_2, growth can only be predicted in this range). This program is thus a suitable means to predict e.g. doubling time under other gas concentrations than the ones that were tested.

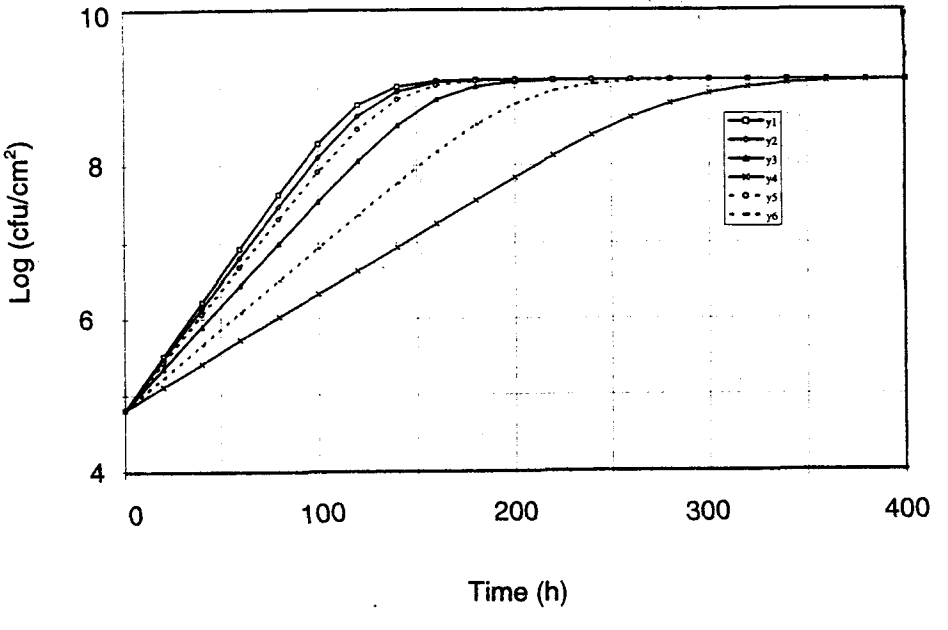

Figure 3. Predicted growth of *Listeria monocytogenes* (using the DModel) under a gasphase atmosphere of 1.5% O_2 and 0, 5, 20, 50, 10 and 35% CO_2 (line y1, y2, y3, y4, y5, y6, respectively). Dotted lines indicate predicted growth under conditions that were not analysed using bacteria plating techniques.

Curve fitting of growth data of spoilage bacteria

Growth data of spoilage bacteria (isolated from mungo bean sprouts and chicory endive) under different CO_2 and O_2 concentrations were also fitted with the Dmodel. Lagtimes were absent and different gas concentrations did not influence the final population densities. The μm generated by the Dmodel is presented in figure 4A and 4B (respectively spoilage bacteria isolated from mungbean sprouts and and chicory endive).

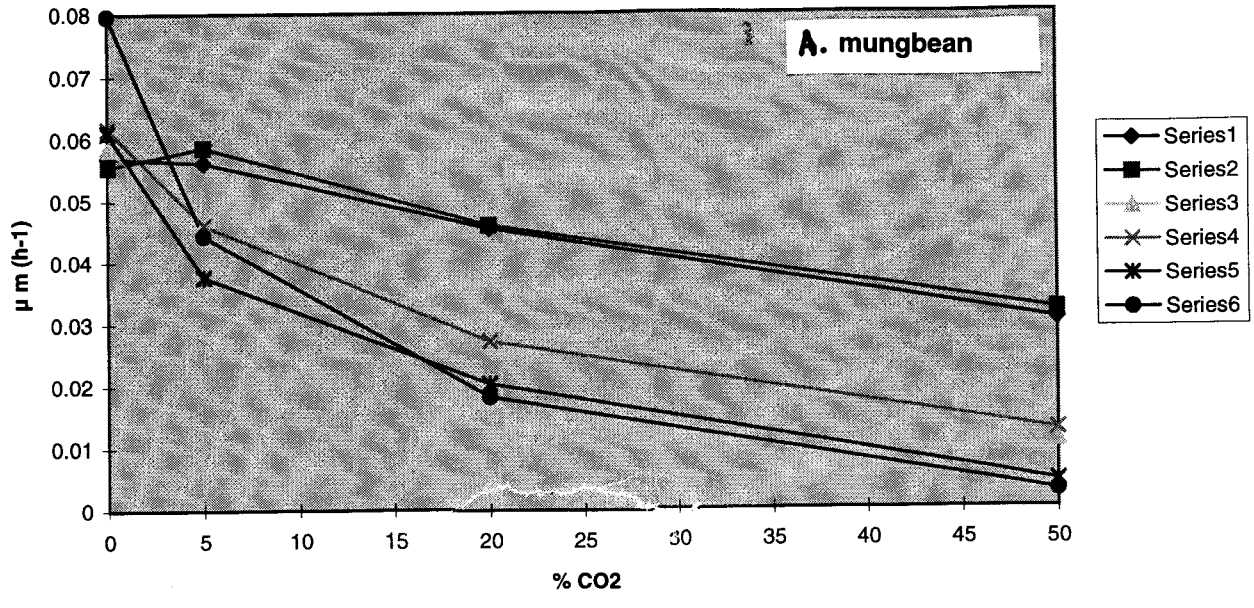

Figure 4A. Maximum specific growth rates as a function of CO_2 concentration at 8°C for surface growth of spoilage organisms isolated from mungbean sprouts: *Pantoea agglomerans* under 1.5% O_2 (series 1) or 21% O_2 (series 2), *Pseudomonas corrugata* under 1.5% O_2 (series 3) or 21% O_2 (series 4), *P. fluorescens* under 1.5% O_2 (series 5) or 21% O_2 (series 6)

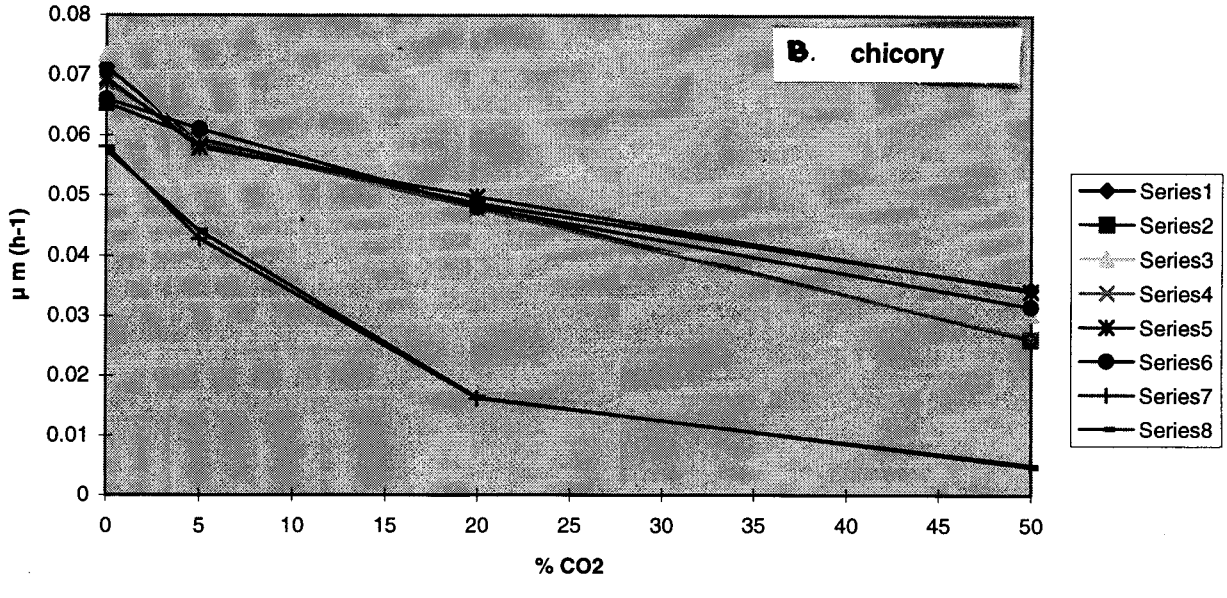

Figure 4B. Maximum specific growth rates as a function of CO_2 concentration at 8°C for surface growth of spoilage organisms isolated from chicory endive: *Enterobacter vulneris* under 1.5% O_2 (series 1) or 21% O_2 (series 2), *P. fluorescens* under 1.5% O_2 (series 3) or 21% O_2 (series 4), *Rahnella aquatilis* under 1.5% O_2 (series 5) or 21% O_2 (series 6) and *P. viridiflava* under 1.5% O_2 (series 7) or 21% O_2 (series 8).

Conclusions

Curve fitting of bacterial growth data with both the modified Gompertz equation and the Dmodel give good fits, rendering maximum specific growth rate, maximum population densities and lagtimes. The advantage of the Dmodel over the Gompertz equation is the possibility of predicting bacterial growth on the basis of interpolation of obtained results.

Data supply / handling for the use of Dmodel:

Files containing growth data should be in a ascii delimited text file with a comma as field separator. In Microsoft excel this is a *.csv file. Use one file for every single growth curve. Indicate the time (h) in the first column, and the growth data (^{10}Log (cfu/ml)) in the second column. Save as '*.csv'. Furthermore, make an indexfile in Microsoft excel indicating all the names of the saved *.csv files. These data are suitable for analysis with the Dmodel.

Example:
worksheet containing data: example.wk1 or example.xls

time (h)	0% CO_2 log cfu/ml	5% CO_2 log cfu/ml	20% CO_2 log cfu/ml
0	4	4	4
10	5	4.5	4.5
20	6	5	5
30	7	6	5.5
40	8	7	6
50	8	8	7
60	8	8	8

Make *.csv files for separate series:
example: filename = 0%CO2.csv

time(h)	log cfu/ml
0	4
10	5
20	6
30	7
40	8
50	8
60	8

Finally create a index file with all the names of the separate growthcurve files listed in one column and save it as *.xls worksheet (for more details please contact M. Bennik).

II) BIOPRESERVATION

The natural microflora of LABs is believed to be a natural antimicrobial system in potential. LABs may exert an antimicrobial effect, due to the production of lactic and acetic acid and possibly bacteriocins. Bacteriocin producing LABs with activity against pathogens such as *L. monocytogenes* and *C. botulinum* were isolated from fresh chicory endive. The most potent bacteriocin producing LABs were *Enterococcus mundtii* and two strains of *Pediococcus parvulus*. The bacteriocin produced by *P. parvulus* was identified and was identical to pediocin PA1, produced by *P. acidilactici* (Marugg et al. 1992, Motlach et al. 1994, Daba et al. 1994). These strains were not capable of producing bacteriocins at a low temperatures. However, *E. mundtii* produces a bacteriocin active against *L. monocytogenes* and *C. botulinum*, that can be produced at low temperatures. This would make *E. mundtii* a suitable strain for application in refrigerated fresh vegetables as an additional safety factor against the outgrowth of gram positive pathogens. Research focussed on the purification and identification of this bacteriocin and the application of the producer organism and the (partially) purified bacteriocin on a product.

IIa Purification and sequence analysis of bacteriocin produced by Enterococcus mundtii

Materials and methods

Purification of the bacteriocin was done with a 1% inoculum of an overnight culture of *E. mundtii* in 2 liters of ATP broth and grown for 20 h at 30°C. Cells were removed by centrifugation (9000 g, 15 min, 20°C) and the culture supernatant was adjusted to pH 6.0 and heated to 70°C for 30 min. A two step ammonium sulphate precipitation was carried out to concentrate the protein in the culture supernatant. In the first step, ammonium sulphate was added (113 g/L) by stirring for 1h at room temperature. The precipitate was dissolved in 30 ml of 50mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5 (fraction A). Ammonium sulphate (360 g/L) was added to the supernatant and after stirring for 1 h at room temperature the precipitated proteins were removed by centrifugation (9000 g, 15 min 20°C). The precipitate was dissolved in 30 ml of 50 mM MES, pH 5 (fraction B). The active fraction B was loaded onto a phenyl-Sepharose column chromatography column, equilibrated with 50 mM MES buffer pH 5.5, containing ammoniumsulphate (0.42 M). The bacteriocin was eluted by a linear gradient (mobile phase: 50 mM MES buffer (pH 5.5) with 0.85 M ammonium sulphate [A], 50 mM MES buffer (pH 5.5) [B]). The active fractions were loaded onto a cationic exchanger equilibrated with 10 mM HCOOH, 10% EtOH in water and eluted by a linear gradient (10 mM HCOOH, 10% EtOH in water to 1 M NaCl, 10 mM HCOOH, 10% EtOH in water). The active fractions were then loaded onto a gelfiltration column with a separation range of 100-7000 Dalton (Pharmacia) equilibrated with 0.15 M NaCl, 0.1% TFA and 20% EtOH, and the bacteriocin was eluted as a single peak. The amino acid sequence was determined by Edman degradation.

Results / discussion

The bacteriocin produced by *E. mundtii* was purified to homogeneity using ammonium sulphate precipitation, HPLC and FPLC techniques. The N terminal amino acid sequence was determined for 39 a.a. The estimated size of the peptide is 44 amino acids, but the last part of the sequence was not clarified. The analysis rendered a unique sequence, that has highest homology with the bacteriocin piscicolin 126 produced by *Carnobacterium piscicola* (80%) (Jack et al. 1996) and sakacin P, produced by *Lactobacillus sake* (70%) (Ticaczek et al 1994). Homology with bacteriocins of other enterococci was not found. The bacteriocin of *E. mundtii* belongs to the Listeria active class IIa bacteriocins (Klaenhammer et al, 1993).

Conclusion

The bacteriocin of *Enterococcus mundtii* is novel and shows highest homology with piscicolin 126 and sakacin P, but not with other bacteriocins from enterococci.

IIb) *Application of bacteriocin producing organisms on MAP vegetables*

The fate of *Listeria monocytogenes* in the presence of bact$^+$ and bact$^-$ LABs was investigated on mungo bean sprouts stored under MA conditions at 8°C. The product was inoculated with pure cultures of *L. monocytogenes* and bact$^+$ or bact$^-$ cultures of *E. mundtii* and combinations of these organisms. Also, mungbeans were dipped in a bacteriocin solution prior to contamination with the pathogen.

Materials and methods

Product and storage conditions
Mungo bean sprouts were obtained directly from a local grower and used in the experiment within 6 hours after harvesting. Temporary storage was at 4°C.

Storage conditions and bacterial inoculations
7 Batches of mungo bean sprouts were stored at 8°C under 1.5% O_2 and 20% CO_2. One batch was dipped in water as a control. One batch was artificially contaminated with a pure culture of bact$^-$ *E. mundtii* DSM 4838, one with bact$^+$ *E. mundtii,* and one with a mix of *L. monocytogenes* LCDC 81-861 and LCDC 81-1087, by dipping the product in a solution of cells. 2 Batches were contaminated with the mix of *L. monocytogenes* and bact$^-$ *E. mundtii* or bact$^+$ *E.mundtii*. Initial loads of *L.monocytogenes* were approximately 10^5 CFU/ml and for *E.mundtii* (bact$^+$ and bact$^-$) 10^6 CFU/ml. In addition, one batch was dipped in a *E.mundtii* bacteriocin solution (420 and after that inoculated with a washed cell suspension of *L. monocytogenes* to give an initial load of approximately 10^4 Listeria per g of product.

Incubation and gasphase composition
Inoculated mungbean sprouts were put in the flow through system that has previously been described (progress report #3) under a constant stream (200 ml/min) of 1.5% O_2, 20% CO_2 and 78.5% N_2, at 8°C. This gasphase composition has been shown to extend the keepability of mungo bean sprouts.

Microbial analysis
Microbiological growth was examined during storage, i.e. total aerobic counts, counts of LABs and *L. monocytogenes*, using standard methods (Mossel and Jacobs-Reitsma, 1990).

Results / discussion

Total mesophylic counts were increasing at the same rate to the same maximum population densities for the 7 different batches of mungo bean sprouts. Counts of LABs were also similar under all combinations tested. *L. monocytogenes* grew equally well on product contaminated with bac$^-$ *E. mundtii* and bact$^+$ *E. mundtii* as compared to product that was not inoculated with LABs. However, dipping the product in a bacteriocin solution before contamination with the pathogen resulted in a strong suppression of the outgrowth of listeria. Figure 5A shows the total aerobic counts, figure 5 B the LABs and figure 5C the listeria counts (CFU/g) on product inoculated with listeria in combination with bact$^+$ or bact$^-$ *E.mundtii,* and the product dipped in bacteriocin solution prior to contamination with Listeria. Although *E. mundtii* can produce bacteriocins at 8°C, the presence of a bacteriocin producing organism did not inhibit growth of *L. monocytogenes.* By contrast, the application of a partial purified bacteriocion in solution proved to be an effective means to suppress the growth of the pathogen, although outgrowth is observed after 7 days. The ineffectiveness of applying the bacteriocin producing strain to suppress the growth of *L. monocytogenes* might be related to the stage of growth at which bacteriocin release takes place, namely the end of the logarithmical stage (previous report). During this period, Listeria will not be inhibited. Additionally, a fraction of the listeria population might be resistant to the bacteriocin. This assumption is supported by the outgrowth of the pathogen after the dip in bacteriocin solution. Furthermore, proteolytic enzymes present on the product (bacterial or plant origin) can cause breakdown of the bacteriocin, resulting in a decreased efficiency.

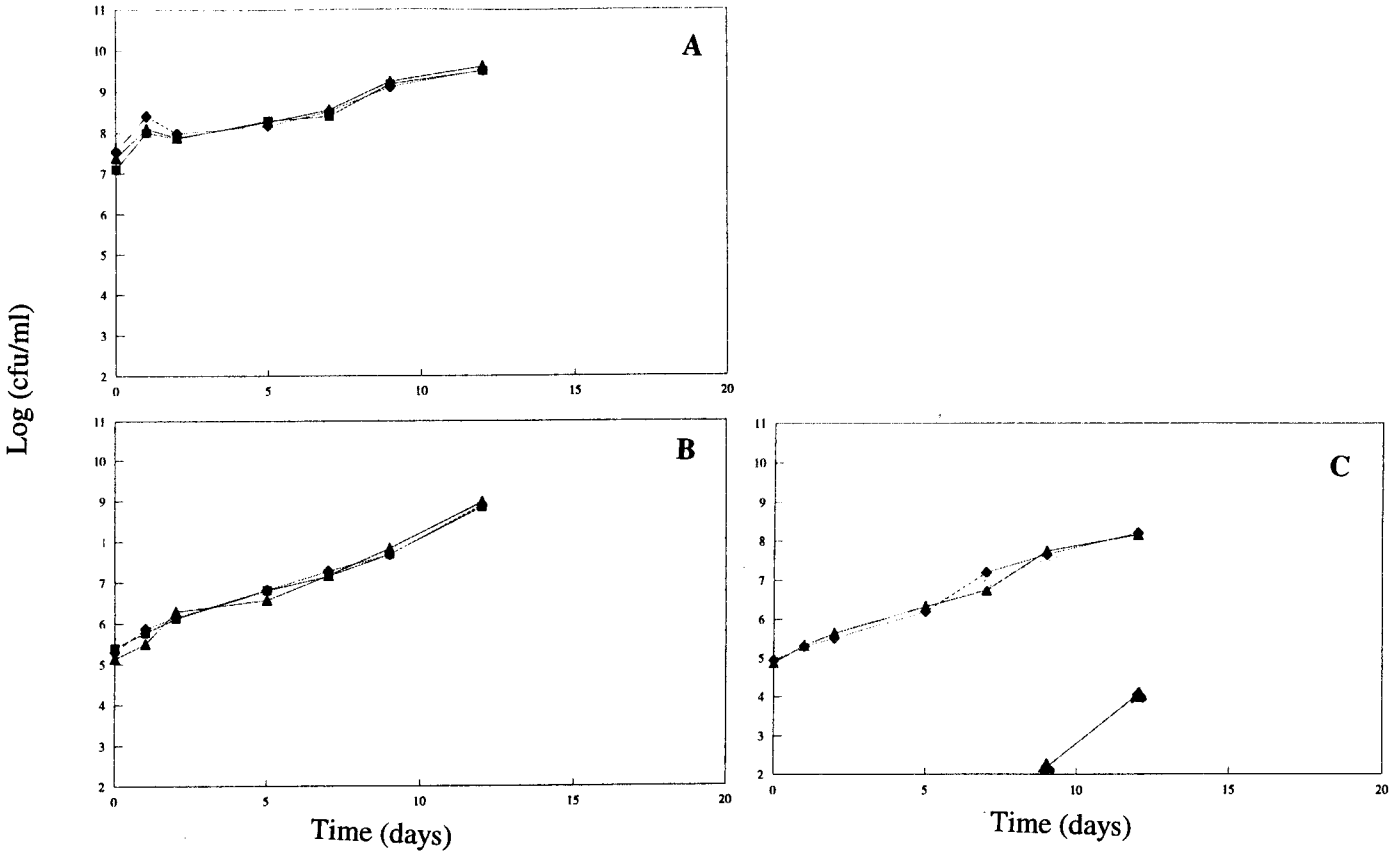

Figure 5 Total aerobic counts (Fig 5a), LABs (Fig 5b) and *L. monocytogenes* (Fig 5c) on mungo bean sprouts inoculated with (■) *E.mundtii* bact$^+$ with a mix of *L. monocytogenes* LCDC 81-861 and -1087, (◆) *E. mundtii* bact- with a mix of *L. monocytogenes* LCDC 81-861 and -1087 and (▲) mix of *L. monocytogenes* LCDC 81-861 and -1087 inoculated after dipping in bacteriocin solution. Storage conditions were at 8°C under 1.5% O_2 and 20% CO_2.

Conclusions

The presence of a bacteriocin producing organism did not inhibit growth of *L. monocytogenes* on mungo bean sprouts, but the application of a partially purified bacteriocin efficiently reduced the outgrowth of listeria up to 7 days during storage.

SUB PROJECT (B) *Systematic investigation of the quality of fresh or freshly processed vegetables under Modified atmospheres*

Modelling gas conditions inside MA packages using

H.W. Peppelenbos

Introduction

In the past years models were developed that relate variations in O_2 and CO_2 concentrations to gas exchange rates of plant products. Both oxidative and fermentative metabolism was incorporated. Meanwhile MA-packaging of carrots took place, generating data on gas exchange and gas conditions inside packages. These data are used for a validation of the gas exchange models. To facilitate the validation, the models were incorporated in existing models describing gas conditions in MA packages. The (theoretical) improvement comprises two aspects:

- a better description of O_2 uptake rates using Michaelis-Menten kinetics;
- the incorporation of fermentative CO_2 production at low O_2 concentrations.

Material and methods

Modelling gas exchange

Gas exchange rates were compared with an O_2 consumption model and a CO_2 production model. For O_2 consumption, and the CO_2 influence on O_2 consumption, a model using a noncompetitive type of inhibition by CO_2 (Peppelenbos and van 't Leven, 1996) was used:

$$VO_2 = \frac{V_mO_2 * O_2}{(K_mO_2 + O_2) * \left(1 + \frac{CO_2}{K_mCO_2}\right)} \quad (1)$$

where V_{O2} is the O_2 consumption rate ($ml.kg^{-1}.h^{-1}$), Km_{O2} is the Michaelis constant for the inhibition of O_2 consumption by O_2 ($\%O_2$), and Km_{CO2} the Michaelis constant for the inhibition of O_2 consumption by CO_2 ($\%CO_2$).

For CO_2 production, a model was used describing both oxidative or fermentative pathways (Peppelenbos et al., 1996):

$$V_{CO2} = V_{O2} * RQ_{ox} + \frac{Vmf_{CO2}}{1 + \left(\frac{O_2}{Kmf_{O2}}\right)} \quad (2)$$

where V_{CO2} is the CO_2 production rate ($ml.kg^{-1}.h^{-1}$), RQ_{ox} is the RQ of oxidative processes, Vmf_{CO2} is the maximum fermentative CO_2 production rate ($ml.kg^{-1}.h^{-1}$) and Kmf_{O2} the Michaelis constant for the inhibition of fermentative CO_2 production by O_2.

Model 2 was extended with an CO_2 inhibition term for fermentative CO_2 production:

$$V_{CO2} = V_{O2} * RQ_{ox} + \frac{Vmf_{CO2}}{1 + \left(\frac{O_2}{Kmf_{O2}}\right) + \left(\frac{CO_2}{Kmf_{CO2}}\right)} \quad (3)$$

where Kmf_{CO2} the Michaelis constant for the inhibition of fermentative CO_2 production by CO_2.

Model validation

Equations 1 to 3 were incorporated in an existing MA-model. The simulation language used was PROSIM. Data on gas exchange of carrots and gas conditions in MA-packed carrots were collected from the University of Limerick. Because not all parameters necessary for the models could be estimated, some were collected from literature (Leshuk and Saltveit, 1991). The MA-model generated gas concentrations, and these values were compared with data of Limerick (carrot cultivar Nairobi, packed in sealed bags of polypropylene, storage temperature 8°C).

Results and Discussion

Almost all the data of packed carrots showed a decrease of the O_2 to values close to 1%. Because no correction was made for argon, the actual O_2 concentration probably approached 0%. This is reflected in the predicted O_2 concentrations as generated by the MA-model (figure 1). It is known the carrots produce considerable amounts of CO_2 at very low O_2 concentrations (Leshuk and Saltveit, 1991). This fermentative CO_2 production was also found in the MA-packed carrots, were CO_2 concentrations continued to rise, even when respiration had almost ceased. This fermentative CO_2 production was accounted for in the model, as it also generated increasing CO_2 concentrations (fig.1). Interestingly a higher maximum fermentation rate had to be used than expected (based on literature) in order to fit the data. More data will be analyzed in the future, but a conclusion is that the incorporation of fermentation in the MA-model improved its functioning.

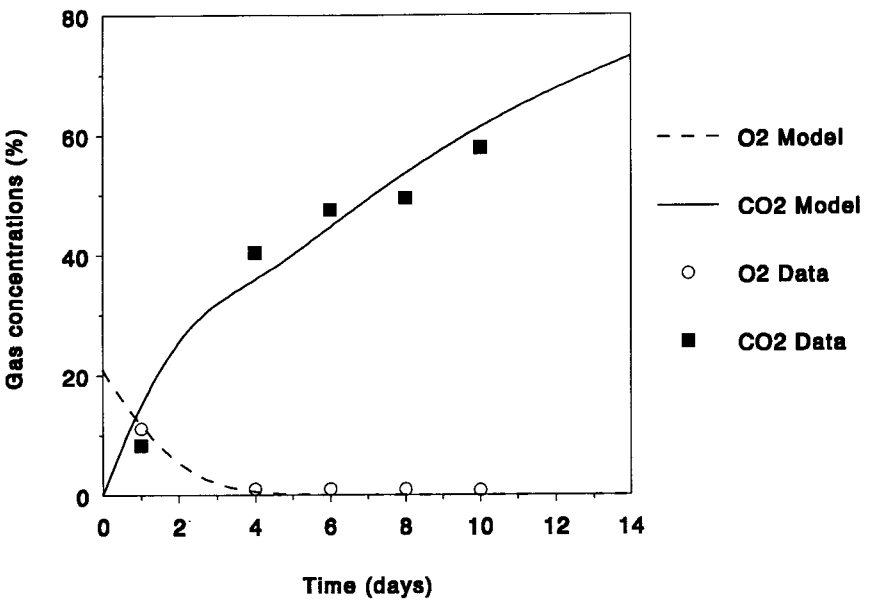

Figure 1: Changes in gas conditions in MA-packages of carrots.

Bibliography

Baranyi, J., Roberts, T.A. & McClure, P. (1993) A non-autonomous differential equation to model bacterial growth. *Food Microbiol.* **10**, 43-59

Bennik, M.H.J., Smid, E.J., Rombouts, F.M. and Gorris, L.G.M. (1995) Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. *Food Microbiol.* **12**, 509-519

Bhunia, A.K., Johnson, M.C. and Ray, B. (1987) Direct detection of an antimicrobial peptide of Pediococcus acidilactici in sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *J. Industrial Microbiol.* **2**, 319-322.

Daba, H., Lacroix, C., Huang, J., Simard, R.E. and Lemieux, L. (1994) Simple method of purification and sequencing of a bacteriocin produced by Pediococcus acidilactici UL5. *J.Appl.Bacteriol.* **77**, 682-688.

Jack, R.W., Wan, J. Gordon, J. Harmark, K. Davidson, B.E. Hillier, A.J. Wettenhall, R.E.H., Hickey, M.W. and Coventry, M.J. (1996) Characterisation of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by Carnobacterium piscicolin JG126. *Appl. Environm. Microbiol.* In press.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic aciid bacteria, FEMS *Microbiol. Rev.* **12**: 39-86

Leshuk, J.A. & Saltveit M.E. (1991) Effects of rapid changes in oxygen concentration on the respiration of carrot roots. Phys. Plant., **82**: 559-568

Martinezbueno, M., Maqueda, M., Galvez, A., Samyn, B., Vanbeeumen, J., Coyette, J. and Valdivia, E. (1994) Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. *J.Bacteriol.* **176**, 6334-6339.

Marugg, J.D., Gonzalez, C.F., Kunka, B.S., Ledeboer, A.M., Pucci, M.J., Toonen, M.Y., Walker, S.A., Zoetmulder, L.C.M. and Vandenbergh, P.A. (1992) Cloning, expression, and nucleotide sequence of genes involved in production of Pediocin PA-1, a bacteriocin from Pediococcus acidilactici PAC1.0. *Appl.Environ.Microbiol.* **58**, 2360-2367.

Mossel, D.A.A. and Jacobs-Reitsma, W.F. *Mikrobiologisch Onderzoek van Levensmiddelen*, Zeist: P.C. Noordervliet B.V. 1990. Ed. 3rd

Motlach, A.M., Bhunia, A.K., Szostek, F., Hansen, T.R., Johnson, M.C. and Ray, B. (1992) Nucleotide and amino acid sequence of pap-gene (pediocin AcH production) in Pediococcus acidilactici H. *Lett.Appl.Microbiol.* **15**, 45-48.

Peppelenbos, H.W. & van 't Leven, J. (1996) Evaluation of four types of inhibition for modelling the influence of carbon dioxide on oxygen consumption of fruits and vegetables. *Posth. Biol. Techn.*, **7**: 27-40.

Peppelenbos, H.W., Tijskens L.M.M., van 't Leven, J., & Wilkinson, E.C. 1996. Modelling carbon-dioxide production of fruits and vegetables. *Posth. Biol. Technol.*, **7** (in press).

Ticaczek, P.S., Vogel, R.F. and Hammes, W.P. (1994) Cloning and sequencing of sakP encioding sakacin P, a bacteriocin produced by Lactobacillus sake LTH673. *Microbiol.* 140: 361-367.

D. 1. Short description of the work planned for the second half of 1996

During the second part of 1996 work will focus on the following topics

1) Modeling of data: validation of microbiological data relevant to improvement of the keeping quality of fresh or minimally processed vegetables stored under MAP conditions, that were also obtained by other participants, using microbiological growth models
2) The generated knowledge during the course of the project in the areas of product physiology and microbiology will be integrated to obtain an optimisation for packaging of different types of produce under the most suitable packaging conditions. Focus will be on both the quality and safety of produce. A detailed workplan will be discussed during the meeting in Nice.
3) Integration of microbiology of MAP vegetables, coatings and bacteriocins. By the use of coatings (eg containing bacteriocins), we want to investigate the potential of applying a combination of anti microbial barriers against spoilage organisms and pathogens on MAP vegetables.

E. Dissemination (*Recent publications and presentations from the ATO-DLO team*)

Bennik, M.H.J., E.J. Smid, F.M. Rombouts, L.G.M. Gorris, 1995. Surface growth of psychrotrophic pathogens under different gasphase compositions in a model system. Food Microbiology 12, 509-519.

Bennik, M.H.J., E.J. Smid & L.G.M. Gorris, 1995. Mild food preservation using biopreservation as an additional safety hurdle. COPERNICUS workshop "Process Optimization and Minimal Processing of Foods, Porto, Portugal.

Bennik, M.H.J., Peppelenbos, H.W., Nguyen-the, C., Carlin, F., Smid, E.J., Gorris, L.G.M. Microbiology of minimally processed, modified atmosphere packaged chicory endive, Postharvest Biology & Technoloy (accepted).

Bennik, M.H.J., W. Vorstman, E.J. Smid, L.G.M. Gorris. The influence of carbon dioxide on the outgrowth of spoilage organisms isolated from MAP vegetables. Journal of Applied Bacteriology *(to be submitted).*

Guilbert, S., N. Gontard & L.G.M. Gorris, 1996. Prolongation of the shelf-life of perishable food products using biodegradable films and coatings. Lebensmittel- Wissenschaft und Technologie 29 (1/2), 10-17.

Ponne, C.T., M. Balk, Ö. Hancioglu, L.G.M. Gorris, 1996. Effect of radio frequency energy on biological membranes and microorganisms. Lebensmittel- Wissenschaft & Technologie 29 (1/2), 41-48.

Gorris, L.G.M., T. Abee & M. Peck. Food matrix influences on pathogen growth. ZFL 47 (1996) 1/2, 62-65.

Gorris, L.G.M., T. Abee & M. Peck. Invloed van de voedselmatrix op groei en overleven van pathogene bacteriën. VMT *(submitted).*

Gorris, L.G.M. & T. Abee. Biopreservation as an additional safety hurdle in prepared salads, ZFL 47 (4), 79-81.

Gorris, L.G.M., 1995. Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild preservation techniques. In: Proceedings COPERNICUS workshop "Process Optimization and Minimal Processing of Foods, Porto, Portugal.

Gorris, L.G.M., 1995. The concept of hurdle technology for mild food preservation. In: Proceedings COPERNICUS workshop "Process Optimization and Minimal Processing of Foods, Porto, Portugal.

Gorris, L.G.M., 1995. Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild preservation techniques. In: Proceedings FI-Europe/GDL Conference 1995, Frankfurt, Germany.

Gorris, L.G.M., 1996. Edible coatings to improve produce quality and shelf-life of ready-to-eat vegetables and fruits. In: "Minimal Processing of Ready Made Foods", Ohlsson, T., Ahvenainen R., Mattila-Sandholm, T., (eds.), Göteborg, Sweden, 18-19 April 1996, pp. 45-58

Peppelenbos, H.W. & van 't Leven, J. (1996) Evaluation of four types of inhibition for modelling the influence of CO_2 on O_2 consumption of fruits and vegetables. *Posth. Biol. Techn.*, **7**, 27-40.

Peppelenbos, H.W., Tijskens L.M.M., van 't Leven, J., & Wilkinson, E.C. 1996. Modelling carbondioxide production of fruits and vegetables. *Posth. Biol. Technol.*, **7** (in press).

F. Highlights during the first half of 1996

Curve fitting of bacterial growth data was compared using the modified Gompertz equation and the Dmodel, an inhouse program of the IFR, Reading, UK. Although both programs are suitable for fitting purposes, the advantage of the Dmodel is the possibility of predicting growth on the basis of interpolation of obtained results. We now chose for the use of the Dmodel for further modeling of data.

The bacteriocin produced by *Enterococcus mundtii* was purified to homogeneity and the amino acid sequence was determined. The bacteriocin is unique, and has highest homology to bacteriocins produced by *Carnobacterium piscicola* and *Lactobacillus sake.*

Application of the bacteriocin producing strain *E.mundtii* on mungbean sprouts to suppress the growth of *Listeria monocytogenes* was not succesfull. However, application of a purified bacteriocin fraction on produce was efficiently suppressing outgrowth of the pathogen for 1 week at 8°C.

The models describing gas exchange were incorporated in a Modified Atmosphere (MA) model. This modified MA model is suitable to describe gas concentrations inside packages even when the most important proces occurring is fermentation.

Participant 2: Wageningen Agricultural University (WAU.DFS)
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A. ***1. (Sub-)Project title***
Physiology of pathogens and spoilage bacteria and biopreservation of foods in combination with novel, mild techniques.

2. Project coordinator
Prof.Dr.ir. Frans M. Rombouts

3. Projects participants and 4. Resources

Ir. A. Verheul	12	months/year
Dr. T. Abee	2.4	" "
Prof.Dr.ir. F.M. Rombouts	0.6	" "
R.R. Beumer	0.6	" "
Ir. M.H. Zwietering	0.6	" "

B. ***1. Description of the research topic or practical problem***
The research in the Department of Food Science will focus on the physiology of spoilage bacteria and pathogens and on the biopreservation of foods.

Physiology of pathogens and spoilage bacteria: Contamination and outgrowth of *Listeria monocytogenes* and non-proteolytic *Clostridium botulinum* are of particular concern in minimally processed chilled foods. These bacteria are able to grow at refrigeration temperatures and heat-resistent spores of *C. botulinum* would survive a minimal heat process and possibly germinate, grow and produce toxin in temperature abused food. Likewise, the growth of psychrotrophic spoilage bacteria needs to be investigated for the optimalization of mild techniques to prevent shelf life.

Biopreservation of food: Bacteriocins are biologically active low-molecular weight proteins produced by certain species of genera such as *Lactobacillus, Lactococcus, Streptococcus* and *Pediococcus*. The bacteriocins nisin and pediocin, produced by *Lactococcus lactis* and *Pediococcus spp.*, respectively, have a broad-spectrum activity towards gram-positive bacteria including pathogenic *Listeria* and *Clostridium* species. These bacteriocins or the producing strains will be included in different foods as an alternative for chemical preservation, and their effects on spoilage bacteria and pathogens will be studied.

2. Goal of the research
Physiology of pathogens and spoilage bacteria: At present nothing is known about the bioenergetics of these pathogens and spoilage bacteria. A thorough understanding of the physiology of these organisms will contribute to the optimalization and development of novel mild preservation techniques.

Biopreservation: Various bacteriocins or bacteriocin-producing strains will be included in different foods as an alternative for chemical preservation. In addition to modified atmosphere, these bacteriocins can possibly minimize the growth risk of gram-positive spoilage bacteria and pathogens thereby increasing the shelf life of fresh and minimally processed foods.

3. Short description of the approach

Basic knowledge on microbial physiology will be obtained by studying the utilization of various sugars (for carbon- and energy) and nitrogen sources (amino acids and peptides) by these food pathogens under different environmental/food conditions. Energy generation, regulation of intracellular pH and osmoregulation will be studied at low a_w, low temperature and under modified atmosphere. Combined with the studies on biopreservation, this information can significantly contribute to the development and optimalization of mild preservation techniques.

C. *1. Overview of the work performed from 1-1-1996 to 1-7-1996*

a) Oligopeptides as the source of essential amino acids for growth of L. monocytogenes

Introduction

Amino acids essential to *L. monocytogenes* include leucine, isoleucine, valine, methionine and cysteine (Premaratne *et al.*, 1991; Beumer *et al.*, 1994). This amino acid requirement cannot be satisfied by complex proteins such as casein (Seeliger and Jones, 1986). Since the amount of free amino acids in food products like vegetables is generally inadequate, the supply of essential amino acids for growth of *L. monocytogenes* in these products has to originate from other sources. In the previous meetings (#3 and #4) we showed that optimal growth of *L. monocytogenes* was dependent on growth of proteolytic bacteria, *i.e. Bacillus cereus* and *Pseudomonas fragi.* This indicates that the pathogen can benefit from the proteolytic activity of other bacteria present in food. Consequently, it does not have to spend energy on the synthesis of protease(s) which may offer a competitive advantage over other microorganisms present in food. As a result of proteolytic breakdown a mixture of amino acids and peptides will become available and therefore it is worthwhile to assess the nutritional value of peptides for growth of *L. monocytogenes.* In the previous meetings (#4 en #5) we showed that di- and tripeptides can supply *L. monocytogenes* with amino acids essential for growth. No extracellular peptide hydrolysis was detected, indicating that peptides are hydrolyzed after being transported into the cell. Evidence was presented that the pathogen is in the possession of a constitutive, proton motive force dependent di- and tripeptide transport system with a broad substrate specificity (Verheul *et al.*, 1995). In this term we focussed on the utilization of oligopeptides as a source of essential amino acids for growth of *L. monocytogenes.*

Methods

Growth experiments were performed in defined minimal medium (DM) (Premaratne *et al.*, 1991) at 37°C whereby one of the essential amino acids was supplied as a oligopeptide (final concentration 0.1 mM). Growth was measured turbidimetrically at 620 nm using a continuous microtiterreader (SLT-340 Labinstruments). For transport assays, cells were grown in DM at 37°C with agitation (200 rpm) in a shaker incubator. Cells were harvested in late-exponential phase (OD_{620nm} = 0.6 à 0.7), washed twice in 50 mM potassium phosphate buffer (pH 6.9) with 5 mM $MgSO_4$ containing 50 µg chloramphenicol per ml and stored on ice until use. Transport assays were conducted at 37°C in 0.25 ml incubation mixtures containing 0.1 ml cell suspensions and 5% (w/v) glucose. Peptides were introduced at the concentration indicated. Peptides (and amino acids) were monitored by determining extracellular concentrations using HPLC after removal of cells as described (Verheul *et al.*, 1995).

Results and Discussion

a) Utilization of peptides by L. monocytogenes during growth
Valine-containing oligopeptides were applied to examine the size restriction range for peptide utilization of *L. monocytogenes*. Removal of the essential amino acid valine resulted in an almost complete inhibition of growth. Upon addition of the dipeptides Ala-Val and Val-Gly growth in DM lacking valine is stimulated (Fig. 1). These results are consistent with those previously obtained with leucine- and methionine-containing dipeptides (report meeting #4).
In addition it appeared that valine-containing tetra-, penta-, hexa-, hepta- and octapeptides can also provide *L. monocytogenes* with the amino acid valine essential for growth. (Fig 1 and 2). The nonapeptides Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr and Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu and the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu were not able to stimulate growth of *L. monocytogenes* in DM without valine (Fig. 2). These experiments suggest that the size limit for peptide utilization in *L. monocytogenes* is 8 amino acid residues. Moreover, it is clear from these results that growth stimulation by valine-containing peptides is not affected by the position of valine in the peptide chain.

b) Uptake of oligopeptides in L. monocytogenes
Peptide uptake was studied via analysis of extracellular fractions after incubation with certain peptides of *L. monocytogenes* for the presence of peptides and amino acids. The dipeptide Ala-Val disappeared rapidly from the external medium (Table 1). The initial rate of Ala-Val uptake was 12 nmol/min/mg protein, which corresponds with the uptake rate of prolyl-alanine in *L. monocytogenes* under these conditions (Verheul *et al.*, 1995). The rate at which oligopeptides disappeared from glucose-energized cell suspension of *L. monocytogenes* varied with the nature of the oligopeptide (Table 1). To discriminate between uptake of oligopeptide followed by uptake of free amino acids and extracellular oligopeptide hydrolysis followed by uptake of free amino acids, the pentapeptide $(Ala)_5$ was incubated with cell extract (CE) or with 200-fold-concentrated cell-free supernatant from DM-grown *L. monocytogenes* cultures. Breakdown of $(ala)_5$ could not be detected in the concentrated supernatant, whereas the concentration of alanine increased during the incubation of $(Ala)_5$ with CE being indicative for peptidase activity in CE. These results are in agreement with previous results obtained with artificial *p*-nitroanilide substrates (Verheul *et al.*, 1995).
The observed increase in several amino acids present in the used oligopeptides during incubation with these peptides is most likely the result of outwardly directed concentration gradients of these amino acids. The fact that an increase in the extracellular concentration is not observed with all amino acid present in these peptides might be explained by differences in the nature of the amino acid transport systems involved and/or differences in rates of metabolism of the amino acids.

c) Toxicity of oligopeptides towards L. monocytogenes
In the course of the growth experiments with essential amino acid containing peptides it appeared that several oligopeptides inhibited the growth of *L. monocytogenes* in DM containing all essential amino acids. Peptides toxic to *L. monocytogenes* include the pentapeptides Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, the hexapeptide Arg-Tyr-Leu-Gly-Tyr-Leu, the heptapeptide Ser-Ile-Gly-Ser-Leu-Ala-Lys, the nonapeptides Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu and Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr and the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. From HPLC analysis it was inferred that the pentapeptides Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu are rapidly taken up by *L. monocytogenes* from the medium, followed by intracellular hydrolysis to the constitutive amino acids. It was tested whether the addition of some amino acids (Tyr, Ser, Thr) to DM affected the growth of *L. monocytogenes*. In addition these individual amino acids were added to a *L. monocytogenes* culture growing

exponentially in DM. The results (shown in Fig. 3) indicate that growth of *L. monocytogenes* in DM plus Tyr was completely inhibited, wheras the growth of the pathogen was not affected by the amino acids Ser or Thr. Addition of any of these amino acids to exponentially growing cells did not result in growth inhibition (Fig. 3). These results might indicate that the toxicity of the abovementioned oligopeptides could be assigned to the appearance of the individual amino acid Tyr that is free in the cytoplasm after peptide uptake and subsequent hydrolysis. However, one of the oligopeptides that efficiently inhibits growth of *L. monocytogenes* lacks the amino acid Tyr (*i.e.* the heptapeptide Ser-Ile-Gly-Ser-Leu-Ala-Lys). In addition it was shown (see section a)) that the tyrosine-containing heptapeptide Arg-Val-Tyr-Ile-His-Pro-Phe, the octapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, the nonapeptides Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu and Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr and the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu did not inhibit the growth of *L. monocytogenes.*
After prolonged incubation of *L. monocytogenes* in DM in the presence of the pentapeptide Tyr-Gly-Gly-Phe-Met (approximately after 70 hours), some cells started growing. These cells were stored at -20°C and subsequently subjected to growth in DM containing the "toxic" peptides Tyr-Gly-Gly-Phe-Met, in DM containing Tyr-Gly-Gly-Phe-Leu, in DM containing Arg-Tyr-Leu-Gly-Tyr-Leu and in DM containing Ser-Ile-Gly-Ser-Leu-Ala-Lys. The growth in these media appeared to be comparable to that in DM with regard to lag time, growth rate and final optical density (data not shown). This might indicate that the toxicity of the different peptides relies on the same mechanism. Another possibility is that we isolated a oligopeptide permease mutant. The basis of the mechanism of the inhibitory oligopeptides remains to be elucidated. However, the finding that certain oligopeptides inhibits the growth of *L. monocytogenes* is of interest, since these peptides may have potential application as antimicrobial substances.

Literature cited

Beumer, R.R., M.C. te Giffel, L.J. Cox, F.M. Rombouts and T. Abee. 1994. "Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium". *Appl. Environ. Microbiol.* **60**, 1359-1363.

Premaratne, R.J., W-J. Lin and E.A. Johnson, 1991. "Development of an improved chemically definded medium for *Listeria monocytogenes*". *Appl. Environ. Microbiol.*, **57**, 3046-3048.

Seeliger, H.P.R. and D. Jones. 1986. "*Listeria*", p. 1235-1245. In: P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt (eds). *Bergey's Manual of Systematic Bacteriology*, vol.2. Baltimore, Williams and Wilkins.

Verheul, A., A. Hagting, M.-R. Amezaga, I.R. Booth, F.M. Rombouts and T. Abee. 1995. "A di- and tripeptide transport system can supply *Listeria monocytogenes* Scott A with amino acids essential for growth". *Appl. Environ. Microbiol.* **61:**226-233.

D. *1. Short description of work planned in the subsequent part of the project*

In the last term we will continue the work on the physiology of *L. monocytogenes.*

E. *Dissimenation (Recent publications from WAU.DFS related to the project)*

Abee, T., A. Coffey and F.M. Rombouts. 1996. "Verhoogde expressie van virulentiefactoren in *Listeria monocytogenes* onder zoutstress: karakterisering van het metalloprotease". Supplement Ned. Tijdschr. Med. Microbiol. **4:**S16.

Coffey, A., F.M. Rombouts and T. Abee. 1996. "Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species". Appl. Environ. Microbiol. **62:**1252-1256

Rombouts, F.M. 1996. "Bederforganismen en pathogenen bij koelkasttemperaturen". Supplement Ned. Tijdschr. Med. Microbiol. **4:**S8.

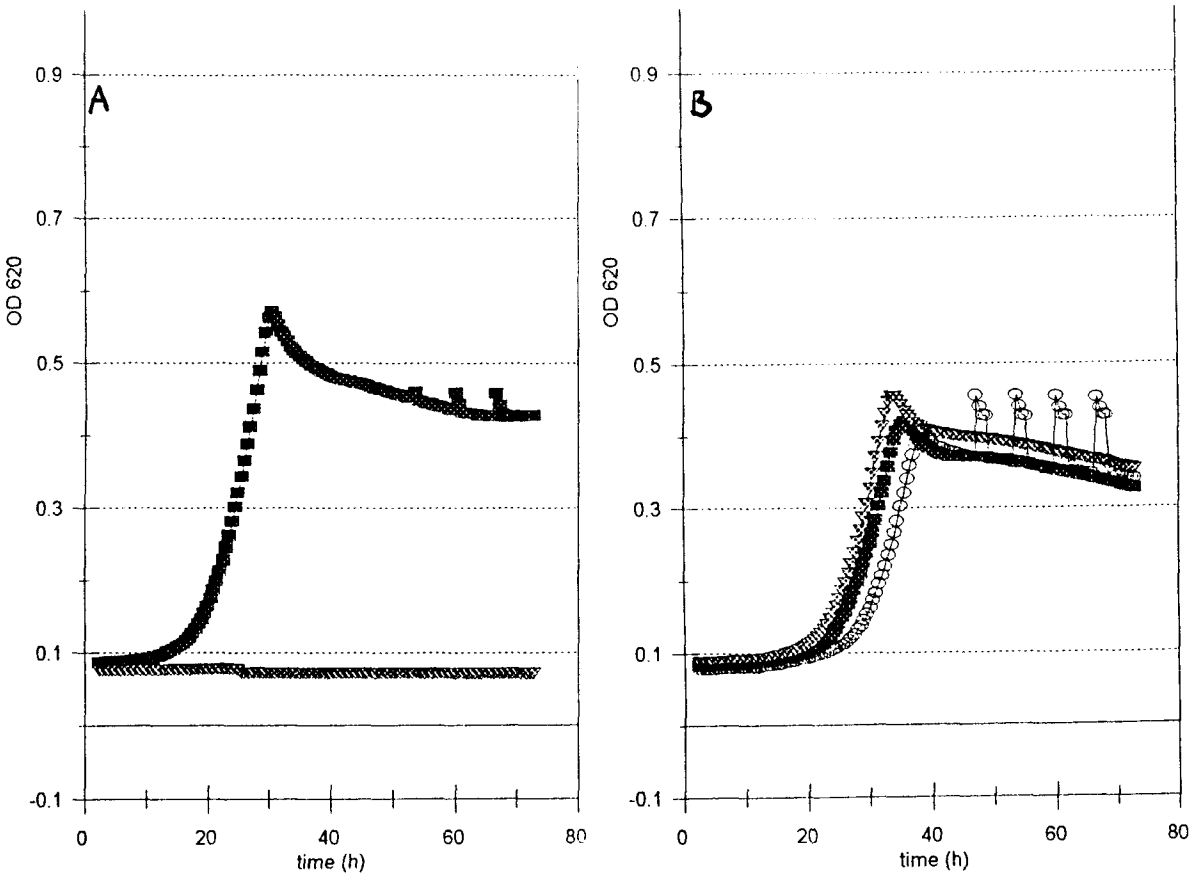
Verheul, A., F.M. Rombouts and T. Abee. 1996. "L-carnitine transport in *Listeria monocytogenes* Scott A en fysiologische stress". Supplement Ned. Tijdschr. Med. Microbiol. **4:**S24

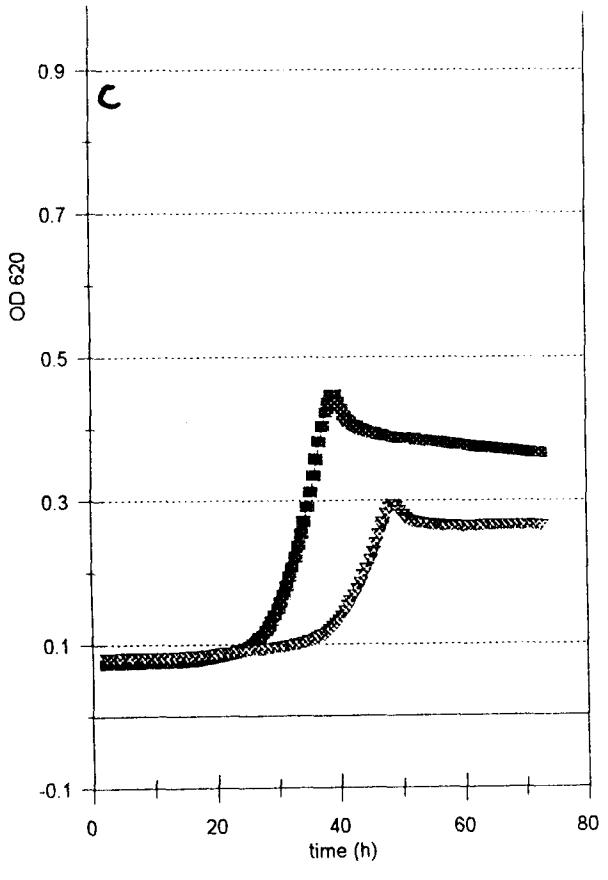
Verheul, A. and T. Abee. 1995. "A novel, ATP-dependent L-carnitine transporter in *Listeria monocytogenes* Scott A is involved in osmoregulation". Book of Abstracts, Physiology of food poisoning microorganisms, Norefjell, Norway, March 27-30 1996.

Verheul, A., N.J. Russell and T. Abee. 1995. "Effect of stress factors on membrane composition and lipid biosynthesis". Book of Abstracts, Physiology of food poisoning microorganisms, Norefjell, Norway, March 27-30 1996.

F. *Progress highlights*

In food products, *L. monocytogenes* has no direct access to amino acids essential for its growth due to unability of the pathogen to produce extracellular proteolytic enzymes. However, *L. monocytogenes* can benefit from the proteolytic activity of other microorganisms present in foods. Therefore, WAU.DFS is interested in the nutritional value of peptides for the growth of *L. monocytogenes*. The di- and tripeptide transport system of *L. monocytogenes*, recently characterized at WAU.DFS, will enable the pathogen to use small peptides as essential amino acid source. WAU.DFS now reported on the utilization of oligopeptides as a source of essential amino acids for growth of *L. monocytogenes*. The size limit for peptide utilization in *L. monocytogenes* was suggested to be 8 amino acid residues. No extracellular oligopeptide hydrolysis could be detected in the supernatant, whereas peptidase activity was significant in cell extract of *L. monocytogenes*, indicating that oligopeptides are hydrolyzed after being transported into the cell.
During the study it appeared that several oligopeptides are toxic to *L. monocytogenes*, although the basis of the mechanism of the inhibitory oligopeptides remained unclear. Several characteristics of these peptides, including their sensitivity to proteolytic breakdown have to be determined to establish whether these peptides have potential application as antimicrobial substances.





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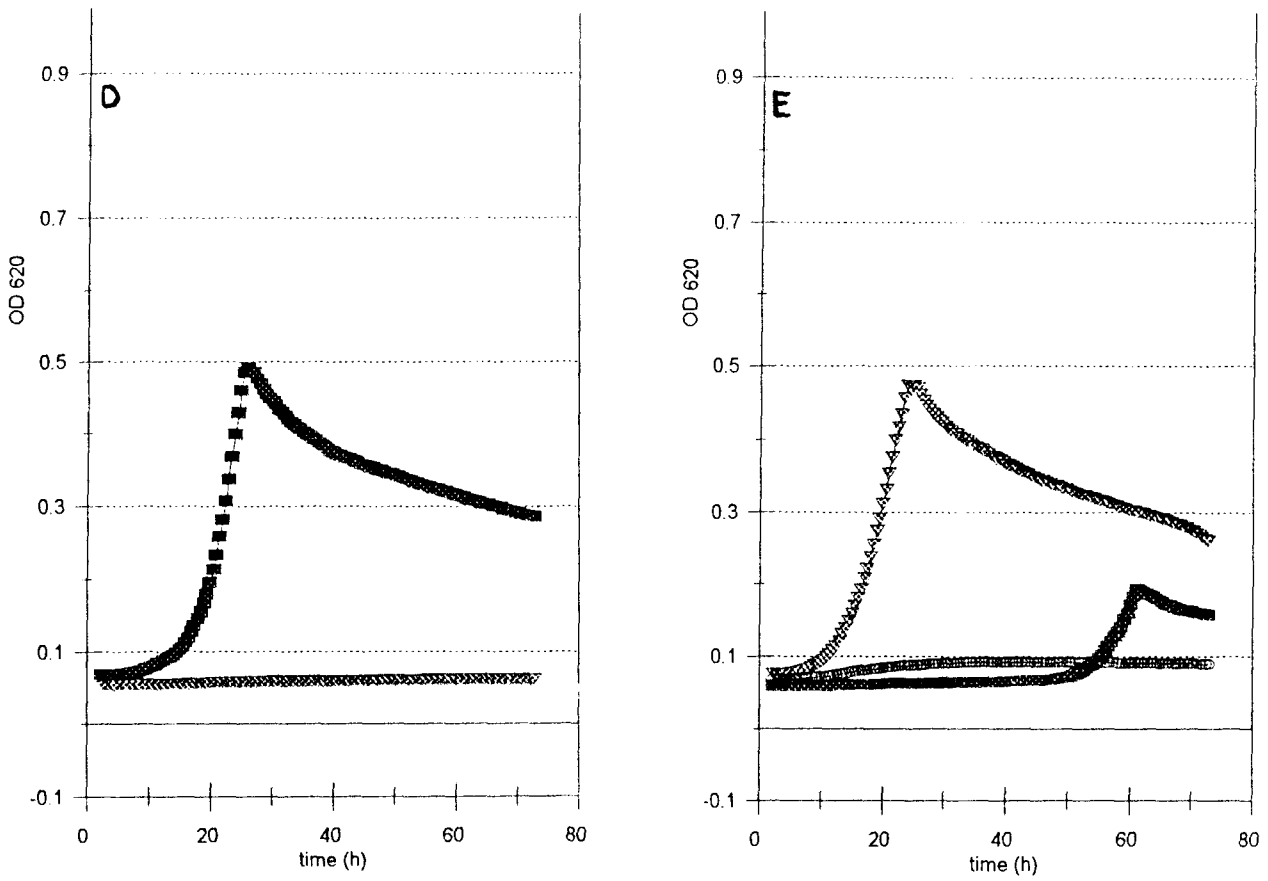

Fig. 1. Valine-containing peptides as source of the essential amino acid valine for growth of *L. monocytogenes* at 37°C. A: *L. monocytogenes* grown in DM (■) and in DM lacking valine (▽); B: *L. monocytogenes* grown in the absence of leucine but with the dipeptide Ala-Val (■), the dipeptide Val-Gly (▽) or with the tripeptide Val-Pro-Leu (○);
C: *L. monocytogenes* grown in the absence of leucine but with the hexapeptide Gly-Ala-Val-Ser-Thr-Ala (■) or with the hexapeptide Val-Gly-Gly-Ser-Glu-Ile (▽);
D: *L. monocytogenes* grown in the absence of leucine but with the heptapeptide Arg-Val-Tyr-Ile-His-Pro-Phe (■) or with the nonapeptide Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (▽);
E: *L. monocytogenes* grown in the absence of leucine but with the nonapeptide Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr (■), the octapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (▽) or with the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (○).
NOTE: OD-values have to be multiplied by a factor 1/0.6 to normalize to a pathlength of 1 cm.

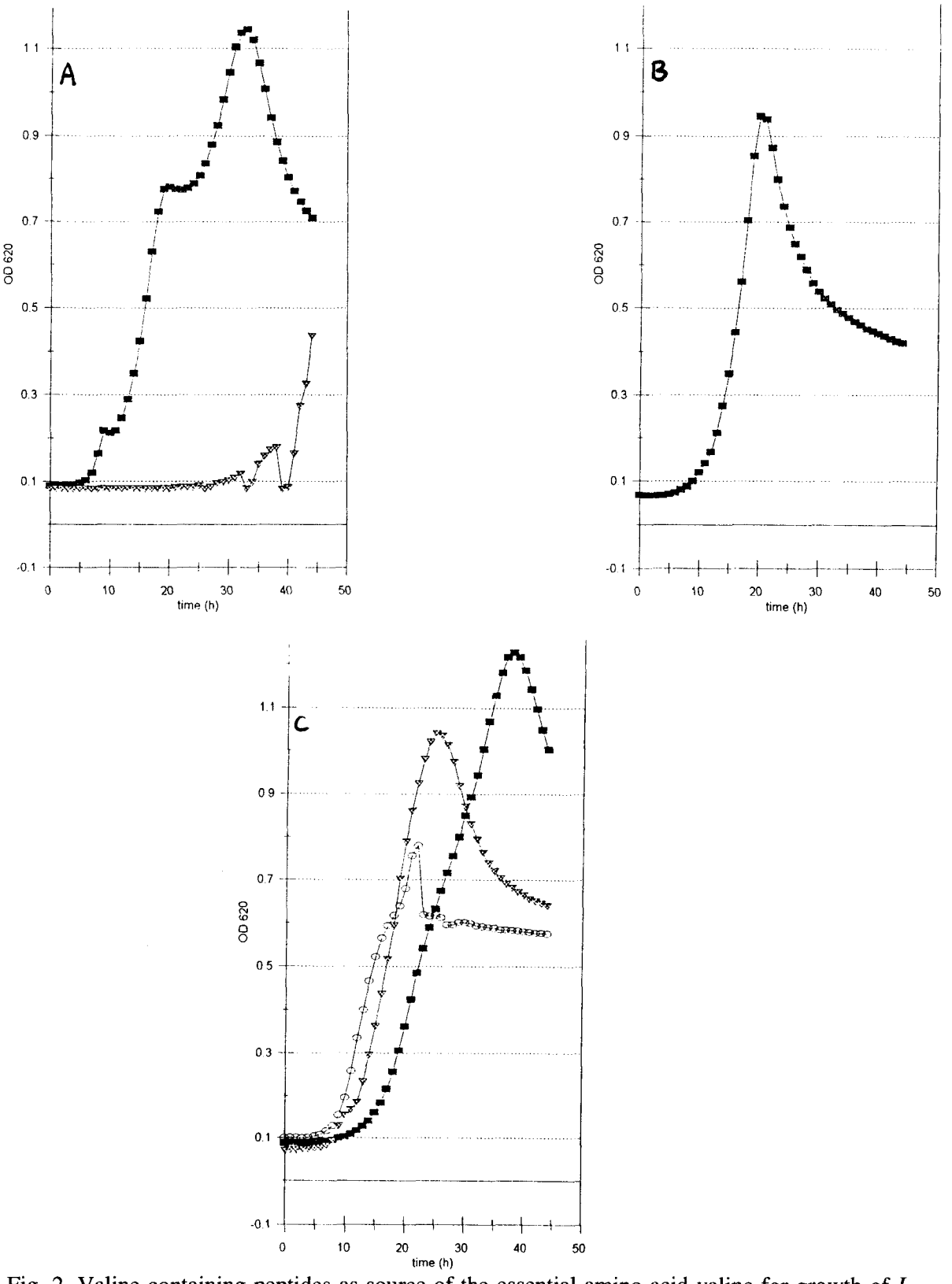

Fig. 2. Valine-containing peptides as source of the essential amino acid valine for growth of *L. monocytogenes* at 37°C. A: *L. monocytogenes* grown in DM (■) and in DM lacking valine (▽); B: *L. monocytogenes* grown in the absence of leucine but with the tripeptide Ala-Val-Leu (■); C: *L. monocytogenes* grown in the absence of leucine but with the tetrapeptide Val-Gly-Asp-Glu (■), the tetrapeptide Val-Ala-Ala-Phe (▽) or with the octapeptide Val-His-Leu-Thr-Pro-Val-Glu-Lys (○).

Table 1. Extracellular concentrations (μM) after incubation of *L. monocytogenes* at 37°C with different peptides during 0, 10 and 30 minutes.

Peptide amino acids	incubation time 0 min	10 min	30 min
Ala-Val	386	40	0
Ala	21	212	225
Val	0	50	162
Val-Gly-Asp-Glu	212	171	126
Val	0	0	8
Gly	0	0	4
Asp	0	3	0
Glu	0	0	0
Val-Leu-Ser-Gly-Gln	242	192	143
Val	0	0	61
Leu	0	26	50
Ser	0	0	11
Gly	0	35	102
Gln	0	0	0
Gly-Ala-Val-Ser-Thr-Ala	263	0	0
Gly + Thr	2	213	434
Ala	0	0	0
Val	0	0	0
Ser	0	14	27
Ala-Ala-Ala-Ala-Ala	109	52	0
Ala	0	273	574
Ala-Ala-Ala-Ala-Ala-Ala	75	not determined	27
Ala	21	not determined	221

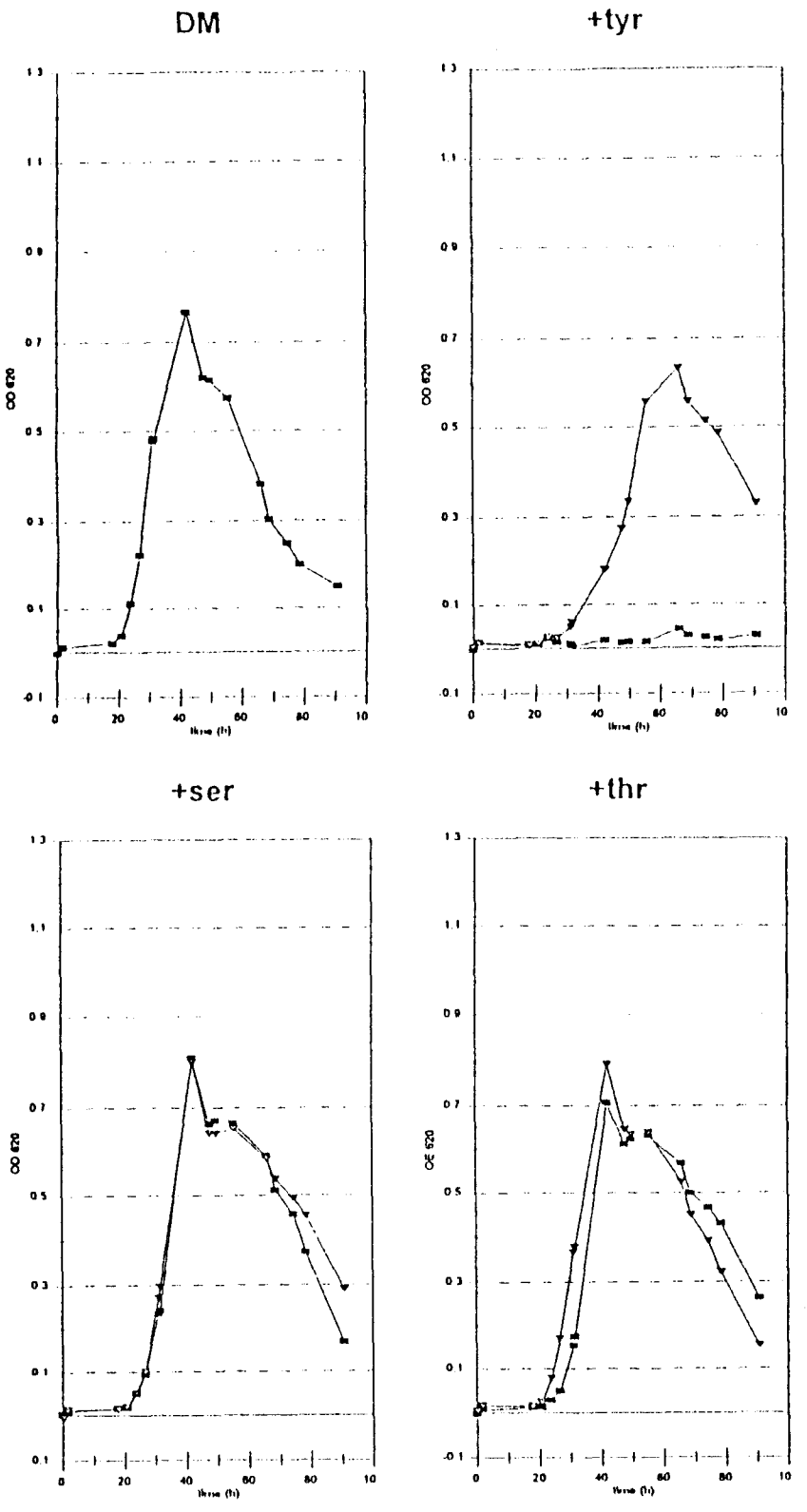

Fig. 3. Growth of *L. monocytogenes* in DM (A), DM plus tyrosine (B), DM plus serine (C), DM plus threonine (D). Amino acid and peptide were added at t=0 (■) or at t=30 h (▽).

Participant 3: Institut National de la Recherche Agronomique (INRA.AV)
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Participant 8: Les Crudettes (FRUID)
Fruidor S.A.
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France

A - 1. Sub-project title

Development of foodborne pathogens in minimally processed fresh salads: effect of storage conditions and of mild preservation techniques.

2. Project coordinator

Dr. Christophe Nguyen-the (INRA.AV)
Ing. Sylvie Le Hesran (FRUID)

3. Project participants and 4. Resources

INRA : 85 Man Months - Dr. C. Nguyen-the, Dr. F. Carlin and Dr. C. Morris (Microbiology); Dr. Varoquaux (Food Science).
FRUID : 16 Man Months (6, 1st year - 10, 2nd year) - Ing. S. Le Hesran (Quality and research development)

B - 1. Topic

Microbiological stability of fresh mildly processed vegetables stored under temperature of refrigeration. Behaviour of psychrotrophic foodborne pathogens and spoilage microorganisms on fresh salad leaves and on two industrial products which consist in mixtures of raw and cooked vegetables. Influence of storage conditions, nature of vegetables and saprophytic microorganisms. Testing mild preservation techniques on the products.

2. Goal

Determination of the various factors influencing the development of psychrotrophic foodborne pathogens on salad leaves, so as to meet the following objectives: (i) to obtain data for a prediction of the growth of foodborne pathogens in function of spoilage development (ii) to determine conditions which would minimize development of foodborne pathogens without reducing quality (iii) to estimate the risk of foodborne pathogens development in already existing industrial products. Identification of microorganisms from salad leaves with antagonistic properties against *L. monocytogenes* and characterization of this antagonisms. Reducing microbial growth to reduce spoilage and improve safety, using mild preservation techniques, in the light of the results obtained in the first part of the work.

3. Short description of the approach

Inoculation of salad leaf pieces with the psychrotrophic pathogen *Listeria monocytogenes* and measure of growth during storage under precisely controlled conditions. Number of *L. monocytogenes* will be determined on each salad leaf pieces, together with the number and the nature of epiphytic microorganisms and the importance of decay. The epiphytic microflora will be identified and tested for its ability to cause spoilage of the product. Interaction between *L. monocytogenes* and representative epiphytic microorganisms will be studied *in vitro* and *in vivo*. A similar but simplified approach will be used to study the microbial stability of the industrial products from "Les Crudettes". The use of coating to improve the microbial stability of the product will be investigated.

C - Overview of the work performed from 1-1-1996 to 1-7-1996 (INRA with CIRAD-SAR)

The work was focussed on the potential application of edible coating as a mean to limit growth of foodborne pathogens. Cooked sweet corn was used as the model food for the following reasons:

* cooked sweet corn supports a very rapid and high growth of microorganisms, including *Listeria monocytogenes*;
* cooked sweet corn is frequently incorporated in ready-to-use mixed salads, particularly a product developped by " FRUIDOR Les Crudettes " that consists in raw green endive mixed with sweet corn. In such mixed salads, the presence of sweet corn increases dramatically the growth potential of *L. monocytogenes*, as compared to raw endive alone (more information can be found in the reports from 1993 and 1994).

Therefore, to reduce the risk of *L. monocytogenes* outgrowth in the mixed salad to a level similar to that of raw endive alone, it is necessary to isolated sweet corn from the other ingredients or to specifically protect sweet corn by preservatives. Edible coatings could be a mean to achieve these objectives by creating a none visible barrier around sweet corn and by concentrating preservatives at the surface of sweet corn. In addition, cooked sweet corn is a good model to test coating on foods with a high moisture content.

Zein was selected among several formulations for its ability to form a continuous and stable coating with a nice aspect on sweet corn (period from 1-7-1995 to 31-12-1995). Zein was applied on sweet corn by pouring the zein solution (10% in ethanol) over the grains placed on a polypropylene grid. Retention of sorbic acid by zein films was shown on a model agar system, using a fungal strain as indicator. Growth of *L. monocytogenes* was measured on: sweet corn, coated sweet corn, a control treated in similar conditions that coated sweet corn but without zein, and the same samples with sorbic acid. *Listeria monocytogenes* was unable to grow on coated sweet corn and on the control, presumably because of the residue of ethanol and the desiccation caused by the coating procedure. Therefore, it had not been possible to test the effect of zein coating combined with sorbic acid to protect sweet corn.

The effect of zein coating could be tested by comparing the minimum inhibitory concentration of sorbic acid against *L. monocytogenes* on sweet corn, for sorbic acid applied alone and for sorbic acid incorporated in the coating. Ideally, coating should reduce the minimum inhibitory concentration. Therefore it is necessary to have an accurate measure of sorbic acid concentration in the sweet corn. Extraction in water, used in the work presented in the previous report, was efficient for none coated sweet corn, but not for sorbic acid incorporated in a zein film.

The main objectives of the work performed in this period were:
* to optimize extraction sorbic acid from zein films for HPLC measure of sorbic acid concentration in coated sweet corn;
* to design a coating technique which limit ethanol residue and desiccation to a level that permit a significant growth of *L. monocytogenes*;
* to measure growth of *L. monocytogenes* on sweet corn with and without zein for various sorbic acid concentration.

1 - Extraction of sorbic acid from zein films and coated sweet corn

Sorbic acid had a very low solubility in water. Extraction procedures frequently use ethanol. This is not possible in our case because ethanol would dissolve zein which might precipitate after while during the HPLC analysis, run in aqueous phase, and thereby damaging the column. We tested aqueous solutions at various pH, and ethanol/water solutions at various pH, with a ratio of Ethanol/water low enough to prevent zein dissolution.

1-1- Maximum ethanol content in the extraction solution.

Dissolution of zein film started at 50% (v/v) ethanol in water. Concentrations higher than 20% gave a very sticky texture to the zein film which tend to clog the Ultraturax blender. A solution with 18% ethanol was eventually chosen.

1-2- Selection of the extraction solution

Conditions tested were: ethanol 18% (v/v) in 0.1M buffer pH 3 to 8, 0.2M buffer at pH 3 to 8, none buffered ethanol 18 (v/v) solutions and water with pH adjusted by addition of HCl or NaOH. None buffered solutions extracted less than 60% of sorbic acid from zein films. Buffered solution at pH 8 (Tris Hcl) extracted about 80% of sorbic acid (according to the concentration of sorbic acid in the zein film). The presence of ethanol 18% did not improve extraction.

Extraction with a TrisHCl buffer, 0.1 M, pH 8, was used in the subsequent work.

2 - Optimization of the coating technique to limit ethanol content and dessication

2-1 Choice of the zein formulation

Zein is commercially available under three forms:
* Zein soluble in ethanol (used in our work so far);
* Zein soluble in ammoniac
* Zein soluble in aqueous formulations

Aqueous formulations are long and difficult to dry (according to the manufacturer) and was excluded. Ammoniac was not selected because residues could be more toxic to *L. monocytogenes* than ethanol.

2-2 Elimination of ethanol by moderate vacuum

Sweet corn was coated, dried and placed in vacuum cabinets. The atmosphere was kept humid by adding distilled water. Ethanol was evaporated at two levels of vacuum, 320mm Hg and 700 mm Hg. Before evaporation, ethanol content in sweet corn was between 6 and 8% (v/w). Even after 7 hours, less than 35% of ethanol was eliminated from sweet corn. This solution was not retained.

Elimination of ethanol could also have been done by steam, but this was not retained either because steam would have also eliminated sorbic acid (steam extraction is the AFNOR method for sorbic acid analysis in foods).

2-3 Modifications of the coating procedure

The coating procedure was modified to reduce to amount of ethanol applied on sweet corn with the zein coating. In addition, the procedure used so far (by pouring the film solution over the grain) needed a long time under hot air to properly dry the coating. Zein tends to adhere more to the grid than to sweet corn and this long drying time was necessary to remove the grains from the polypropylene grid without tearing the coating.

A first attempt was made to reduce the time needed to dry the coated sweet corn (and therefore to reduce desiccation) by selecting a support with a lower adhesion to zein. Waxes, paraffin, Teflon, polyethylene, glass, stainless steel, parafilm, were tested without success and behaved similarly to the polyethylene grid. A this stage it was decided to applied the film solution in a different way.

A classical procedure for coating is to incorporate slowly the film solution to the product rotating in a tumbler. This was not successful with zein and sweet corn because grains remained stuck together and could not be separated without tearing the coating.

Alternatively, the zein solution was sprayed onto the sweet corn placed on the polypropylene grid under a flow of laminar, sterile air. An amount of zein solution sufficient to cover the grains surface was applied on the upper side, and after drying, the grains were turned upside down to be treated again. The objectives was to prevent the accumulation of zein solution between sweet corn and the grid so as to avoid adhesion of the grains to their support, and in the same time to obtain a continuous coating. Coatings were observed after staining with commassie blue under a stereomicroscope, as described in the previous report. Three types of sprayer were tested: coarse (normally used for pesticides), medium (normally used for perfumes) and fine (laboratory " Spray-Gun " apparatus). The coarse sprayer delivered to much zein solution in a single spray and sweet corn remained stuck on the grid. The mist produced by the spray gun apparatus was too fine and the coating produced was not continuous but rather reticulated, even after 5 coating layers. The medium sprayer met the objective, but three layers were necessary to obtain a continuous coating.

2-4 The coating technique selected

For the subsequent parts of the programme, coating sweet corn with zein was done as follow: sweet corn grains were placed on the polypropylene grid; the 10% (w/v) zein solution in ethanol was sprayed until the upper side of the grains was covered, but before the solution start dripping over the grains; sweet corn was left 10 min under a sterile laminar air flow for drying; two additional layers of zein were sprayed and the same procedure applied for the other side of the grains.

The amount of ethanol in the coated sweet corn was between 2.5 and 3.5% (v/w), and the loss in fresh weight, due to desiccation during coating, was of 16% (against 40% with the procedure used in the previous report).

3 - Multiplication of *Listeria monocytogenes* on coated sweet corn

3-1- Choice of controls for zein-coated sweet corn

A proper control to test the effect of zein-coating on growth of *L. monocytogenes* should contain the same concentration in ethanol and should have undergone a similar desiccation. This latter requirement could be easily achieved by letting the control sweet corn under the laminar air-flow for the same time as the coated sweet corn. To obtain a similar ethanol content was more difficult because for similar volumes of solutions sprayed, ethanol content in the sweet corn was lower for ethanol alone than for the zein-ethanol solution (1.5 to 2 % instead of 3 to 3.5% of sweet corn fresh weight). Six successive sprays of ethanol on each side were necessary to have the same concentrations in sweet corn as in coated sweet corn (for a coating done in 3 successive sprays). After each spray of ethanol, sweet corn was dried for 5 min, instead of 10 min for zein, to obtain

the same total time under the laminar air flow. Sorbic acid can be added to ethanol and applied on sweet corn in a way similar to sorbic acid-containing coating. This control will be used to test the effect of coating containing sorbic acid, i.e. to test the retention of sorbic acid on the surface of sweet corn.

Alternatively, to test whether the barrier effect of zein against diffusion of nutrients has significantly delayed growth of *L. monocytogenes*, comparison was made between growth of *L. monocytogenes* on zein-coated sweet corn (without sorbic acid) and growth on the same batch of coated sweet corn mashed before inoculation to break the coating.

3-2- Effect of coating on growth of Listeria monocytogenes

Listeria monocytogenes Scott A was inoculated on: sweet corn, zein-coated sweet corn, zein-coated sweet corn, mashed to break the coating, and incubated at 10°C as described previously. Coating did not contained sorbic acid.

After 8 days, numbers of *L. monocytogenes* in logcfu/g of sweet corn were:
* 10.0 ± 0.0 for sweet corn,
* 7.2 ± 0.1 for zein-coated sweet corn
* 7.9 ± 0.2 for mashed coated sweet corn,
for an initial number of 4.0.

The zein-coating reduced growth of *L. monocytogenes* on sweet corn, but most of this effect was due to the ethanol content and the dessication, and not to the barrier properties of zein. The difference between coated sweet corn and mashed coated sweet corn is statistically significant, but is too low to have any practical interest. However, multiplication of *L. monocytogenes* on zein-coated sweet corn is possible with the procedure presented above, and work is under way to test the application of sorbic acid in the zein-coating. It should then be possible to conclude as to the potential interest of zein-coating to applied low concentrations of preservative on a food with high-humidity. In any case, the technology of zein coating should be improved before industrial development to avoid desiccation and the presence of ethanol residues in the product.

D - Work planned for the last term of the programme

The effect of sorbic acid in Zein coating on the growth of *L. monocytogenes* on sweet corn will be tested, by comparison with sorbic acid applied alone. In both cases, growth of *L. monocytogenes* will be measured for a range of sorbic acid concentration. The effect of coating with and without sorbic acid will be eventually tested in the mixed salad containing raw endive and sweet corn.

E. Dissemination of results

Papers

VAROQUAUX P., ALBAGNAC G., NGUYEN-THE C., VAROQUAUX F., 1995. Modified atmosphere packaging of fresh beansprouts. Journal of the Science of Food and Agriculture, 70, 224-230.

CARLIN F., NGUYEN-THE C., ABREU DA SILVA A., COCHET C., 1996. Effects of carbon dioxide on the fate of *Listeria monocytogenes*, of aerobic bacteria and on the development of spoilage in minimally processed fresh endive. International Journal of Food Microbiology, in press.

CARLIN F., NGUYEN-THE C., MORRIS C.E., 1996. The influence of the background microflora on the fate of *Listeria monocytogenes* on minimally processed fresh broad leaved endive (*Cichorium endivia* var. *latifolia*). Journal of food Protection, 59 (7); in press.

CARLIN F., PECK M.W., 1996. Growth and toxin production by non-proteolytic and proteolytic *Clostridium botulinum* in cooked vegetables at refrigeration temperatures. Applied and Environmental Microbiology, accepted for publication.

CARLIN F., PECK M.W., 1996. Metabiotic association between non-proteolytic *Clostridium botulinum* type B and foodborne *Bacillus* species. Sciences des Aliments, in press.

BENNIK, M.H.J., PEPPELENBOS, H.W., NGUYEN-THE, C., CARLIN, F., SMID, E.J., GORRIS, L.G.M., 1996. Microbiology of minimally processed, modified atmosphere packaged chicory endive. Postharvest Biology & Technoloy, accepted for publication.

Communications or posters in meetings and symposiums

CARLIN F., PECK M. W., 1996. Croissance et production de toxine par *Clostridium botulinum* non proteolytique dans une gamme de légumes cuits. Colloque de la Société Française de Microbiologie, Section Microbiologie des Aliments, 21 et 22 Mars, Paris, France.Oral presentation and proceedings.

id. previous reference. Second european sympsiun on sous vide, Leuven, April 10-12, 1996. Poster and proceedings.

CARLIN F., NGUYEN-THE C., 1996. Fate of *Listeria monocytogenes* on minimally processed fresh vegetables. Proceedings of the 15th Environmental Health Conference, Sligo (Ireland), May 8-11, 1996, pp.31-35. Text and oral presentation.

DEL CAMPO J., NGUYEN-THE C., 1996. Nature des interactions entre *Listeria monocytogenes* et la microflore saprophyte des feuilles de salade. Colloque de la Société Française de Microbiologie, Section Microbiologie des Aliments, 21 et 22 Mars, Paris, France.

F. Progress highlights

Participant 4: Centre Coopération Intern. Rech. Agronomique Dévelop.
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France

A. ***1. (Sub-)Project title:***

A) Retention of antimicrobial agents in edible films and coatings
   1. Measurement of diffusion coefficients
   2. Microbiological tests

B) Gas permeability properties of edible and biodegradable films. Influence of relative humidity and film composition on oxygen and carbon dioxide permeabilities.

2. Project coordinator:
Prof.Dr. Stéphan Guilbert

3. Project participants and 4. Resources:
Prof.Dr. S. Guilbert (responsable scientist; Dr. es Sciences); 5 man-months (m-m)
Dr. D. Dufour (scientist, Ph.D.; microbiology); 10 m-m
Dr. A.L. Wack (scientist, Ph.D.; mass transfers studies); 10 m-m
Dr. N. Gontard (scientist, Ph.D.; edible films studies); 24 m-m
N. Zakhia (scientist; microbiology and quality); 14 m-m
M.C. Lahon (technician); 14 m-m

B. ***1. Description of the research topic or practical problem:***
Ready-to-eat vegetable products are highly perishable due to product physiological and microbiological processes. Stabilization of the post-harvest/post-processing quality, to render long(er) shelf lives, now is persued by wrapping or packaging produce in plastic foils. Because this non-biodegradable material is discarded into the environment subsequent to its use, alternative, preferably biodegradable, materials should be developed. This project proposes to design these biodegradable, even edible, films or coatings on the bases of natural plant compounds such as proteins (*e.g.* gluten) or waxes. Successfull design of these biodegradable materials will reduce the load of plastics into the environment, while in the same instant making better use of the available natural resources.

2. Goal of the research:
To achieve the use of gas-barrier EPSLs or active EPSLs (including antimicrobial agents) as wrapping materials of natural origin for application with perishable, fresh or minimally processed foods in order to improve their surface microbial stability while maintaining the product quality. (joint effort of CIRAD.SAR and other participants).

3. Short description of the approach.
To develop edible protective superficial layers (EPSL)s in the form of films or coatings, possibly containing preservatives, and test their diffusion barrier properties (their watervapour, O_2, CO_2 permeability) and ability to protect ready-to-eat, chilled fruits and vegetables.

C. Overview of the work performed from 1-1-1996 to 30-6-1996

C. Overview of the work performed from 01-01-96 to 30-06-96

1- EXPLOITATION OF GAS SELECTIVITY OF WHEAT GLUTEN FILM: INFLUENCE ON QUALITY AND RESPIRATION RATE OF MUSHROOMS DURING STORAGE.

The objectives of this study were to measure permeabilities of gluten films to oxygen and carbon dioxide and to examine the effect of these films as packaging materials on the respiration rate and on the overall quality of fresh mushrooms.

Material & methods

Vegetal material

Fresh mushroom "champignon de Paris" *Agaricus Bisporus* were calibrated and selected in the bud state, and stalks were eliminated. 200 g were equilibrated at the required temperature (10 or 20°C) before experiments.

Tested films

- Wheat gluten film- Prepared as described in previous report in order to obtain 60 or 100mm thick films.
- Hydrophilic synthetic film- Pantec Osmolux films provided by Elf Atochem (Serquigny, France) were made of polyether block amide.
- Microperforated synthetic film with various perforation density and thus gas permeability were provided by Société Franco-Suisse de Façonnage du papier (Illfurt, 68 France)

All films were previously equilibrated in a 100% relative humidity atmosphere for 1 week before experiments.

Oxygen and carbon dioxide % measurement in gas.

Measurements were made by taking a 50µl sample of jar internal gas which was analysed by gas chromatography with catharometric detector. Carbon dioxide and water were separated on a Porapak Q column (Chromapack). Oxygen and nitrogen were separated on a molecular sieve.

Mushroom respiration rate measurement

Respiration rate was evaluated before the beginning of experiments and at the end of each experiment using the following procedure: 200 g of mushroom were placed in a totally hermetically closed jar equipped with a septum and stored at the desired temperature (10 or 20 °C). The evolution of internal carbon dioxide and oxygen % as a function of time were used for respiration intensity calculation:

$$RIg = Sg.\ V.\ 273.\ 10/(273+T).22.4.\ M$$

RIg is Respiration Intensity for O_2 or CO_2 in mmol. $Kg^{-1}.\ h^{-1}$; Sg is O2 consumption or CO2 production in $\%.h^{-1}$; V is the free volume in l.; T is experiment temperature in °C; M is mushroom weight in kg.
Respiration ratio is given by the ratio: $RICO_2/RIO_2$.

Mushroom storage experiments:

200 g of mushrooms were placed in a 1 liter glass jar equipped with a septum gas sampling and hermetically closed with a 50.3 cm^2 film disc. The jar was then placed in 92 % relative humidity and 10 or 20 °C ventilated chamber.
Oxygen and carbon concentration in jar atmosphere were measured each 2 hours during the first day and then two times a day for the following 5 days at 10°C, and for the following 2 days at 20 °C.

The following quality parameters were evaluated at the experiment beginning and each day:
-weight loss
- color with an Unterlab colorimeter CR20
- "Carpophore" opening with 4 stages notation: (1) in bud; (2) tight "voile", (3) teared "voile and (4) open state.

Results and discussion

Experiments are in progress

2- MODELLING OF DIFFUSION MECHANISM OF A SOLUTE INCORPORATED IN THIN LAYER FILMS PLACED ON MODEL FOOD (Fickian diffusion, one dimensional, non concentration dependent diffusion coefficient)

The use of edible protective surface layers has been proposed as one of several hurdles aiming to protect food products (Guilbert *et al.* 1988). Efficiency of such treatment has been proved in microbial studies using an high moisture model food and wheat gluten based films containing sorbic acid with *Penicilium notatum* and *Micrococcus luteus* as the challenge micro-organism (see previous report and De Regt 1995). The diffusion barrier properties of these coatings were identified as a potential means of stability improvement. Diffusion mechanism of sorbic acid incorporated in edible wheat gluten and lipid based films has been investigated in a previous study (see previous report and Redl *et al.* 1996). Fickian diffusion was identified as being the predominant mechanism of release and diffusion coefficients were determinate ranging from 7.5 10^{-12} m2/sec to 2.4 10^{-16} m^2/sec for a gluten and a beeswax film respectively. The mechanism of sorbic acid migration in model foods such as aqueous gels containing 1.5 % agar has been studied by Guilbert *et al* (1986), a diffusion coefficient of 9 10^{-10} m^2/sec was identified. This means that the diffusion coefficients of sorbic acid in model food and in possible surface layers are available. But no analytical solution of the diffusion equation of a surface layer containing a solute posed on food with a different diffusion coefficient can be found. The problem has been solved by using an explicit numerical method with finite differences. A two compartment model with symmetric conditions of giving and receiving compartment was developed (figure 1). By varying diffusion coefficient and geometry of the receiving compartment the coating of a model food with an active film can be simulated.

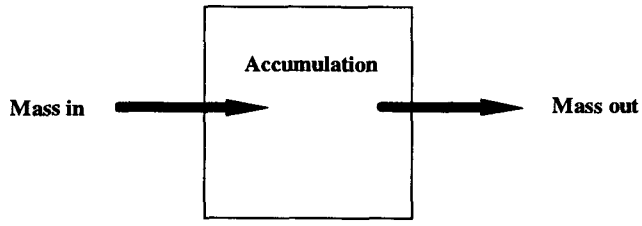
Theoretical considerations

The mathematical theory of diffusion in isotropic substances is based on the hypothesis that the rate of transfer of diffusing substance through unit area of a section is proportional to the concentration gradient normal to the section, i.e. Fick's first law (Crank 1975)

$$F = -D \frac{\partial C}{\partial x}$$

with: F the rate of transfer per unit area of section, C concentration of diffusing substance, x the space co-ordinate and D the diffusion coefficient.

By assuming that the concentration of the solute varied linearly within each interval of Dz during the short increment of time Dt, the mass balance of slice i during time variation of Dt can be written as follows:



$$0 = D_f \cdot \Delta t \cdot \frac{c_{i-1,j} - c_{i,j}}{\frac{\Delta z_{i-1}}{2} + \frac{\Delta z_i}{2}} + \left(c_{i,j-1} - c_{i,j}\right) \cdot \Delta z_i - D_f \cdot \Delta t \cdot \frac{c_{i,j} - c_{i+1,j}}{\frac{\Delta z_i}{2} + \frac{\Delta z_{i+1}}{2}}$$

with: D_f Diffusion coefficient of solute in film, D_g Diffusion coefficient of solute in model food, Dz, Dt space and time variation, c concentration of solute, j time index, i space index.

On the surface of the compartments no concentration gradient is imposed, which is the mathematical condition for zero flow across the boundary. The flow across the interface is calculated introducing an interfacial compartment with interface concentration c_g but no accumulation term. This means a system of 2n+1 equation with 2n+1 unknown variables can be established (table 1).
table 1
For each time j the concentration profile can be calculated from values of the preceding time (j-1).This is done by writing the equation system in Matrix form

$$A \cdot c_{i,j} = c_{i,j-1}$$

with: A constant matrix of the equation system

and inverting the constant matrix using a mathematics calculation program (MATHCAD 5.0 Plus).

$$c_{i,j} = A^{-1} c_{i,j-1}$$

To reduce the number of variables but nevertheless achieve sufficient accuracy the dimension of slices is getting progressively smaller close to the interface symmetrically in the two compartments
for i=1 ... n $Dz_i = a\, Dz_{i-1}$
with: a model parameter, ranging fro 0 to 1, e. g. a = 0.8

Results and discussion

A typical concentration evolution within time is shown in figure 2, simulating a gluten acetylated monoglycerides film of 100 µm thickness with a diffusion coefficient of 3.2 10^{-12} m²/sec (previous report and Redl *et al.* 1996) posed on model food of 50 mm thickness with a diffusion coefficient of 9 10^{-10} m²/sec (Guilbert *et al* 1986). It can be seen in figure 2 that interfacial concentration drops immediately below 5% of initial concentration in film. Diffusion mechanism appears to occur in two steps: in a first step (0 - 60 min) the mass transfer resistance is clearly located in the surface layer and the diffusion coefficient of the model food does not interfere significantly. This step is very close to conditions were film is immersed in a large water compartment and surface concentration can be considered as being zero. In a second step, approximately after 60 min, the diffusion resistance is located in the model food. In figure 3 the concentration of sorbic acid in model food is shown with a different scale. It appears that sorbic acid has only penetrated the first 5 mm of the 50 mm thick model food and that equilibrium is far from being reached.

Experimental studies of sorbic acid migration incorporated in a gluten beeswax film posed on 1.5% Agar gel at 4°C were conducted by Geai F., (1996). A comparison of the experimental points with theoretical modelling is shown in figure 4.
The theoretical evolution of surface concentration within time is shown in Figure 5 with similar conditions as above. Surface concentration drops below 10% of initial concentration after 1 h of depose.
In the case of a pure AM film, where diffusion coefficient was found to be 2.7 10^{-13} m²/sec, that is 3000 times lower than in model food, (figure 6) surface concentration drops below 10 % of initial concentration after about 15 hours.
In the case of a pure beeswax film, where diffusion coefficient was found to be particularly low (2.4 10^{-16} m²/sec) surface concentration remains above 50% of the initial concentration even after 180 days (figure 7).

Conclusion

Surface concentration of sorbic acid on a coated food as modelled above, due to Fickian diffusion does not match exactly with microbial results previously obtained (see previous reports) and can not explain the observed considerable retardation of microbial growth of gluten composite films.
Such a discrepancy might be explained with the experimental conditions used for microbiological tests were samples were contaminated at time zero. Sorbic acid, even if the retention is only significant for a few hours, can inhibit initial microbial growth and then retard microbial growth for a long period. Another hypothesis is that Fickian diffusion is not the only migration mechanism, but that a partition coefficient reigns the equilibrium distribution of sorbic acid in the lipid phase and the hydrophilic phase of the film or coating. A small amount of sorbic acid, highly concentrated in the lipid phase, would be very effective and could therefor explain the observed microbial results.

Literature cited

De Regt H. 1995. Stabilisation de Produits végétaux faiblement transformés à l'aide des agents antimicrobiens: étude microbiologique préliminaire sur aliment modèle. Rapport de DEA, Science des Aliments, Université de Montpellier II.

Geai F., 1996; Diffusion de l'acide sorbique dans les films comestibles à base de gluten de blé appliqués sur aliment modèle. Rapport de stage, 2ème annee d'Ingeniorat. Université de Technologie de Compiégne.

Giannakopoulos, A. and Guilbert, S. 1986a. Determination of sorbic acid diffusivity in model food gels. Journal of Food Technology. 21: 339.

Guilbert, S. 1988. Use of superficial edible layer to protect intermediate moisture foods : application to the protection of tropical fruit dehydrated by osmosis. In "Food Preservation by Moisture Control," C.C. Seow, T.T. Teng and C.H. Quah (eds.), p. 199. Elsevier Applied Science Publishers, Ltd, New York.

Redl A. Gontard N., Guilbert S. 1996. Determination of sorbic acid diffusivity in edible wheat gluten and lipid based films. Journal of Food Science. 61 (1), 116-120.

D. Description of the work planned for the subsequent part of the project

01-07-96 to 31-12-96

- Further experiments on model gel to validate the sorbic acid diffusion mathematical model
- Further experiments to evaluate the impact of wheat gluten film gas selectivity on vegetal food quality preservation during storage.
- Evaluation of ethylen permeability of wheat gluten film.

E. Dissemination

-*Zakhia, N., Gontard, N., Guilbert, S., 1994. Edible and biodegradable films to improve the shelf-life of minimally processed fruits and vegetable: Recent advances of the FLAIR EC-R-1 CT92-0125 project reaserch. "New Food Packaging" congress, 18/04/94, Copenhague.
-Guilbert, S., Gontard, N., 1994. Edible and biodegradable food packaging. International symposium "Interaction: Food / Food packaging material". 8-10/06/94, Lund, Sweeden.
-*De Savoye F., Dalle Ore F., Gontard N. and Guilbert S., 1994. Improvement of Fresh fruits and vegetables shelf-life and quality: surface retention of preservative agents using edible films and coatings. In: the international coloque "Le froid et la qualité des légumes frais", 7-9 Septembre, Brest, France.
-Gontard N., Guilbert S., 1994. Bio-Packaging : technology and properties of edible and/or biodegradable material of agricultural origin. In: "Food Packaging and Preservation", M. MATHLOUTI ed., Blackie Academic and Professional, pp 159-181.
-Cuq B., Gontard N. and Guilbert S., 1995. Edible films and coatings as active layers. In: "Active Food Packaging", M. Rooney, editor; Blackie Academic and Professional, pp 111-142.
-Coma V., Couturier Y., Pascat B., Bureau G., Guilbert S., Cuq J.L., 1994. Estimation of the biodegradability of packaging materials by screening test and a weight loss method. Packaging Technology and Science, 7 (1), 27-37.
-Gontard N., Duchez C., Cuq J.L., Guilbert S. , 1994, Edible composite films of wheat gluten and lipids: water vapor permeability and other functionnal properties., International Journal of Food Science and Technology, 29, 39-50.
-Guilbert S., Gontard N., 1994. Edible and biodegradable food packaging. In: "Interaction Food - Food Packaging Material", M. JAGERSTAD ed., Royal Society of Chemistry, London.
-Coma V., Couturier Y., Pascat B., Bureau G., Guilbert S., Cuq J.L., 1994. Biodegradability of packaging materials using a respirometric method. Packaging Technology and Science, 7 , 87-98.
-Guilbert S., Gontard N., Raoult-Wack A.L., 1995. Superficial edible films and Osmotic Dehydratation: Application of Hurdle Technology Without Affecting the Food Integrity. In: "Food Preservation by Moisture Control", J. WELTI-CHANES ed., Technomic publishing Co, USA, in press.
-Coma V., Couturier Y., Pascat B., Bureau G., Cuq J.L., Guilbert S., 1995. Estimation of the biofragmentability of packaging materials by an enzymatic method. Enzyme and Microbial Technology, in press.
-Gontard N., Marchesseau S., Cuq J.L., Guilbert S. , 1995. Water vapor permeability of edible bilayer films of wheat gluten and lipids., International Journal of Food Science and Technology, in press.
-Cuq B., Aymard C., Cuq J.L., Guilbert S., 1995. Biopackaging based on myofibrillar proteins: Study and ajustement of the film formulation and final functional properties, Journal of food science, in press.
-*Redl A., Gontard N., Guilbert S., 1995. Determination of sorbic acid diffusivity in edible wheat gluten based films. Journal of food science, in press.
-*Guilbert S., Gorris L. Gontard N., 1995. Prolongation of the shelf-life of perishable food products using biodegradable films and coatings. Lebensmittel Wissenschaft und Technologie, in press.
-Marquie C., Aymard C., Cuq J.L., Guilbert S., 1995. Biodegradable packaging made from cottonseed flour: Formation and improvement by chemical treatments with gossypol, formaldehyde and glutaraldehyde. Journal of agricultural food chemistry, submited 03/95.
-Cuq B., Gontard N., Cuq J.L., Guilbert S., 1995. Fonctional properties of myofibrillar protein-based biopackagings: Influence of the film thickness. Journal of food science, submited 04/95.
-*Gontard N., Thibault R., Cuq B., Guilbert S., 1995. Influence of relative humidity and film composition on O_2 and CO_2 permeability of edible films. Journal of agricultural and food chemistry, in press.
-Guilbert S., 1995. Biopackaging : Tecnologia y Propiedades de Polimeros Biodegradables. International workshop "Polimeros Biodegradables, Avances y Perspectivas", Buenos-Aires, 25-27 april.
-Guilbert S., 1995. Films de proteinas. International workshop "Polimeros Biodegradables, Avances y Perspectivas", Buenos-Aires 25-27 april

-Gontard N., 1995. Films de gluten. International workshop "Polimeros Biodegradables, Avances y Perspectivas", Buenos-Aires 25-27 april
-Guilbert S., 1995. Propiedades y aplicaciones de "edible films". International workshop "Polimeros Biodegradables, Avances y Perspectivas", Buenos-Aires 25-27 april
-Gontard N., 1995. Filmes proteicos : obtençao, propriedades e aplicaçoes. International symposium "Proteinas em alimentos", Sao-Paulo 18-20 april
-Guilbert S., 1995. Texturizaçao e filmes protéicos : estagio atual. International symposium "Proteinas em alimentos", Sao-Paulo 18-20 april
-Guilbert S., Gontard N., 1995. Technology and applications of edible protective films. International symposium "New shelf life technologies and safety assessments", Helsinki, 18-19 may
- *Mujica Paz H., Gontard N. 1996. Oxygen and carbon dioxide permeability of wheat gluten film: effect of relative humidity and temperature. Submitted at Journal of Agricultural and Food Chemistry.

* *research directly supported by the project*

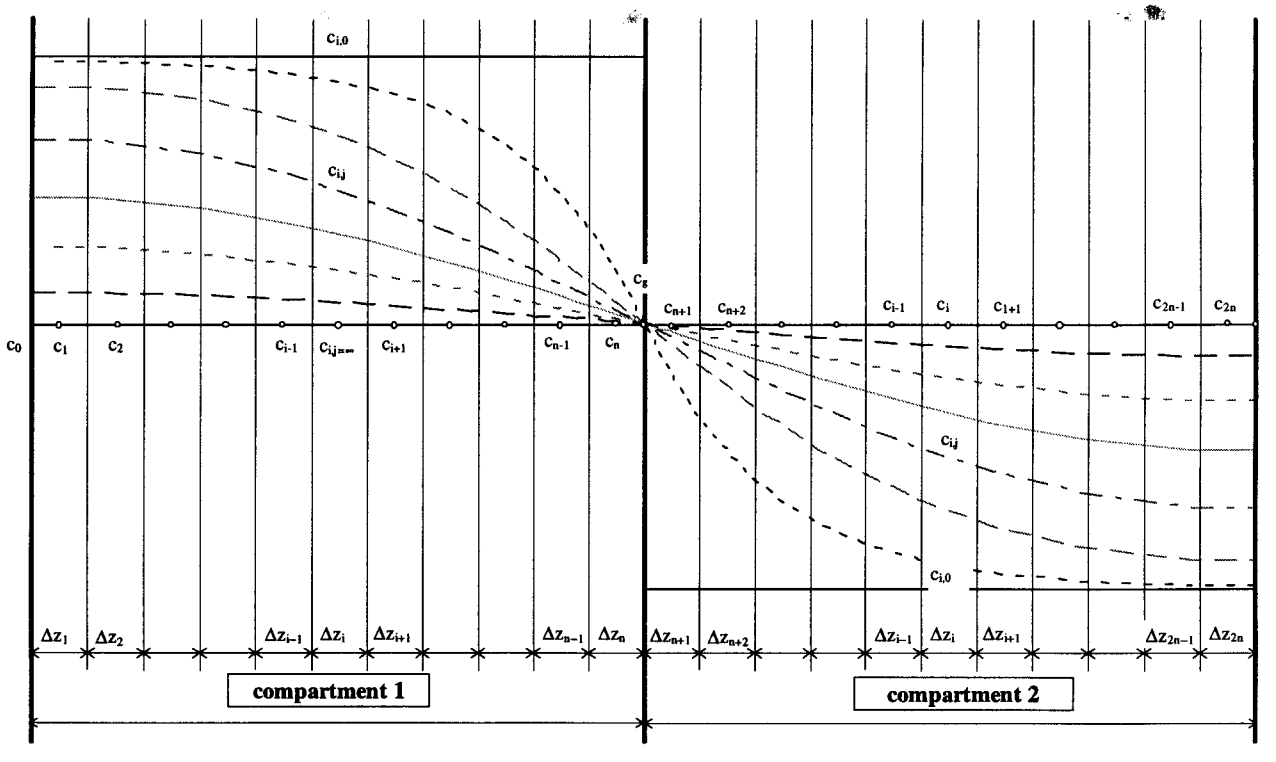


Figure 1. Two compartment model of Fickian Diffusion. with: Δz space variation, c concentration of solute, j time index, i space index.

Table 1. Equation system of Fickian diffusion in a two compartment model; with: D_f Diffusion coefficient of solute in film, D_g Diffusion coefficient of solute in model food, Δz, Δt space and time variation, c concentration of solute, j time index, i space index.

	Mass in	accumulation terme	Mass out	
i=1		$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$	$-D_f\cdot\Lambda t\cdot\frac{c_{1,j}-c_{2,j}}{\frac{\Lambda z_1}{2}+\frac{\Lambda z_2}{2}}$	=0
i=2.... n-1	$D_f\cdot\Lambda t\cdot\frac{c_{i-1,j}-c_{i,j}}{\frac{\Lambda z_{i-1}}{2}+\frac{\Lambda z_i}{2}}$	$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$	$-D_f\cdot\Lambda t\cdot\frac{c_{i,j}-c_{i+1,j}}{\frac{\Lambda z_i}{2}+\frac{\Lambda z_{i+1}}{2}}$	=0
i = n	$D_f\cdot\Lambda t\cdot\frac{c_{n-1,j}-c_{n,j}}{\frac{\Lambda z_{n-1}}{2}+\frac{\Lambda z_n}{2}}$	$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$	$-D_f\cdot\Lambda t\cdot\frac{c_{n,j}-c_{g,j}}{\frac{\Lambda z_n}{2}}$	=0
interface	$D_f\cdot\Lambda t\cdot\frac{c_{n,j}-c_{g,j}}{\frac{\Lambda z_n}{2}}$		$-D_g\cdot\Lambda t\cdot\frac{c_{g,j}-c_{n+1,j}}{\frac{\Lambda z_{n+1}}{2}}$	=0
i= n+1	$D_g\cdot\Lambda t\cdot\frac{c_{g,j}-c_{n+1,j}}{\frac{\Lambda z_{n+1}}{2}}$	$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$	$-D_g\cdot\Lambda t\cdot\frac{c_{n+1,j}-c_{n+2,j}}{\frac{\Lambda z_{n+1}}{2}+\frac{\Lambda z_{n+2}}{2}}$	=0
i= n+2.... .2n-1	$D_g\cdot\Lambda t\cdot\frac{c_{i-1,j}-c_{i,j}}{\frac{\Lambda z_{i-1}}{2}+\frac{\Lambda z_i}{2}}$	$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$	$-D_g\cdot\Lambda t\cdot\frac{c_{i,j}-c_{i+1,j}}{\frac{\Lambda z_i}{2}+\frac{\Lambda z_{i+1}}{2}}$	=0
i=2n	$D_g\cdot\Lambda t\cdot\frac{c_{i-1,j}-c_{i,j}}{\frac{\Lambda z_{i-1}}{2}+\frac{\Lambda z_i}{2}}$	$+\left(c_{i,j-1}-c_{i,j}\right)\cdot\Lambda z_i$		=0

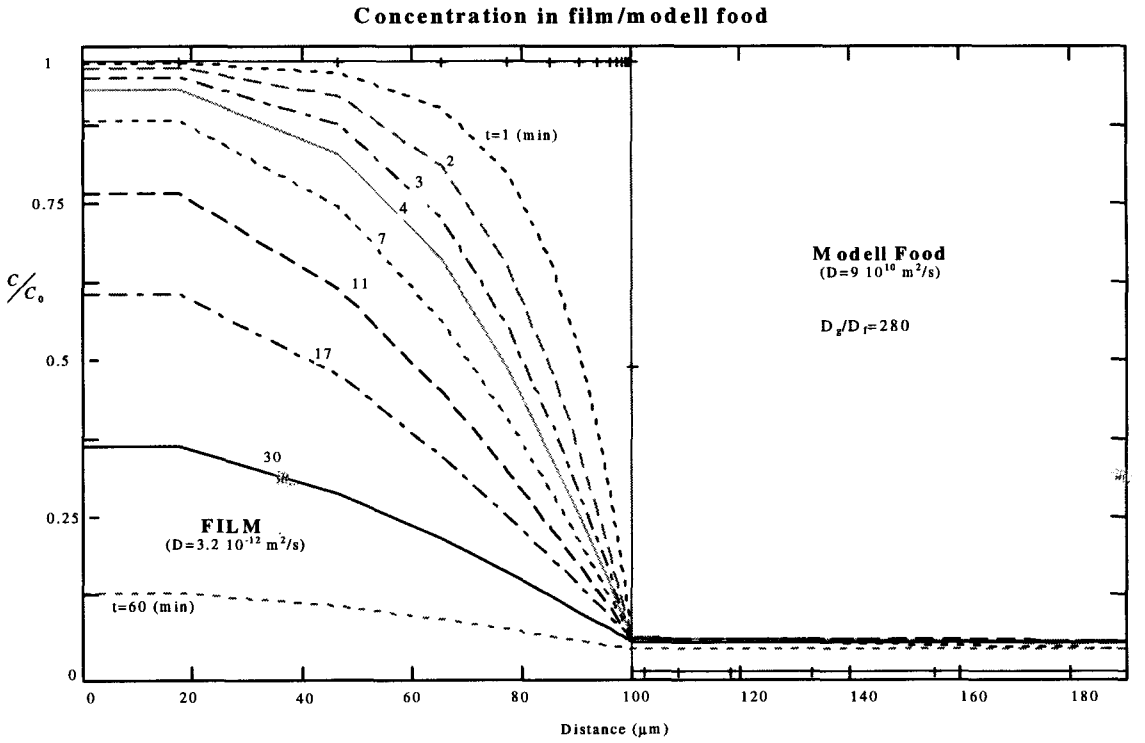


Figure 2. Theoretical evolution of concentration profile of sorbic acid in surface layer and model Food. Case of a gluten -Actylated monoglyceryde film placed on Agar gel. D_f... Diffusion coefficient of sorbic acid in film D_g... Diffusion coefficient of sorbic acid in gel

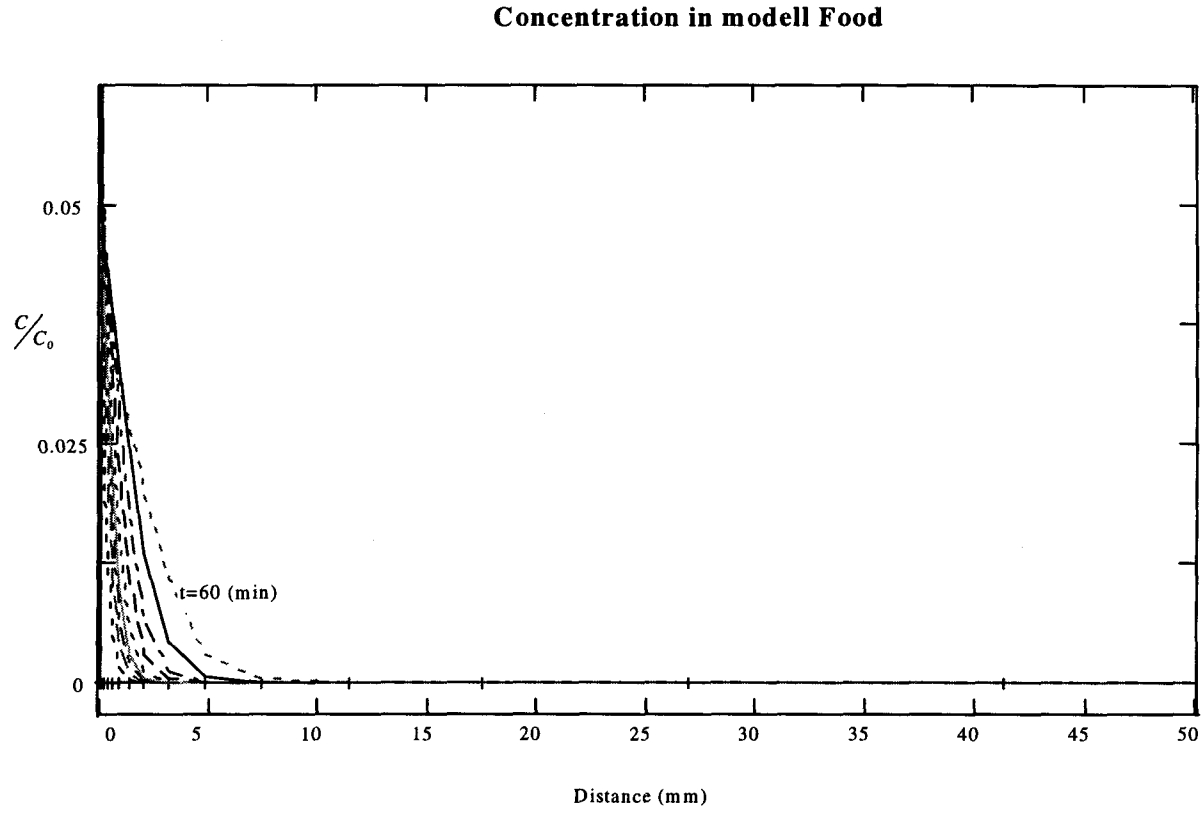


Figure 3. Theoretical evolution of concentration profile of sorbic acid in model Food. Case of a gluten -Actylated monoglyceryde film placed on 1.5% Agar gel.

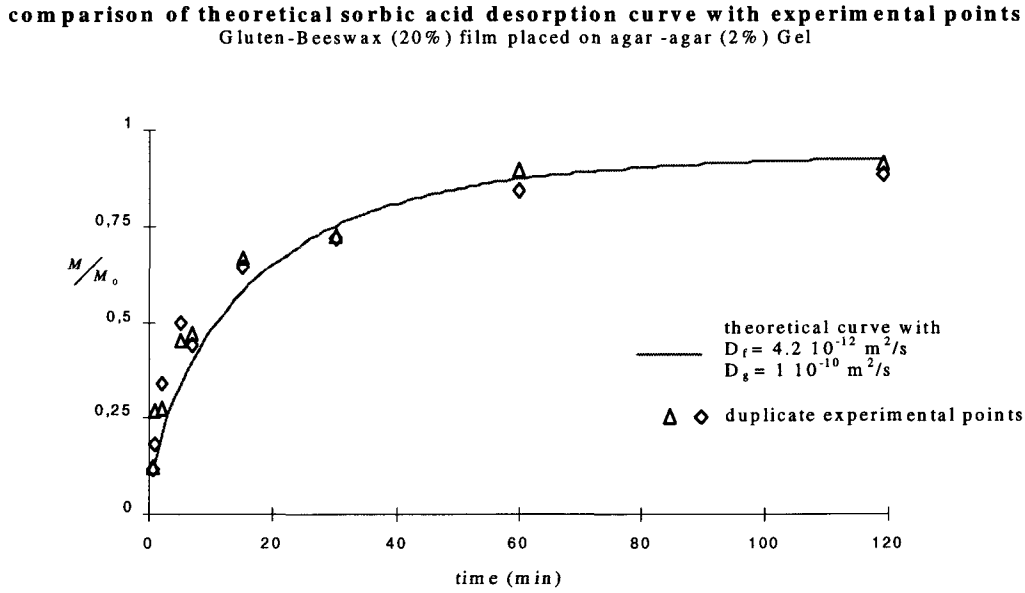


Figure 4 Comparison of theoretical desorption curve with experimental points. D_f... Diffusion coefficient of sorbic acid in film D_g... Diffusion coefficient of sorbic acid in gel M... Mass of sorbic acid diffused into model food M_0...Mass of sorbic acid incorporated in film (M_0=0.04 mg/cm^2)

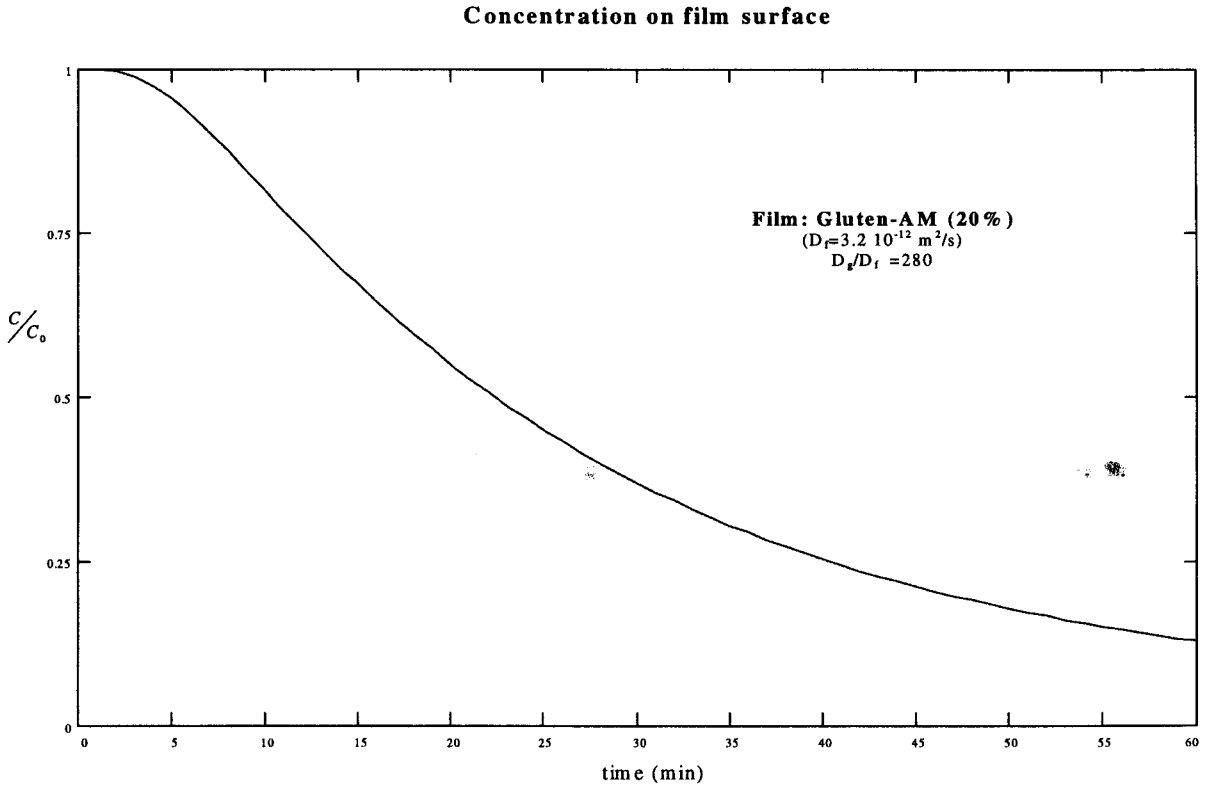


Figure 5 Evolution of surface concentration of sorbic acid incorporated in a Gluten Actylated monoglyceryde v film placed on 1.5% agar gel within time

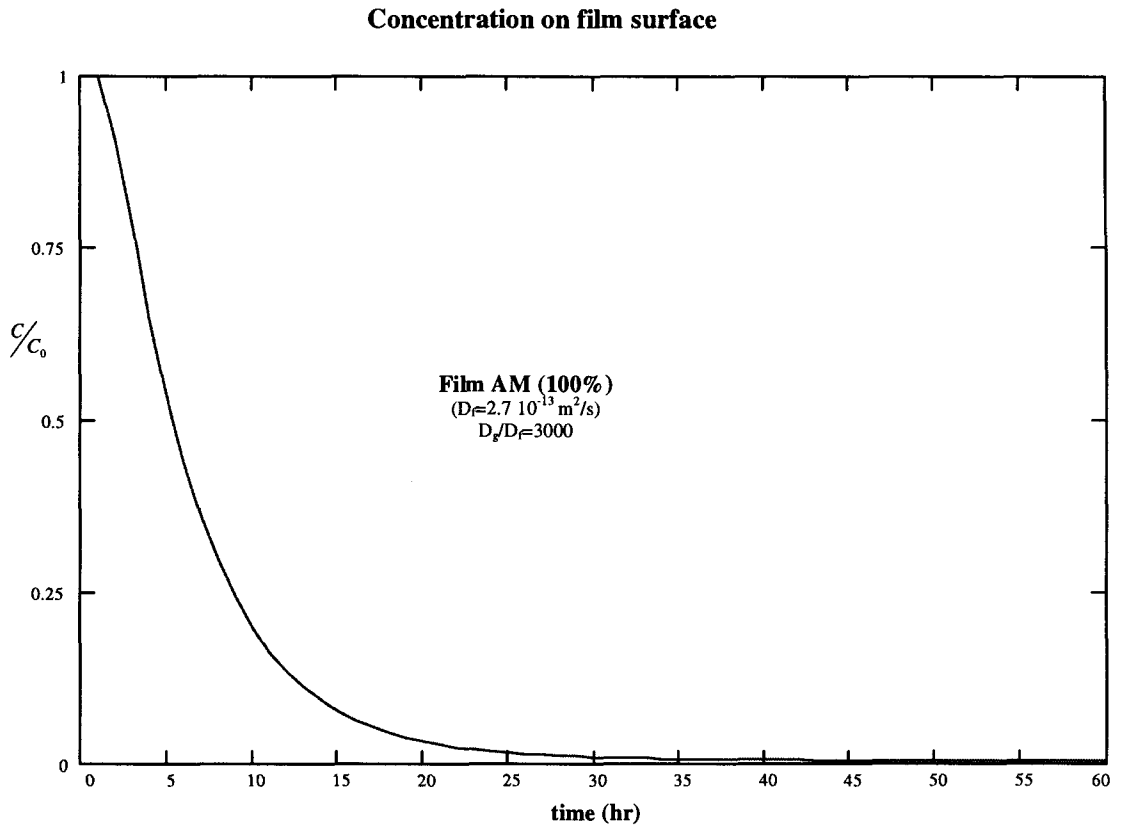


Figure 6. Evolution of surface concentration of sorbic acid incorporated in a Actylated monoglyceryde film placed on 1.5% agar gel within time

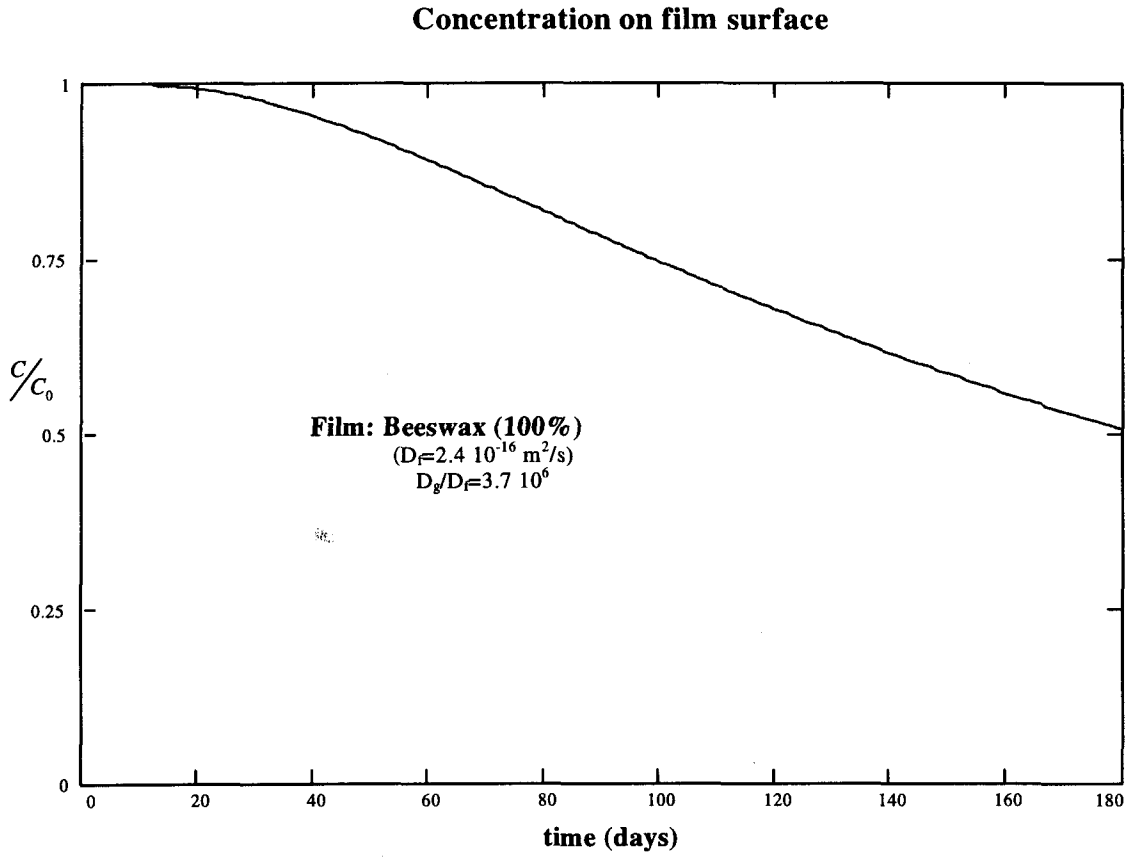


Figure 7. Evolution of surface concentration of sorbic acid incorporated in a beeswax film placed on 1.5% agar gel within time

6

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Resources
18man-months/year

PART A:

(Sub-)Project titles:

1. Effect of gaseous atmospheres on the food pathogens (*Salmonella enteritidis*) and microbial composition of sliced tomato salad [task 3, 4 and 5]

2. Correlation of the physico-chemical and organoleptic changes in ready to eat shredded and sliced carrots [task 6]

3. Development and evaluation of different model system (carrot and tomato juice) for the study (growth, modelling) of food spoilage and pathogenic bacteria (Task 10)

PART B:

b1. Description of the research topic

It is well known that the visual appearance and shelf - life of many Minimally Processed Fresh (MPF) vegetables such as carrots, chicory, broccoli (Barth *et al.* 1993; Gorris 1994) could be enhanced with the use of vacuum or modified atmosphere packaging (VP/MAP).
The quality of these products is better maintained under moderately high carbon dioxide concentration (5-20%) and moderately low oxygen concentration (2-5%) (Nguyen-the & Carlin 1994). With this man-made food ecosystem the prevention of spoilage of these products by Gram negative organisms such as pseudomonads or Enterobacteriaceae is well established (Nguyen-the & Carlin 1994). Surprisingly, although the microbiological changes on VP/MAP ready to eat vegetables are well established, the chemical changes accompanying the growth of bacteria on these products during storage, either aerobically or anaerobically, are not equally investigated. For instance it has been shown (Carlin *et al.*, 1989,1990) that the spoilage of retail packs of fresh

carrots is the result of metabolic products, such as lactic acid and acetic acid, of the dominant lactic acid bacteria (Carlin *et al.* 1989, 1990). However the type of their metabolites as well as the rate of formation depend upon environmental conditions (Marshall 1992). This is the case with other foods (cheese, meat, fish) stored under VP/MAP where lactic acid bacteria are also dominant organisms (Drosinos 1994; Kakouri & Nychas 1994; Kakouri *et al.*, 1996). Concern has been expressed, however, by regulatory authorities, food industry groups (Anon 1988) that MAP may represent an undue safety hazard. Such concern for safety is pertinent in the light of increased evidence of survival and growth of pathogenic bacteria at refrigeration temperature (Palumbo 1987).

For this reason

- sliced tomatoes were inoculated with *Salmonella enteritidis*. These products were stored aerobically and on map (5% CO_2/ 95% N_2) and the microbiological as well as the physicochemical profile was investigated.

- the microbiological data obtained so far, from stored carrots, were modelled with the modified Gompertz equation [logN=A+C*exp[-exp(BM-B*X)]

- factors such as sampling period, packing were studied (analysis of variance and principal components analysis)

- a medium was developed able to be used for the study of survival/growth of pathogens (eg. *Salmonella* spp., *Listeria* spp.). This conductance method will allow us to model the growth of these two pathogens

b2. Goal of the research

The goal of this part of the project is

- to evaluate the effect of gaseous composition on the pathogenic flora (*Salmonella enteritidis*), on ready to eat salads (sliced tomatoes).
- to correlate the physicochemical changes with organoleptic characteristics on shredded carrots.
- to study the effect of different gaseous atmospheres on the maximum specific growth of spoilage organisms grown on shredded carrots
- to 'create' a medium relative equal to vegetables in order to investigate the growth (using an impedance instrument) of specific spoilage and pathogenic bacteria

b3. Short description of the approach

Fresh tomatoes were inoculated with *S enteritidis* with two different inoculum sizes and stored under map conditions at 4 and 10°C. The results from our previous experiment with carrots stored at 4 and 10° were modelled using the modified equation of Gompertz.
Efforts were made for developing the most appropriate medium for Salmonella in order to model the growth this organism.

PART C:

OVERVIEW OF THE WORK PERFORMED FROM 1-1-1995 to 31-5-1995

- Fresh sliced tomatoes flushed with the following mixtures of gases a. air, ; b. 5% CO_2/95% N_2 Inoculated with *Salmonella enteritidis,* (two different inoculum sizes were used) and stored at 4 and 10°C

- Sterile or non sterile carrot juice was used in combination with various carbohydrates and amino acids or casein hydrolysate media inoculated with *Salmonella enteritidis*

DETAILED DESCRIPTION OF THE WORK DONE IN THE LAST SIX MONTHS

Materials and Methods

1. Microbiological analysis

sample preparation (survey)

Fresh tomatoes bought from a local market. The samples were transported to our laboratory within 30 minutes. On their arrival, the product were examined microbiologically as follows; Samples (25g) of products were weighed out aseptically, sterile quarter strength Ringer's solution (225 ml) added and mixed with a stomacher (Lab Blender 400, Seward Medical, London) for 60s at room temperature. Decimal dilutions in ¼ strength Ringer's solution were prepared and duplicate 1 ml or 0.1 ml samples of appropriate dilutions mixed or spread on the following agar media for the isolation of particular groups of bacteria or yeasts: on Plate Count Agar (PCA; Oxoid) for total viable count (TVC), incubated at 25° C for 72h; on MRS pH 6.2 (Oxoid) for lactic acid bacteria, overlayed with the same medium and incubated at 25°C for 96h under anaerobic

Enumeration of pathogens

Salmonella enteritidis were enumerated with xylose lysine decarboxylase agar. Incubation was at 37°C for 24 h. Culture media and selective supplements were supplied by Oxoid, UK.

Studies in food model system

Sterile and non sterile fresh carrot juice. Inoculation with Salmonella enteritidis was followed.

Bacterial Strain

A strain of *Salmonella enteritidis* PT4 was provided by Professor R.G. Board (University of Bath, UK). It was maintained on slopes of nutrient agar (Oxoid, Hampshire, UK) at 4°C and subcultured weekly to prepare an inoculum. An overnight culture was grown in 50 ml nutrient broth (Oxoid) at 37° C, harvested by centrifugation, washed twice with saline and resuspended to give a cell density of 10^9 cell/ml.

Growth media

Fresh carrot juice (Vita mat, Rotor AGCH-3138, Vetendort, Swiss) non sterile or sterilised by

autoclaving was used in combination with NZA, SPYE, L-cysteine, L-lysine, arginine and glycerol to obtained a good growth of *Salmonella*. Partion of the fresh juice was centrifuged also. The supernatant was used either sterile or not in combination with the above mentioned constituents. This experimentation was very basic and important in order to achieved a smooth and clear curve growth of *Salmonella*.

Experimental procedure

Broths were dispensed (2.5ml final volume) into sterile closed glass reaction cells. The broths were added to the Malthus (Radiometer International, Copenhagen, Denmark) reaction cells. Reaction cells were pre-incubated at 37°C for 1 h before inoculation in order to reach the experimental temperature. The cells were then inoculated with 0.1ml of an overnight culture of *Salm. enteritidis* at 37° C in the growth media and incubated statically in the water bath of a Malthus 2000 system maintained at 37°C. Changes in the conductance of the medium during growth were monitored every six minutes by the Malthus 2000.

Growth monitoring

Bacterial growth was monitored by conductance measurements using the Malthus 2000 instrument. This system detects changes in conductance caused by bacterial metabolism in the growth medium. The analyser monitors conductance changes every 6 min and records the data. Changes are expressed in micro Siemens (μS) and shown graphically as conductance curves. The conductance detection time (DT), expressed in hours, reflects microbial metabolism also. It is defined as the time interval between the start of conductance monitoring and the beginning of the acceleration phase of the signal.

2. Physico-chemical analysis

Sample preparation

After the microbiological examination a liquid portion (20ml) was filtered and the clear filtrate, after the addition of TriFluoroAcetic acid (TFA), stored at -80°C and then used for HPLC analysis.

HPLC analysis of L-*lactic and acetic acid.*

The profile of the organic acids (treated with TFA) of uninoculated and inoculated tomatoes were analysed in a Spectra Physics High Performance Liquid Chromatography consisting of a Spectra Physics P2000 two pump system, with a Rheodyne 7125 injector (fitted with a 20 μl loop), connected with a Spectra Focus UV/VIS detector using Low Inertia Scanning Technology (similar to Photodiode array), supplemented with the appropriate Spectra focus software running in IBM 80386 OS/2 computer. The column used was a 300 x 7.8 mm Aminex HPX-87H 5μm. The compounds were separated isocratically with buffer (0.009N H_2SO_4 in distilled water). Peak width was 12, peak threshold 600 and 0.034 AUFS. The whole spectra (190-330) of the chromatograms was analysed with the above mentioned software.

Results, Discussion and Conclusions

Microbiological Changes

The results obtained from the microbiological analysis of tomato are shown in Fig 42.1 and 42.2 for 10° and 4°C respectively.

The main conclusion drwan from the above mentioned experiement is that *Salmonella enteritidis* survive but did not grow at 10°C while at 4°the decrease of the population of this organism was evident (especially when samples inoculated with relative high loads of *S enteritidis*).This could be occurred either due to the inhibitory action of map or due to decrease of pH (Figs 42.1c & 42.2c). The last one could be also due to the production of lactic acid and acetic acid as can be seen in the section of physicochemical results. In general the combined action of map, lactic acid metabolims and temperature can consider to contribute significantly the inhibition of Salmonella.

Legends

Fig. 42.1 Changes in the pH (c, air,□; map, ○) total viable counts (a,b,□) lactic acid bacteria (a, b,○) *S. enteritidis* with low (a, b,✱) or high (a, b,●) inoculum of fresh tomatoes, stored at 10°C under air (a), and 5% CO_2 / 95% N_2 (b) gaseous conditions.

Fig. 42.2 Changes in the pH (c, air,□; map,○) total viable counts (a ,b,□),) lactic acid bacteria (a, b, ○) *S. enteritidis* with low (a, b,✱) or high (a, b, ●) inoculum of fresh tomatoes, stored at 10°C under air (a), and 5% CO_2 /95% N_2 (b) .

b. Carrots

The microbiological analysis of carrots revealed that in these products the most important organisms were the lactic acid bacteria, pseudomonads and yeasts, regardless the storage conditions used. This is the main conclusion from Figs 42.4, 42.5 and Tables 42.1 & 42.2, which furhermore summarises the maxiumum specific growth in total viable count, lactic acid bacteria, and pseudomonads during storage of refrigerated (4° C) or temperature abused (10° C) shredded carrots in air, 4.9% CO_2/ 2.1% O_2/ 93% N_2 and 5% CO_2 / 95% N_2. It is well known that there is a CO_2-dependent retardation at chill temperatures (Eklund & Jarmund, 1983). This was also the case in our studies where the microbiological counts increasing albeit slowly, during storage. Indeed, in our experiment performed on carrots the size of total viable counts reached its greatest value after 17 days in samples stored under 4°C while it needed only 6 days when these samples stored at 10°C. Lactic acid bacteria were the dominant organisms at 10°C regardless the packaging system used after 6 days of storage. It needs to be stressed that in samples stored at 4°C under 4.9% CO_2/ 2.1% O_2/ 93% N_2 and 5% CO_2 / 95% N_2, the population of lactic acid bacteria were found to be the highest among counts on cfc and yeasts although they present only 10% to 1% othe total viable counts.

Pseudomonads and yeasts have been reported to be members of the microbial flora of minimally processed vegetables (Nguyen-the & Prunier 1989; Carlin *et al.*, 1989; Torriani & Massa, 1994; Albrecht *et al.*, 1995). However the role of pseudomonads in the spoilage of shredded carrots has been under dispute (Carlin *et al.*, 1989). In this study although counts on cfc medium were members of the initial flora of shredded carrots, their contribution to the final flora was always significantly as the lactic acid bacteria.

Fig 42.3 The growth of Total Viable count (dash-dot-dotted line), Lactic acid bacteria (solid line) and pseudomonads (dashed line) on carrots stored with two different films (film 1: a,b and film 2: c,d) under aerobic (a,c) or 5%CO_2/95% N_2 (b,d) at 4°C

Fig. 42.4 The growth of Total Viable count (□) Lactic acid bacteria (○) and pseudomonads (▲) on shredded carrots stored under different storage conditions at 4°C.

Fig. 42.5 The growth of Total Viable count (□) Lactic acid bacteria (○) and pseudomonads (▲) on shredded carrots stored under different storage conditions at 10°C.

Table 42.1 The maximum specific growth (μ maximum) of Total Viable Counts, Lactic acid bacteria and pseudomonads grown on carrots under different storage conditions at 4°c.

T°	map	TVC	LAB	PSE
4	AIR	0.706	0.387	0.387
	5%: 5.2%: 89.8% CO_2 :O_2: N_2	0.348	0.367	0.256
	5%: 95% CO_2:N_2	0.342	0.628	0.269
4	AIR	0.982	0.266	0.825
	5%: 95% CO_2:N_2	0.765	0.721	0.501
4	AIR	0.653	0.589	0.625
	5%: 95% CO_2:N_2	0.847	0.587	0.763
4	AIR	0.540	0.391	0.406
	4.9%: 2.1%:93% CO_2 :O_2: N_2	0.318	0.290	0.272
	5%: 95% CO_2:N_2	0.330	0.410	0.224

Table 42.2 The maximum specific growth (μ maximum) of Total Viable Counts, Lactic acid bacteria and pseudomonads grown on carrots under different storage conditions at 10°c.

T°	map	TVC	LAB	PSE
10	AIR	1.02	2.19	-
	20%/80% CO_2:O_2	1.41	2.669	-
	100% CO_2	6.47	14.97	-
10	AIR	0.987	0.781	0.708
	4.9%: 2.1 %: 93% CO_2 :O_2: N_2	0.884	1.230	0.336
	5%: 95% CO_2:N_2	0.572	1.38	0.470
10	AIR	0.880	0.361	0.981
	5%: 95% CO_2:N_2	0.491	0.400	1.187
10	AIR	0.547	0.304	0.711
	5%: 95% CO_2:N_2	0.691	0.229	0.808

In most cases the maximum specific growth of lactic acid bacteria, at low temperature regardless the packaging system used, was greater than those of pseudomonads. Results obtained from the storage at 10°C were contradictory. In general it was found that there was a great and significant differences among carrots treated similarly. Many factors could be accoutn for these differences. One could be consider that is due to the fact that the modified Gompertz equation is not the appropriate one. The different initial level, the contribution of specific flora etc. For this reason a model food system should be used alternatively in order to evaluate and to validate the results obtained from the real food.

The analysis of variance between types of and sampling day (1st and last day of storage) revealed that there was a significant effect on the counts of microbial flora as indicated in Table 42.3

Table 42.3 The effect (P- probability level) of packaging system and the sampling day on the Total viable count (TVC), lactic acid bacteria (LAB), pseudomonads (PSE), acetic acid (AA), lactic acid (LA), and citric acid (CA)

FACTOR	TVC	P	LAB	P	PSE	P
pack (a)	5.27	0.001	3.488	0.01	1.140	0.341
sampling (b)	53.47	0.000	34.629	0.0000	4.790	0.030
a * b	5.28	0.001	3.488	0.010	1.134	0.343

FACTOR	AA	P	LA	P	CA	P
pack (a)	1.579	0.184	9.807	0.000	8.625	0.000
sampling (b)	413.8	0.000	1328.9	0.000	130.5	0.000
a * b	1.58	0.183	9.523	0.000	0.490	0.743

When the Principal Components analysis was performed using TVC, PSE, LAB, AA, LA, CA, T (temperature) and pack as factors, it was revealed that the acids were the most important factors that

Table 42.4 Matrix to be factored

	tvc	pse	lab	la	aa	ca	t	pack
tvc	1.000							
pse	0.155	1.000						
lab	0.525	-0.028	1.000					
la	0.507	0.106	0.543	1.000				
aa	0.477	0.065	0.553	0.946	1.000			
ca	0.443	0.127	0.468	0.771	0.773	1.000		
t	0.076	-0.152	0.230	0.133	0.232	0.405	1.000	
pack	-0.018	0.099	0.070	0.037	-0.014	0.094	-0.219	1.000

The percent of total variance explained by components was 46.352 (1) and 16.598(2)

THE USE OF CONDUCTANCE IN MODELLING

Table 42.5 The effect of different factors on the Detection Time of Salmonella enteritidis as study with the Malthus 2000

juice		medium		amino acid		inoculum	detection time (dt)
sterile	no sterile	nza	spye	cysteine	lysine		
-	+	-	-	-	-	-	5
-	+	-	-	-	-	+	2.9
-	+	-	-	+	-	-	8.2
-	+	-	-	+	-	+	2.4
-	+	-	-	+	+	+	2.3
-	+	+	-	-	-	-	6.5
-	+	+	-	-	-	+	1.9
-	+	-	+	-	-	-	3.1
-	+	-	+	-	-	+	1.2
+	-	-	-	-	-	-	NG
+	-	-	-	-	-	+	1.9
+	-	-	-	+	-	-	NG
+	-	-	-	+	-	+	2.3
+	-	+	-	-	-	-	NG
+	-	-	+	-	-	-	NG
+	-	+	-	-	-	+	1.0
+	-	-	+	-	-	+	1.2
-	-	+	-	-	-	-	NG
-	-	-	+	-	-	-	NG

Similar results were obtained when centrifuged non-sterile fresh juice carrot was used (results not shown).

The examination of the above Table revealed that the most appropriate medium for the study of *Salmonella enteritidis* and *Listeria monocytogenes* is this that could contain either NZA and cysteine. Typical growth curves are shown in the Appendix. As this study is in progress the addition of arginine improved even better the growth curve of this organism, and particularly its sharp growth at the stage of detection time. This is a very important advantages when both these two organisms should be tested in a single Malthus tube.

Physicochemical changes

a. Tomatoes

The pH changes occurring during the storage of the above mentioned cases are described in the Figs 42.1c and 42.2c

It is well known that the critical changes during microbial spoilage take place in the aqueous phase. The water phase contains numerous non-volatile substances such as amino acids, organic acids and sugars (eg. glucose) which are utilised by almost all the bacteria of the tomatoes microflora. It is well known that vegetables contains relative higher amount of sugars which are available to unlimited level for microbial growth. The chromatographic profile of low molecular weight components from fresh tomatoes were shown in previous reports.The profile obtained from this particular experiment is similar with the previous one. Moreover it is evident that a similar acid profile was obtained from carrots.

Table 42.3 The contribution of organic acids (identified and unknown peaks) eluted from an Aminex HPX-87H column, found to be present initially and during the storage of fresh tomatoes under different packaging conditions at 4° C.

		air			5% CO_2 / 95 % N_2	
		days of sampling				
rt	**name**	**0**	**9**	**19**	**9**	**19**
5.89	-	0.065	0.664	0.803	0.84	1.1
6.54	citric	7.73	8.84	7.42	7.9	9.7
7.02	tartaric	0.186	0.493	0.49	0.835	0.9
7.63		-	0.223	0.431	-	0.132
7.9		-	0.863	0.477	-	0.34
8.12		1.46	1.59	1.46	2.69	1.28
9.42	succinic	2.52	3.21	3.7	3.9	3.45
10.45	lactic	0.24	5.94	7	5.43	7.6
11.34		0.33	0.494	0.86	0.49	0.74
12.43	acetic	0.15	0.24	0.37	0.31	0.40
14.16	propionic	1.18	1.24	1.59	0.99	2.25
14.93		-	1.048	1.30	-	1.60
17.7		3.34	2.57	2.60	5.45	5.80

It was evident that the two most important acids (lactic; peak g & acetic; peak I) increased during the storage period at 4°C. This increase coincide with our previous comment that these two acids as well as the unknown yet peak a could be due to lactic acid bacteria metabolism. In our previous study of naturally inoculated samples acetic acid increased during the growth phase of lactic acid bacteria. Acetate is an end-product which lactic acid bacteria would be expected to produce in various quantities, under both aerobic or anaerobic conditions (Kandler 1983). It needs to be noted also that peak h (RT 11.1) could be an important metabolic product of lactobacillus which could be used as a potential indicator. The production of this metabolite could be beneficial from the spoilage point of view. Indeed it is well established that acetic acid has greater antimicrobial action than lactic acid (Reddy *et al.*, 1975) and inhibited the growth of *Pseudomonas fluorescens* (Frey & Hubert, 1993), a bacterium suspected to participate in spoilage of shredded carrots (Carlin *et al.*, 1989). On the other hand the accumulation of this compound in fresh shredded carrots could affect the odour, or induce softening on texture (Ngugen-the & Carlin, 1994). The increase of acetic acid has been reported in other products (meat, poultry, fish, dairy products) stored under such man-made ecosystems (VP/MAP) (Marshall 1992; Kakouri & Nychas 1994, Kakouri *et al.* 1996).

b. Carrots

During our study with carrots the analysis of water soluble acids revealed that a similar pattern was obtained regardless the packing method used for preservation . It was evident however that there was a significant difference as far as the intension of the changes. For example the lactic acid increased rapidly in the case of samples stored at 4°C under air or 5%CO_2 / 5% O_2 / 90% N_2 and then decreased (Fig. 42.6). In contrast when carrots were stored at 10° C the lactic acid increased through out the storage period. Pyruvic acid decreased at 10°C regardless the packing system used while at 4° increased. The concentration of acetic acid increased rapidly under aerobic conditions at 10°C and less extensive in samples stored with map conditions (Fig.42.7).

Fig. 42.6 Changes of the lactic acid (L), acetic acid (A), citric acid (C), pyruvic acid (P) and succinic acid (S) on carrots stored under different storage conditions at 4°C

Fig. 42.7 Changes of the lactic acid (L), acetic acid (A), citric acid (C), pyruvic acid (P) and succinic acid (S) on carrots stored under different storage conditions at 10°C

The unknown peak with RT 11.1 could also play an important role as spoilage indicator. Indeed this peak increased rapidly at 10°C and to a lesser extent at 4°C in both cases regardless the packaging system used. The intension of increased however differs significantly. This compound is due to microbial metabolism as have been shown in our previous work with sterile carrots.

Fig.42.8 Changes of the unknown peak with RT 11.1 during the storage of carrots at 4 and 10°C under different storage conditions air (○), CO_2/ O_2/ N_2 (□) and CO_2/N_2 (▲)

E. Dissemination

1. N, Kakiomenou, CC. Tassou and Nychas, G.J.E Storage of shredded carrots with modified atmospheres:possible role of microbial metabolites as indicator(s) of spoilage (*Inter. J. Food Sci. & Technol.*)

2. Tassou, CC. Kakiomenou, N. Nychas, G.J.E Metabolic products of *Lactobacillus mesenteroides* sub *mesenteroides* during its growth on sterile carrots stored under aerobic or map condition (submitted Food Microbiology).

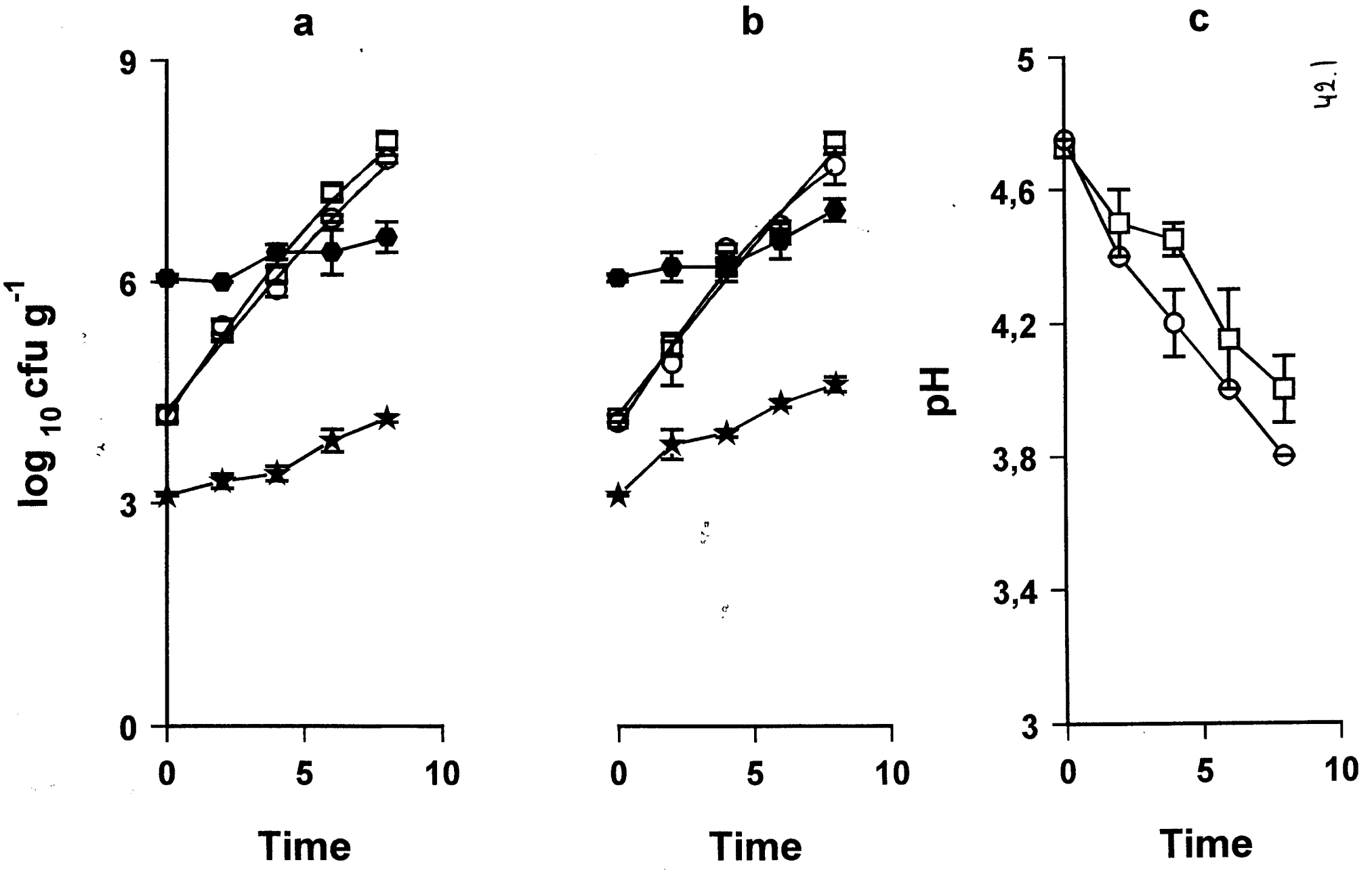
3. Growth/survival of foodborne bacteria on ready to eat carrots stored with map [N, Kakiomenou, Tassou, and Nychas, G.J.E] Poster: In "Food Micro" Budapest 23-27 August 1996

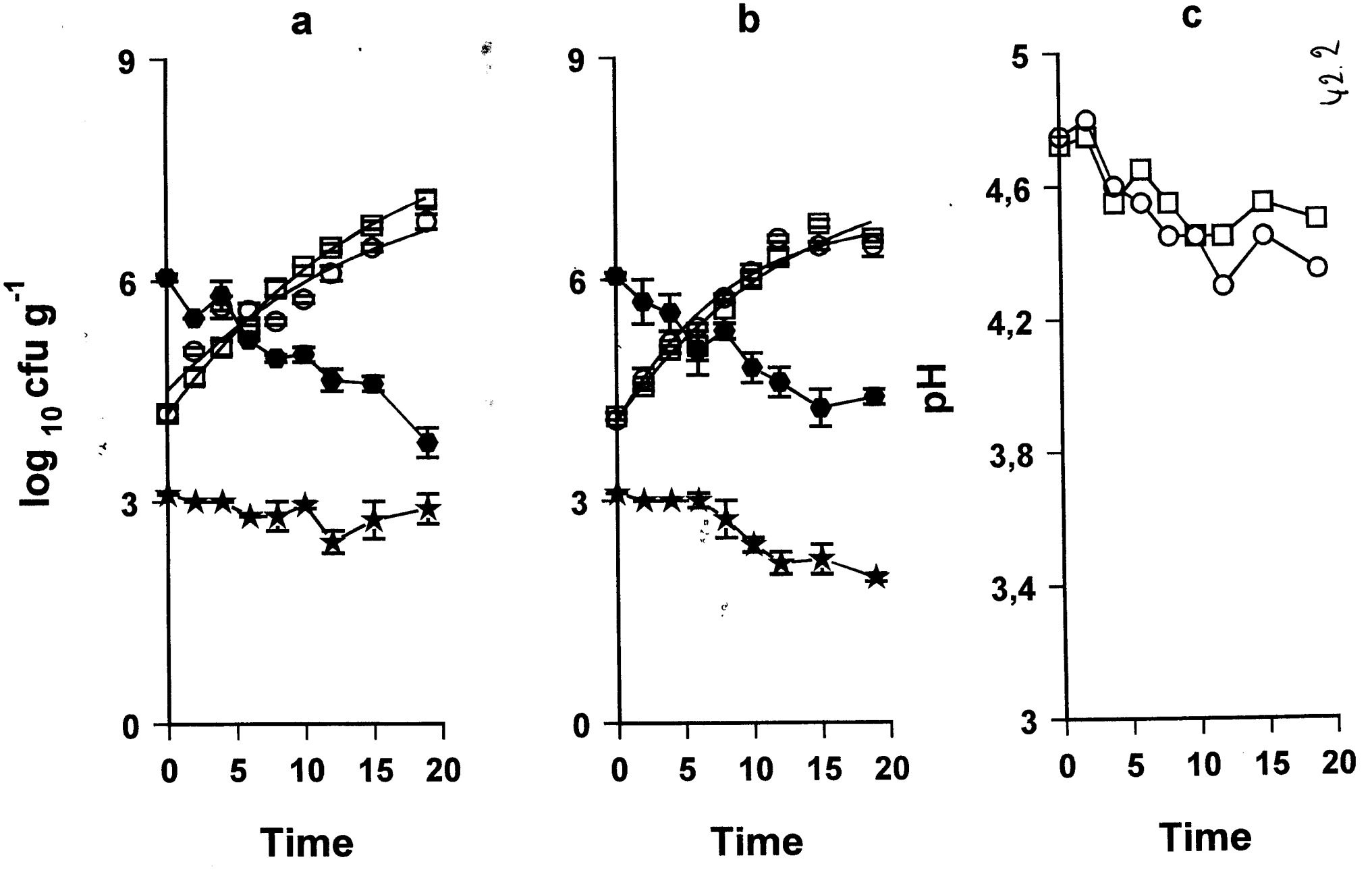
Part D:

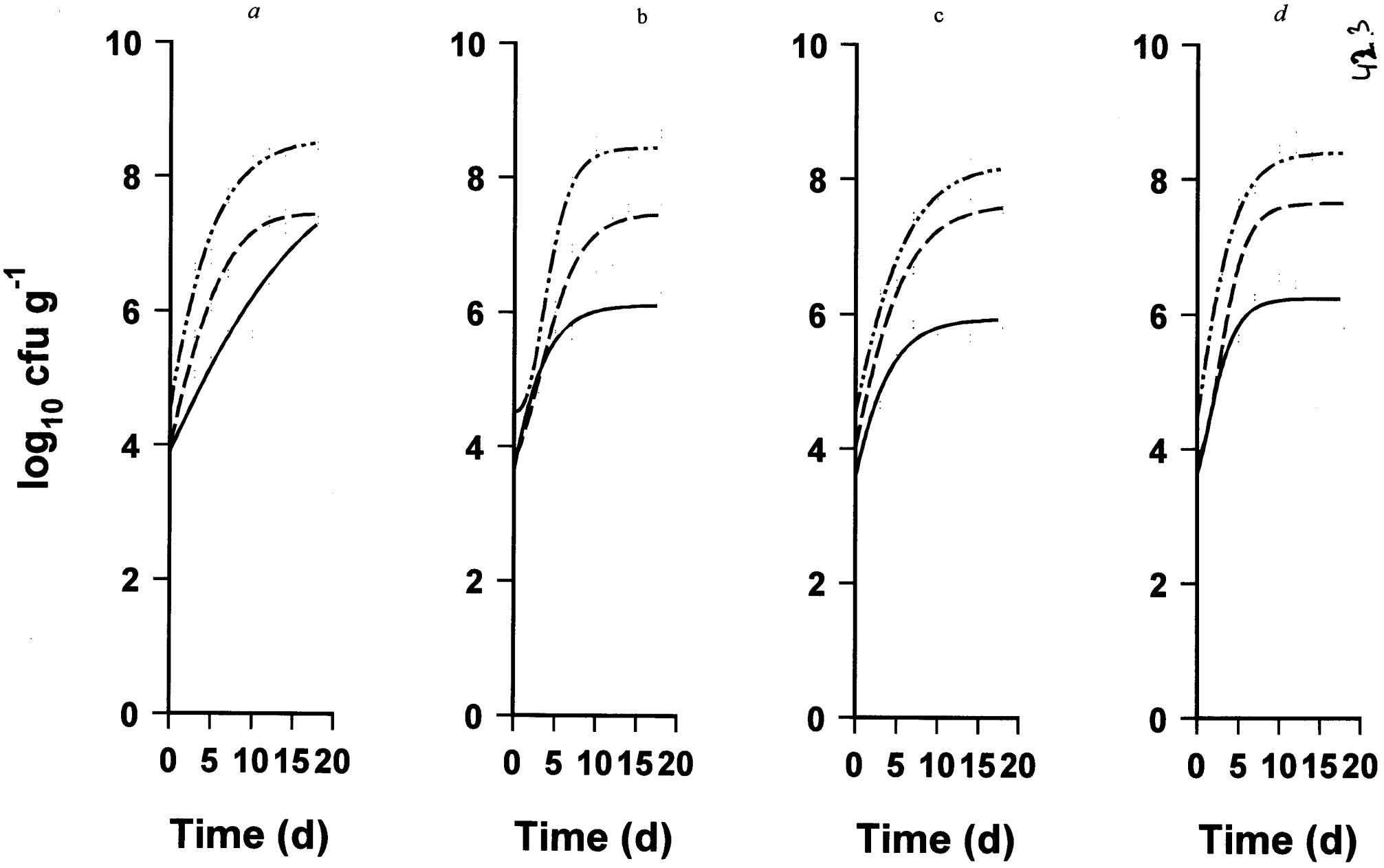
1. evaluation of pH, water activity against food pathogens (*Salmonella enteritidis* and *Listeria monocytogenes*)

2. modelling

Fig 42.1




a
b
c
9
6
3
0
5
4,6
4,2
3,8
3,4
3
0
5
10
15
20
Time
log 10 cfu g-1
pH
42.2


a
b
c
d
10
8
6
4
2
0
0 5 10 15 20
Time (d)
log10 cfu g-1

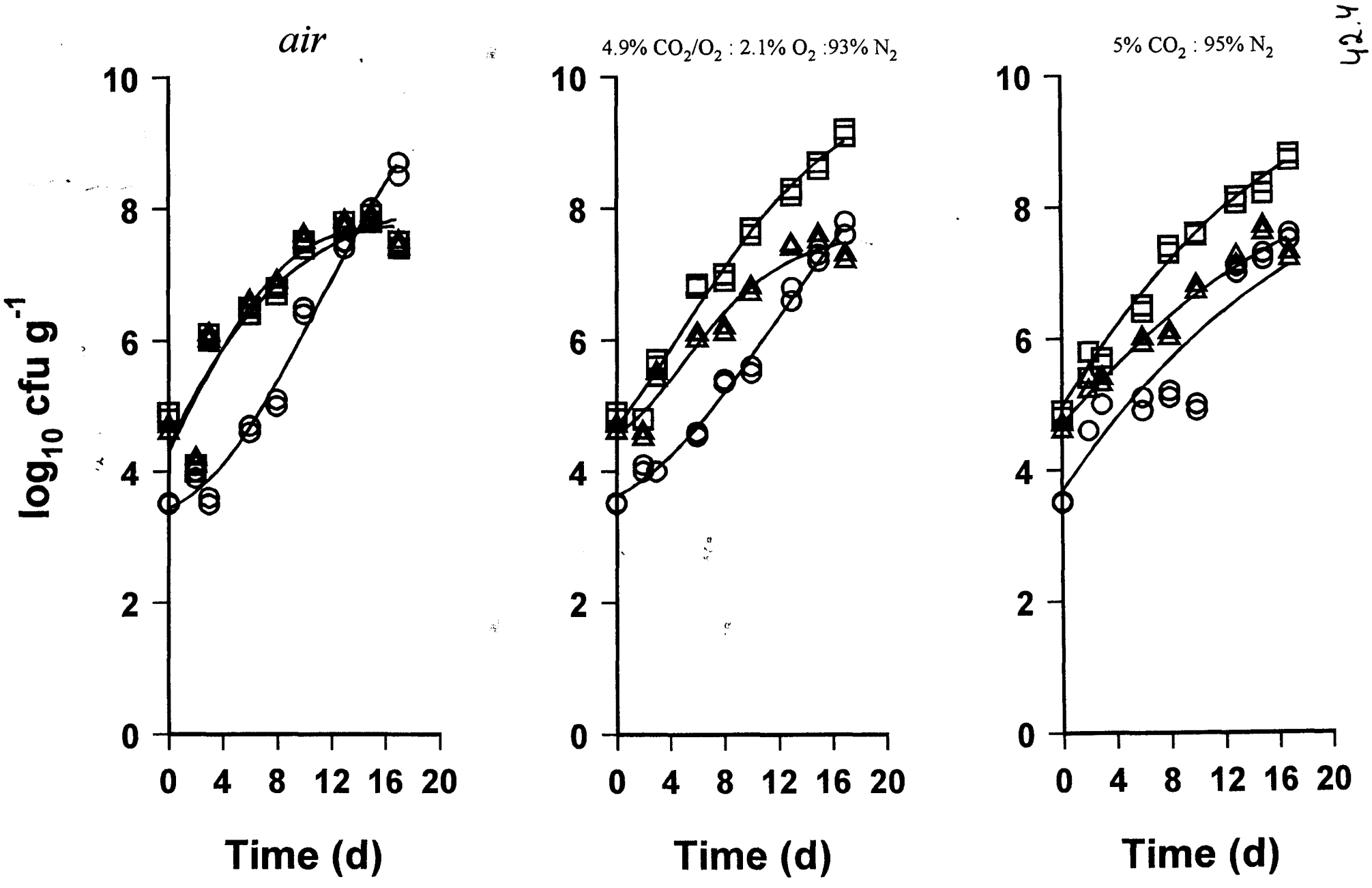
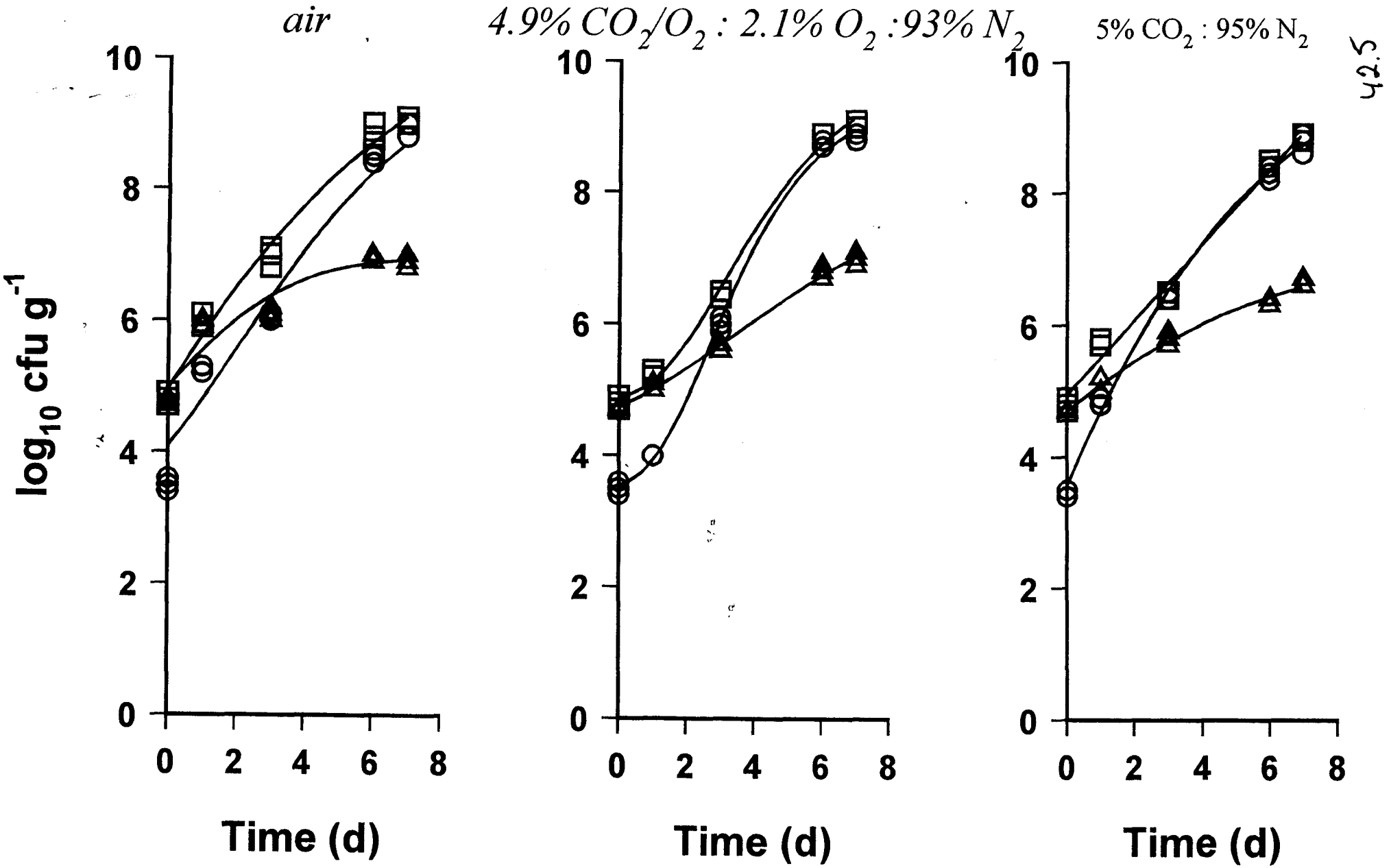

air
4.9% CO_2/O_2 : 2.1% O_2 :93% N_2
5% CO_2 : 95% N_2
$\log_{10}$ cfu g^{-1}
0
2
4
6
8
10
0 4 8 12 16 20
Time (d)

Fig 42


air
4.9% CO2/O2 : 2.1% O2 :93% N2
5% CO2 : 95% N2
log10 cfu g-1
Time (d)
Time (d)
Time (d)
0
2
4
6
8
10

42.5

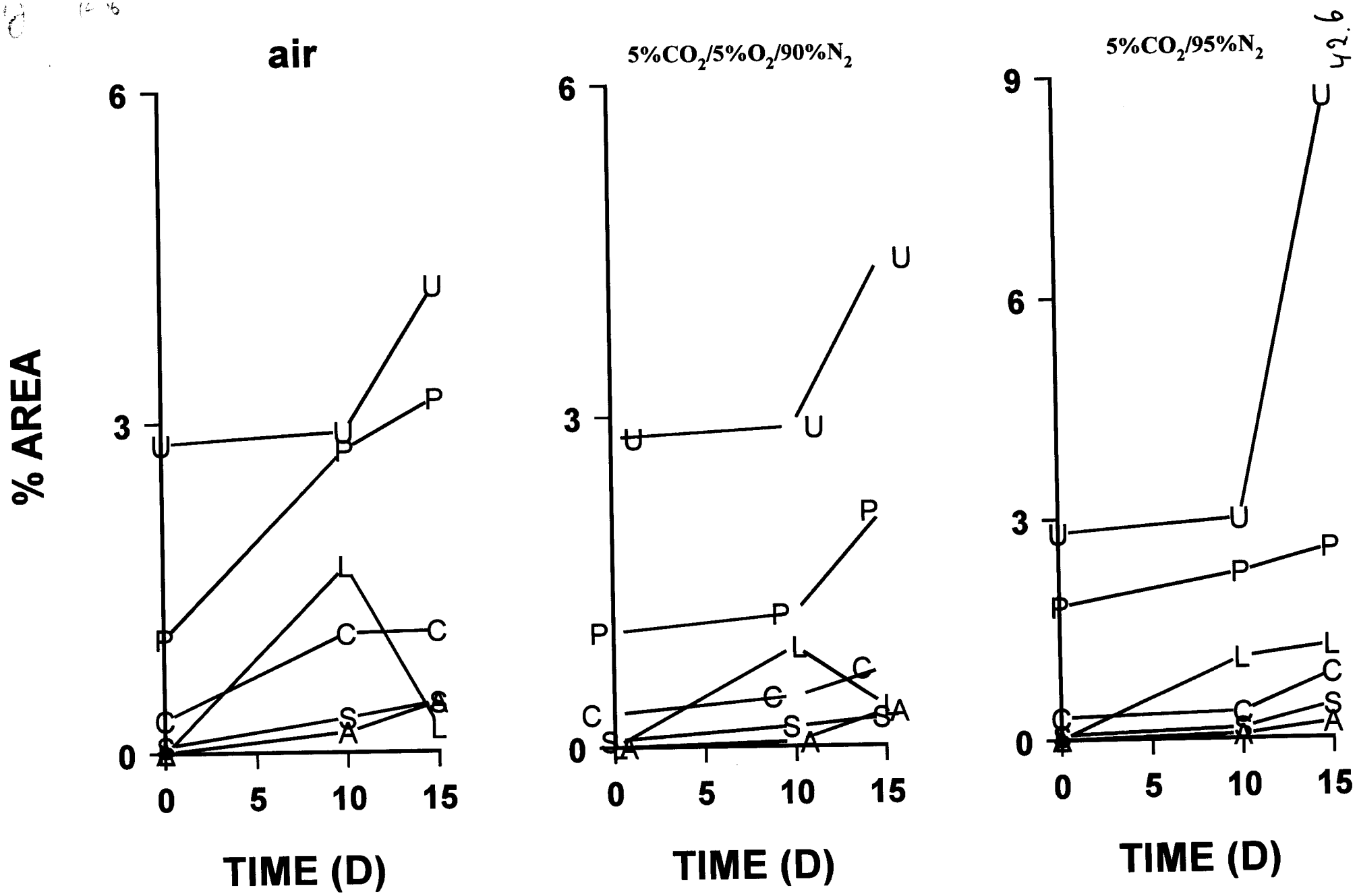
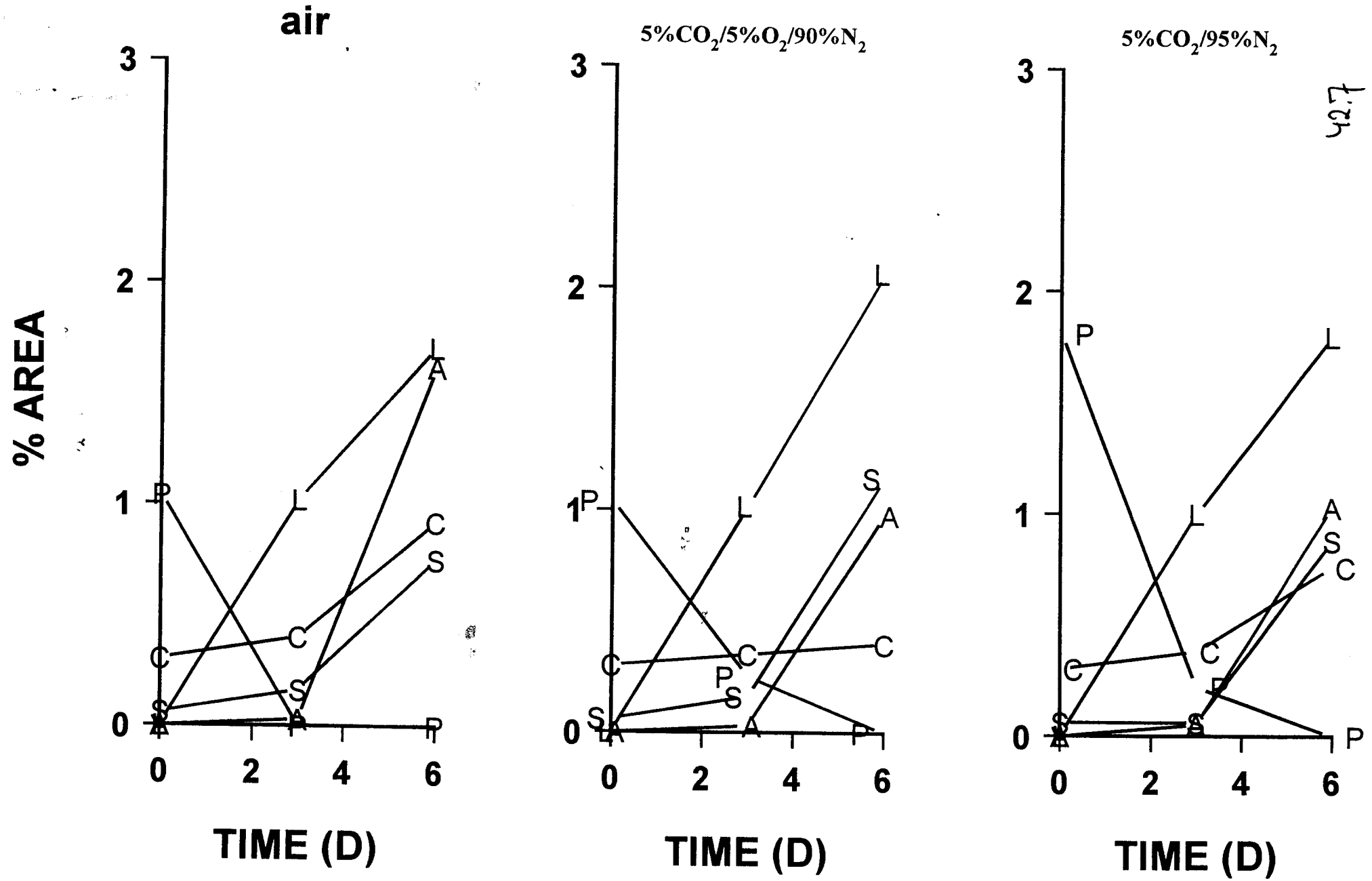

air
5%CO2/5%O2/90%N2
5%CO2/95%N2
% AREA
TIME (D)
TIME (D)
TIME (D)
0
3
6
9
0
5
10
15
U
P
L
C
S
A

Fig 42.7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5%CO2/5%O2/90%N2
5%CO2/95%N2
% AREA
TIME (D)
TIME (D)
TIME (D)
0
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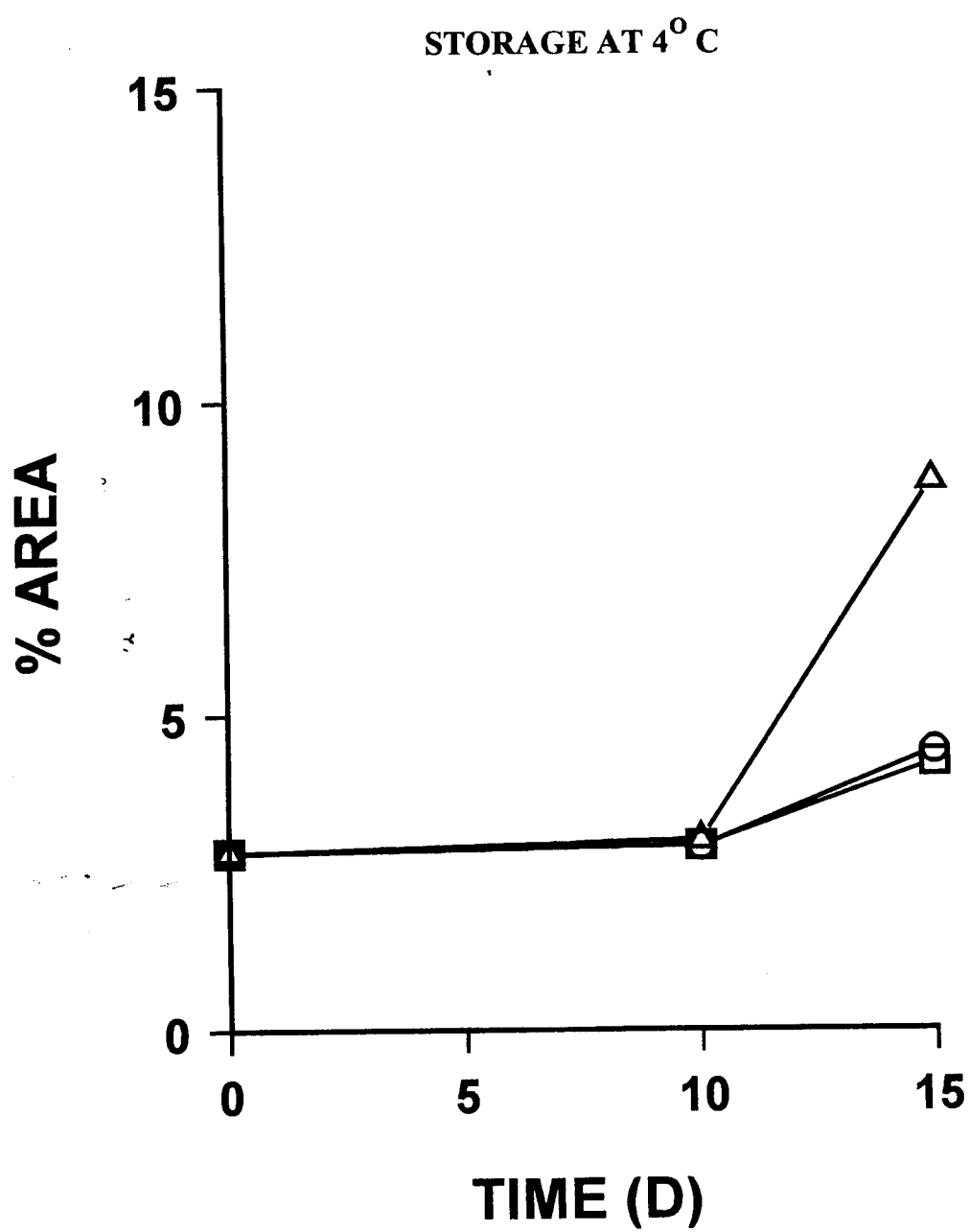
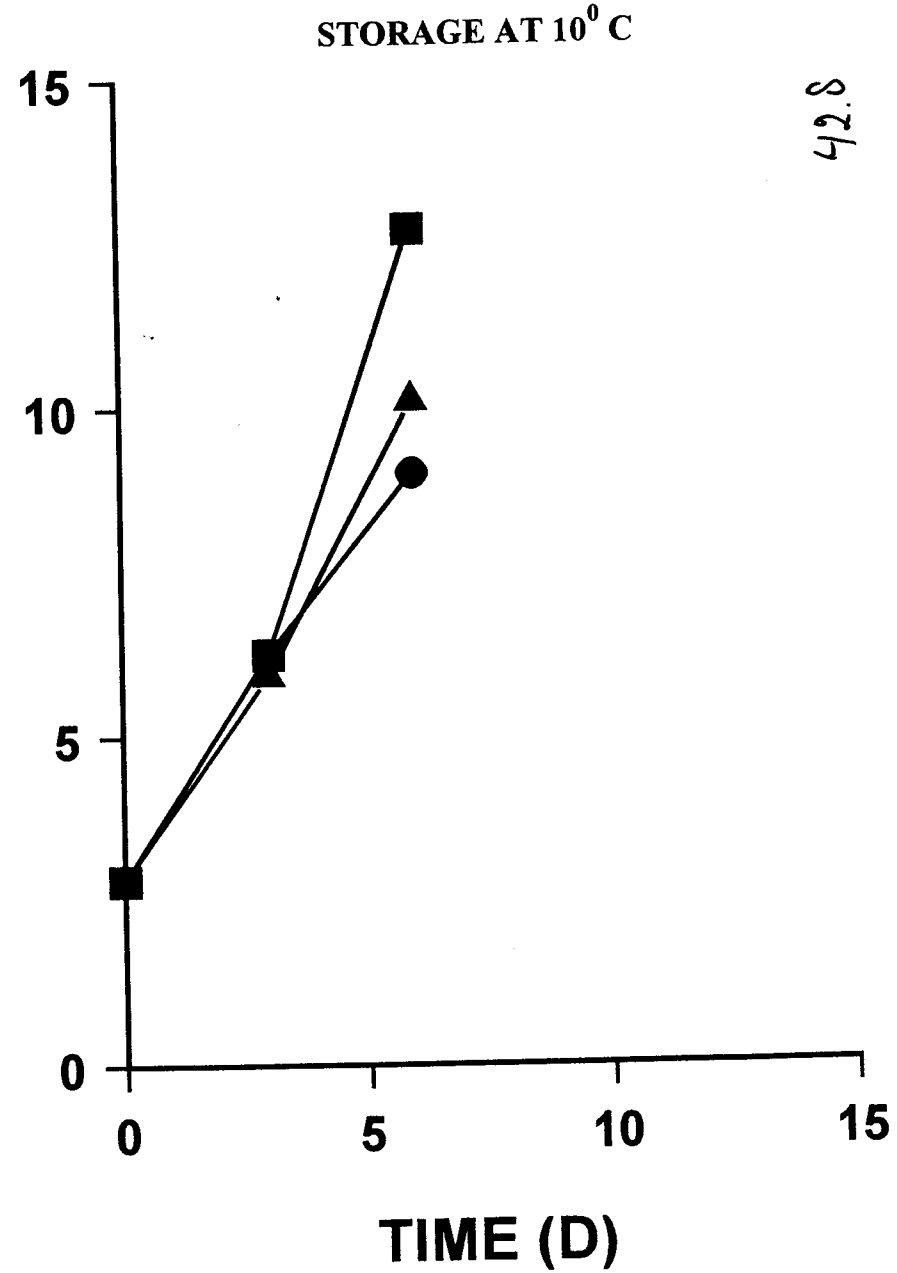
3
0
2
4
6
P
L
A
C
S


STORAGE AT 4° C
% AREA
15
10
5
0
0
5
10
15
TIME (D)


STORAGE AT 10° C
15
10
5
0
0
5
10
15
TIME (D)

42.8

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A1- Project sub-title:
Use of mild preservation techniques to reduce the risk of growth and toxin production from spores of non-proteolytic *Clostridium botulinum* in refrigerated ready-to-eat foods.

A2- Coordinator:
Michael W. Peck

A3- Project participants:
Michael W. Peck (9.6 person-months)
Sandra C. Stringer (45 person-months)
June Plowman (3 person-months)

B1- Description of the problem:
Non-proteolytic strains of *Cl. botulinum* produce heat-resistant spores which are capable of growth at refrigerated temperatures and produce a powerful neurotoxin. This bacterium therefore poses a safety risk in ready-to-eat foods that rely on refrigeration for preservation, particularly those that have received a mild heat-treatment (e.g *sous-vide* foods and other REPFEDs).

B2- Goal of the research:
This project will develop the use of mild preservation techniques to reduce the risk of growth and toxin production from spores of non-proteolytic *Cl. botulinum* in refrigerated ready-to-eat foods.

B3- Short description of the approach:

Studies in laboratory media.
Initially studies will be carried out in laboratory media. Spores will be heated in saline or an anaerobic broth, with recovery in a defined medium in the presence of mild preservative factors. The ability of gas atmospheres and other mild preservative factors to inhibit heat-damaged and unheated spores of non-proteolytic *Cl. botulinum* will be determined.

Studies in Foods.
The ability of the gas atmosphere and other mild preservative factors to inhibit growth from spores of non-proteolytic *Cl. botulinum* will be determined in model foods.
A) The effect of gas atmosphere and other suitable mild preservative factors on growth from heat-damaged spores of non-proteolytic *Cl. botulinum* will be determined in a model vegetable/egg-containing food.
B) The effect of gas atmosphere and other mild preservative factors on growth of non-proteolytic *Cl. botulinum* will be determined in relation to the normal epiphytic flora of salad vegetables.

C1- Short overview of research performed January 1996 to June 1996:

A. THE ABILITY OF HEATED VEGETABLE JUICE TO INCREASE THE THERMAL RESISTANCE OF SPORES OF NON-PROTEOLYTIC *CL. BOTULINUM.*

Introduction

The measured thermal resistance of non-proteolytic *Cl. botulinum* spores depends on the conditions used for recovery. One very influential factor is the presence of lysozyme. This enzyme is thought increase the measured thermal resistance by replacing the heat-damaged germination system. After diffusing through the spore coat lysozyme degrades peptidoglycan in the spore cortex, allowing hydration of the core and inducing spore germination. We have recently shown that vegetable juice can also increased the measured heat-resistance of non-proteolytic *Cl. botulinum* spores (see report from 7th meeting). The mechanism was thought to be similar to that of hen egg-white lysozyme.

To induce spore germination in food packs after heating the lytic activity of the vegetable juice must itself survive the heat-treatment. We examined the effect of heating vegetable juice on its ability to increase the viable count of heat-damaged spores of non-proteolytic *Cl. botulinum.*

Method

Heat-damaged spores were stripped of their spore coats and incubated on media containing vegetable juice heated at 75°C for between 0 and 10 min.

Preparation of heat-treated juice
Juice was extracted from four vegetables, flat bean, broccoli, cabbage and potato using a domestic centrifugal juicer (Kenwood 6000). The juice was cooled, centrifuged for 30 min at 24000**g** and the supernatant was filtered through a 0.65 μm nitrocellulose filter. Flat bean juice, broccoli juice and cabbage juice, each without added hen egg-white lysozyme (HEWL), potato juice with added HEWL and 1 and 10 μg HEWL ml^{-1} solution were subdivided into 150 ml portions in glass bottles. The bottles were heated at 75°C in a waterbath for between 0 and 10 min the cooled rapidly. The actual temperature of the liquid in each bottle was recorded every 6 seconds using thermocouple connected to a data logger.

Heated juice was centrifuged (24000**g**, 30 min, 2°C) and the supernatant was passed through 0.65, 0.45 and 0.2μm pore size nitrocellulose membrane filters (Whatman, Maidstone, U.K.). Aliquots of the clarified juice (100 ml) were filter sterilized (0.2μm pore size nitrocellulose filters, Whatman) and stored frozen at −18°C until required.

Assay of Lysozyme activity
Four hundred µl of a suspension containing lyophilized cells of *Micrococcus lysodeikticus* ATCC 4698 (Sigma, Poole, UK.)(1 mg ml^{-1}, in 0.05M Na phosphate buffer, pH 6.2), 500µl sodium phosphate buffer (0.05M, pH 6.2) and 100µl of sample or standard HEWL solution were added to a 1 ml cuvette, mixed, and the decrease in absorbance at 600nm monitored over a 5 min period at 30°C. Lysozyme activity was quantified as the weight of HEWL required to give the same activity.

Plate preparation
All media were based on PYGS medium (peptone, yeast extract, starch, glucose). Double strength PYGS agar (100 ml) was heat sterilized (121°C; 15 min), cooled to 50°C, mixed with an equal volume of filter sterilized vegetable juice or HEWL solution (warmed to 50°C) and poured into petri dishes. All plates were stored in gas jars (Oxoid, Basingstoke, U.K.) under a headspace of hydrogen/carbon dioxide (90:10, v/v) for 48 hours before use.

Spore preparation
Spores of *Cl. botulinum* 17B were produced using a two-phase medium under an atmosphere of nitrogen/hydrogen (90:10, v/v) as described by Peck *et al.* (1992). The solid phase consisted of double strength Robertson's cooked meat (300 ml), agar (4.5 g) and glucose (0.3 g). The liquid phase was distilled water (40 ml). Exponentially growing culture (5 ml) was inoculated into the water phase. After incubation at 30°C for 5 days, spores from the liquid phase were harvested by centrifugation (10,000**g**, 4°C, 15 min) and washed ten times with ice cold sterile saline (80 ml; 0.85%, w/v). Following washes five, six and seven, the spores were resuspended in 5 ml ice cold sterile saline and treated in an ultrasonic bath (Sonicleaner model 6442 AE Lucus/Dawe Ultrasonics, London) for 10 min.

Washed spores were treated with thioglycolate to remove the spore coat and thus increase the proportion sensitive to lysozyme. Spore suspension (250 µl) was injected into 1M thioglycolate solution, pH 10.0 (10 ml), preheated to 45°C. After 10 minutes, the spores were washed by centrifugation (10,000**g**, 15 min, 4°C) five times in 0.1M Na phosphate buffer, pH 7.0. Washed spores were heated for 3 min at 90°C in 0.1M Na phosphate buffer, pH 7.0, enumerated on PYGS agar and stored refrigerated until required.

Enumeration
Heat-damaged, thioglycolate treated spores were enumerated on the 36 different media previously prepared. The spores were serial diluted were prepared in PYGS using strict anaerobic technique and plated onto each type of medium in triplicate. Inoculated plates were incubated at 30°C in anaerobic culture jars under a headspace of hydrogen/carbon dioxide (90/10, v/v). Colonies were enumerated after eight days.

Results and discussion
The viable counts of the heat-damaged thioglycolate treated spores recovered on PYGS agar containing different vegetable extracts are shown in Figure 1. All the extracts heated at 75°C for 10 min increased the number of spores leading to colony formation above that number obtained on PYGS agar. This shows that pasteurising heat-treatments will not prevent vegetable juice increasing the measured heat-resistance of non-proteolytic *Cl. botulinum* spores. The number of spores able to form colonies on plates containing broccoli juice was slightly reduced when the juice had been heated at 75°C for 8 or 10 min. The number of colonies formed on agar containing cabbage or broccoli juice initially increased with increasing heat-treatment. This increase is likely to reflect the destruction of heat sensitive inhibitory compounds.

The lysozyme activity of the vegetable extracts, as measured in the *M. lysodeikticus* assay, is shown in Table 1. Activity in the 1 μg HEWL ml^{-1} solution, flat bean juice and cabbage juice were below the limit of detection of the assay. The activity in the broccoli juice and potato with added HEWL decreased rapidly at 75°C. The 10 μg HEWL ml^{-1} solution was the most stable.

The vegetable juices with the greatest activity in the *M. lysodeikticus* assay tended to result in the greatest number of colonies with 10 μg HEWL ml^{-1} being the most active and cabbage juice the least active. However, the number of colonies was not directly related to the measured lysozyme activity. Although heating the broccoli juice and potato juice containing HEWL at 75°C reduced activity in the lysozyme assay it did not reduce the number of spores leading to colony formation. Other samples were shown to increase the number of spores able to form colonies without containing measurable lysozyme activity. The limit of detection for the assay as used in this study was 0.5 μg HEWL ml^{-1}. Heat-damaged spores of non-proteolytic *Cl. botulinum* were shown to be sensitive to as little as 0.1 μg HEWL ml^{-1} (Peck *et al.* 1992). It is therefore possible that lysozyme activity below the level of detection in the *M. lysodeikticus* assay induced germination of heat-damaged spores.

Summary/ Highlights
Vegetable juice can increase the measured heat-resistance of spores of non-proteolytic *Cl. botulinum*. Heating active juices at 75°C for 10 min failed to destroy this effect. This implies the survival of non-proteolytic *Cl. botulinum* spores in pasteurised vegetable products would be greater than predicted from studies where spores were recovered in the absence of lytic enzymes.

Time at 75°C (min)	Lysozyme activity (μg HEWL ml^{-1} equivalents)					
	10μg HEWL	1μg HEWL	Potato +HEWL	Flat Bean	Cabbage	Broccoli
0	9	<0.5	7	<0.5	<0.5	6
1	6	<0.5	7	<0.5	<0.5	5
2	8	<0.5	5	<0.5	<0.5	4
4	6	<0.5	4	<0.5	<0.5	3
6	6	<0.5	4		<0.5	2
8	6	<0.5	3		<0.5	1
10	6	<0.5	3		<0.5	1

Table 1. The effect of heating at 75°C on the lysozyme activity of vegetable juice or HEWL solution. The lysozyme activity was assayed by lysis of *Micrococcus lysodeikticus* cells.

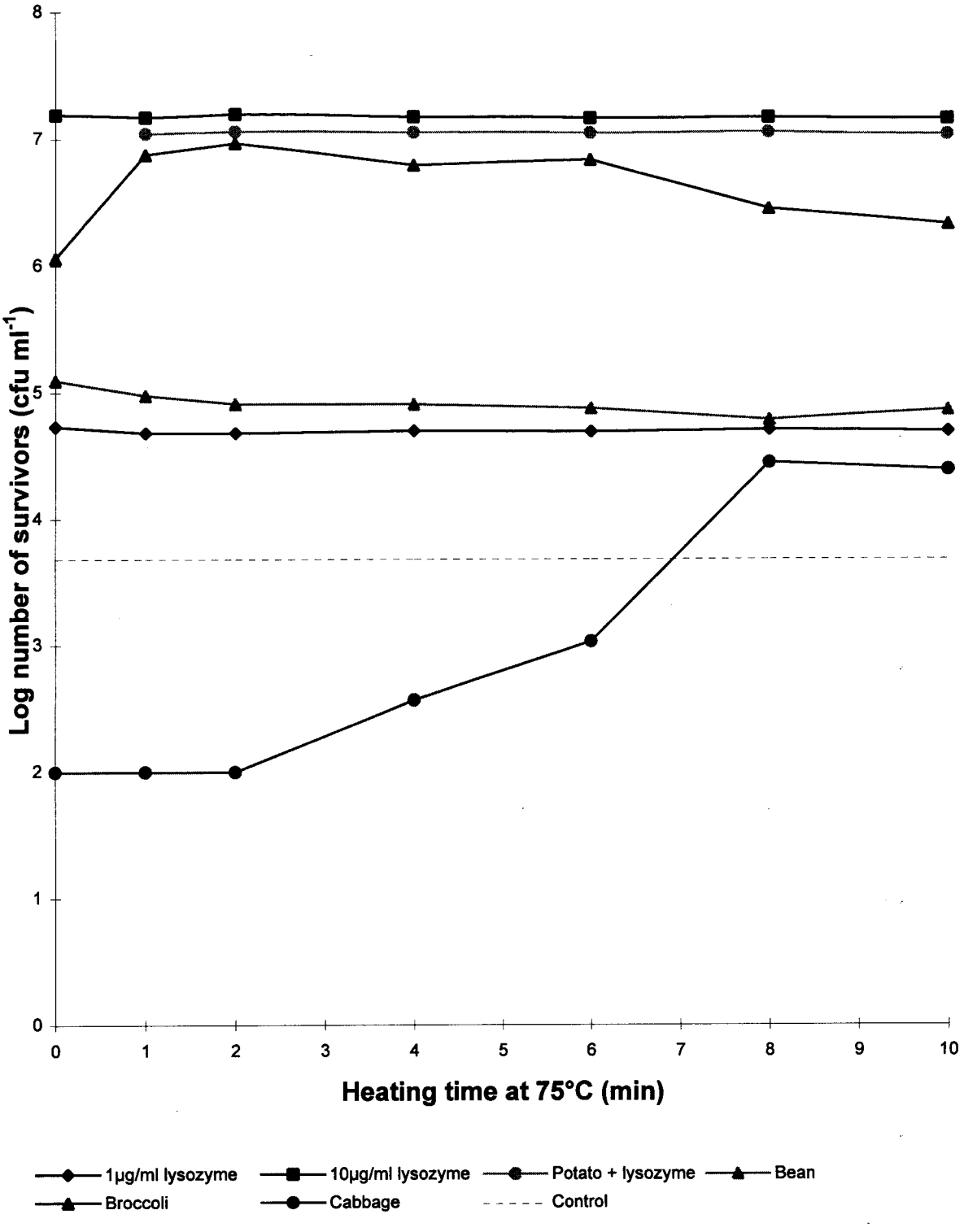


Figure 1. The effect of adding unheated or heated vegetable juice or HEWL solution to recovery media on the number of colonies formed by heat-damaged spores of non-proteolytic *Cl. botulinum*. The spores were heated at 90°C for 3 min and plated on PYGS agar containing unheated HEWL solution or vegetable extract or solution or extract heated at 75°C for 1, 2, 4, 6, 8 or 10 min. The control is standard PYGS agar.

B. The effect modified atmospheres on growth from unheated and heat-damaged spores.

Introduction

We previously reported the effect of ten modified atmospheres on growth from unheated and heated spores of non-proteolytic *Cl. botulinum* (see report of 6th meeting). All atmospheres with less than 2% oxygen failed to significantly reduce the number of spores leading to turbidity. These atmospheres included 100% carbon dioxide and 100% argon, gasses that have been reported to be inhibitory. It has been suggested that argon shows a greater inhibitory affect in the presence of carbon dioxide than in its absence (personal communication). It was therefore decided to compare the growth from unheated and heated non-proteolytic *Cl. botulinum* spores in atmospheres consisting of carbon dioxide and argon, carbon dioxide and nitrogen as well as argon, carbon dioxide and nitrogen in isolation. The spores were incubated both in the presence and absence of hen egg-white lysozyme (HEWL) as heat-treatment will affect difference targets in these two situations.

Method

Peptone, yeast extract, starch and glucose (PYGS) broth was boiled to remove dissolved oxygen and cooled under the required modified atmosphere. Once cooled, the pH was adjusted to 6.8 with hydrochloric acid or potassium hydroxide, the medium was dispensed as 10 ml aliquots under the same atmosphere and sterilised (121°C, 15 min). The modified atmospheres were: 100% nitrogen; 100% carbon dioxide: 50% carbon dioxide & 50% nitrogen; 50% carbon dioxide & 50% argon; 100% argon. If required, filter sterilised HEWL (48000 unit mg^{-1}, Sigma, Poole, UK.) was added to PYGS (PYGS+lysozyme) after sterilisation to a final concentration of 10 $\mu g\ ml^{-1}$.

Washed spore suspension was prepared as described in section A. The spores were heated using a submerged tube method as described by Peck *et al.* (1992) except a 100 µl of spore suspension (containing $2.3x10^8$ spores) was added to 9.9 ml PY broth. Spores were subjected to one of 7 heat-treatments. They remained unheated or were heated at 75°C or 90°C for 2, 5, or 10 min.

Serial dilutions (10-fold) of unheated and heated spores were prepared in dilution PYGS using strict anaerobic technique. Aliquots (100 µl containing between 0.23 and $2.3x10^5$ spores) of appropriate dilutions were inoculated into five replicate PYGS broths for each treatment using disposable sterile 1 ml syringes and 26-gauge needles. Spores heated at 75°C were recovered in the absence of added lysozyme while spores heated at 90°C were recovered in PYGS+lysozyme. Inoculated vials were incubated at 10°C and the number of turbid vials noted at regular intervals. The number of spores resulting in turbidity at each observation time was calculated from five tube most probable number (MPN) tables.

Results and discussion

For all treatments, spores incubated under 100% nitrogen led to turbidity before those under atmospheres containing carbon dioxide or argon (Table 2). The most effective modified atmosphere was 100% carbon dioxide, which delayed turbidity the most for all treatments. However, any inhibitory effect was small and atmospheres containing carbon dioxide and/or argon did not reduce the number of spores able to result in turbidity within 4 weeks compared with 100% nitrogen (Figure 2). Although argon did not greatly delay growth from spores of non-proteolytic *Cl. botulinum*, it was as effective as using 50% carbon dioxide.

Summary

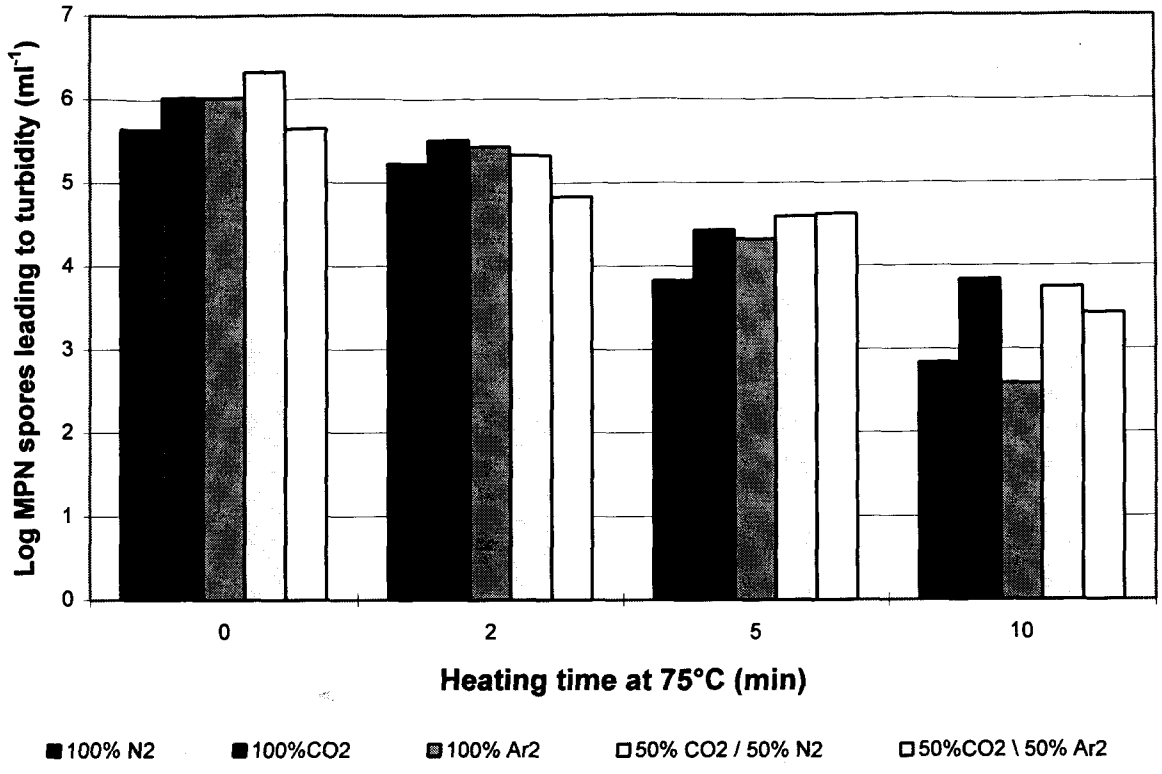
High levels of carbon dioxide and argon marginally increased the time for spores of non-proteolytic *Cl. botulinum* to reach turbidity but did not reduce the number of spores leading to turbidity within four weeks.

Atmosphere	Shortest time to turbidity (Days)							
	Heating time at 75°C				Heating time at 90°C			
	0	2	5	10	0	2	5	10
100% Nitrogen	7	9	9	9	7	10	10	10
100% Carbon dioxide	12	14	14	12	10	12	14	16
100% Argon	7	9	12	12	7	12	12	14
50% Carbon dioxide 50% Nitrogen	7	9	12	12	7	12	12	14
50% Carbon dioxide 50% Argon	7	12	12	12	8	12	12	14

Table 2. Time (days) for the first vial containing up to 2.3×10^5 unheated or heat-damaged *Cl. botulinum* 17B spores to become turbid in PYGS broth flushed with four different gases incubated at 10°C. The vials originally contained 100% nitrogen.

REFERENCE

Peck, M.W., Fairbairn, D.A. and Lund, B.M. (1992) The effect of recovery medium on the estimated heat-inactivation of spores of non-proteolytic *Clostridium botulinum*. *Letters in Applied Microbiology* **15**, 146-151.



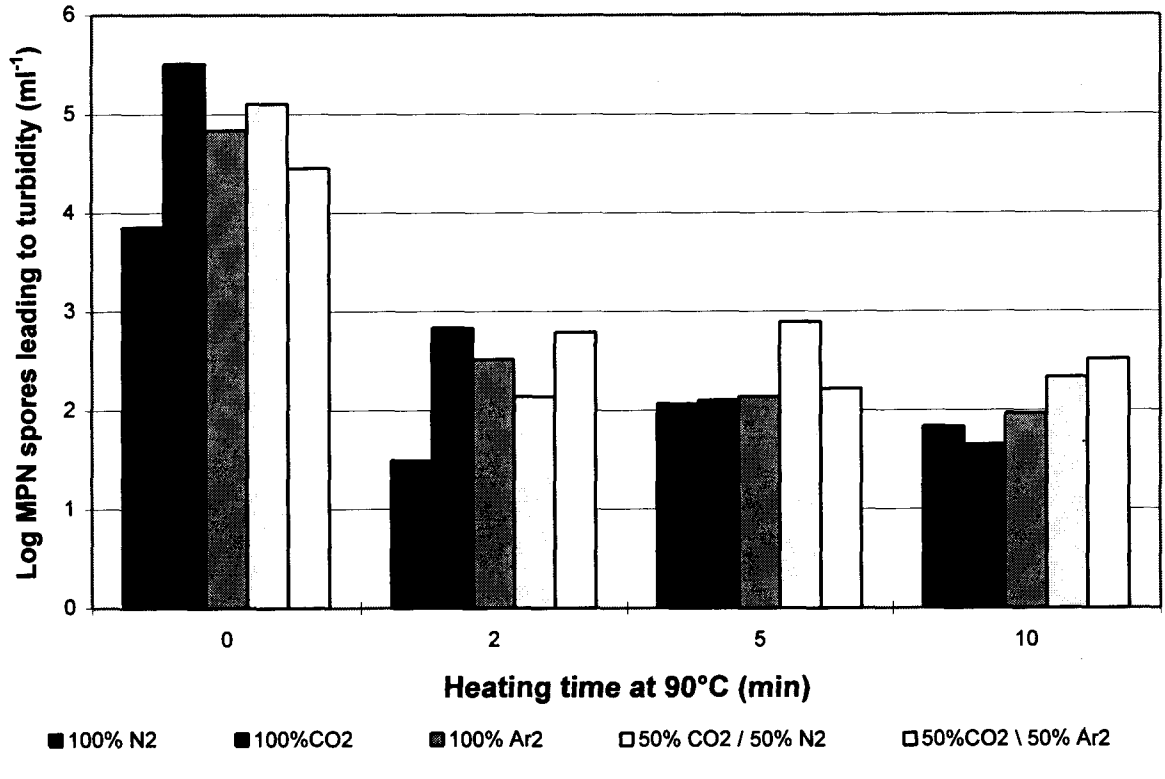


Figure 2. The MPN of unheated or heated spores of non-proteolytic *Cl. botulinum* able to lead to turbidity within four weeks when incubated in PYGS broth prepared under modified atmospheres. The atmospheres were: 100% nitrogen; 100% carbon dioxide: 50% carbon dioxide & 50% nitrogen; 50% carbon dioxide & 50% argon; 100% argon.

D1- General description of the experimental approach

(i) Experimental plan for the entire project

Seven areas of research may be identified (major effort 1-5):

1. Production of spores and confirmation of their heat-resistance.

2. Determination of the effect of preservative factors on growth from heat-damaged and unheated spores of non-proteolytic Cl. botulinum (carried out in laboratory media).

3. Determination of the effect of gas atmospheres on growth from heat-damaged and unheated spores of non-proteolytic *Cl. botulinum* (carried out in laboratory media).

4. Determination of the effect of gas atmosphere and other preservative factors on growth from heat-damaged spores of non-proteolytic *Cl. botulinum* in a model, vegetable/egg-containing, food.

5. Behaviour of non-proteolytic *Cl. botulinum* in relation to gas atmosphere and the epiphytic flora of salad vegetables.

6. Evaluation of the safety, with respect to non-proteolytic *Cl. botulinum*, of novel preservation systems developed by other labs.

7. Development of predictive models.

(ii) Experimental plan for next six months (7-96 to 12-96)

1. The thermal destruction of lytic activity in vegetable juice will be determined at different temperatures and the z-value determined (June).

2. The modified atmosphere experiment outlined in section B will be followed for 10 more weeks. After 6 weeks vials will be examined for the presence of toxin and transferred to an incubator at 30°C to assess the potential for further growth at a higher temperature (June/July).

3. Sterile anaerobic vegetable juice will be prepared using a low pressure boiling method. The ability of spores to lead to growth within each juice will be assessed (July). Any juices that support growth and contain lytic activity will be inoculated with *Cl. botulinum* spores and subjected to varying heat-treatments (August/September).

4. An assay for lytic activity based on the change in optical density that occurs as spores germinate is being developed. This assay would allow rapid assessment of the ability of numerous vegetable extracts to induce spore germination (October/November).

E. Dissemination

Stringer, S.C., Fairbairn, D.A. and Peck, M.W. (1996) Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. *Journal of Applied Bacteriology* (submitted).

Stringer, S.C. and Peck, M.W. Vegetable juice aids the recovery of heated spores of non-proteolytic *Clostridium botulinum*. *Letters in Applied Microbiology*. In press.

Carlin, F. & Peck, M.W. 1996. Metabiotic association between non-proteolytic *Clostridium botulinum* type B and foodborne *Bacillus* species. Sciences des Aliments (in press)

Carlin, F. & Peck, M.W. 1996. Growth of, and toxin production by, non-proteolytic *Clostridium botulinum* in cooked vegetables at refrigeration temperatures. Applied and Environmental Microbiology (submitted).

Peck, M.W. and Stringer, S.C. (1996) *Clostridium botulinum*: Mild preservation techniques. In "Proceedings of Second European Symposium on *Sous-vide*". pp. 181-197. ALMA, Leuven.

Stringer, S.C., Fairbairn, D.A. and Peck., M.W. (1996) The combined effect of heat treatment and recovery temperature on growth from spores of non-proteolytic *Clostridium botulinum*. In "Proceedings of Second European Symposium on *Sous-vide*". pp. 379-386. ALMA, Leuven.

Carlin, F. and Peck, M.W. (1996) Croissance de *Clostridium botulinum* non protéolytique et production de toxine à température de réfrigération dans des légumes cuits. In "Proceedings of Second European Symposium on *Sous-vide*". pp. 311-316. ALMA, Leuven.

Carlin, F. & Peck, M.W. 1996. Production de toxine et croissance de *Clostridium botulinum* a temperature de refrigeration dans les legumes cuits. Abstract and poster presentation at Actualities en Microbiologie de Aliments, Sociéte de Francaise de Microbiologie. Paris, France.

Stringer, S.C. and Peck, M.W. (1996) The apparent heat-resistance of non-proteolytic *Clostridium botulinum* spores is increased by endogenous lysozyme activity of vegetable extracts. Poster presentation at and abstract in Proceedings of 83rd Meeting of the International Association of Milk, Food and Environmental Sanitarians. In press.

Stringer, S.C. and Peck, M.W. (1996) Effect of NaCl on growth from heated non-proteolytic *Clostridium botulinum* spores. Poster presentation at and abstract in Proceedings of Food Micro 96. In press.

Stringer, S.C., Fairbairn, D.A. and Peck, M.W. (1996) Quantitative assessment of the effect of heat treatment and subsequent incubation temperature on growth from spores of non-proteolytic strains of *Clostridium botulinum*. Poster presentation at and abstract in "Biomedical Aspects of Clostridial Toxins: International Conference Oxford - 1996".

Gorris, L.G.M., Abee, T. & Peck, M.W. 1996. Food matrix influences on pathogen growth. ZFL (International Food Manufacturing) **47** 62-65.

Gorris, L.G.M., Abee, T. & Peck, M.W. 1996. Invloed van de voedsel matrix op groei en overleven van pathogene bacterien. VMT [Voedingsmiddlelentechnologie] (in press)

Participant 7: Department of Life Sciences
University of Limerick
Plassey Technological Park
Limerick, Ireland

A. 1. (Sub-) Project titles:

(a) Accuracy of oxygen measurement
(b) Effects of raw materials and preparation
(c) Results of sub-projects (a) and (b) will be applied in optimisation of product-packaging film compatibility

2. Project Coordinator:
Prof David O'Beirne

3. Project Participants and 4. Resources:
Prof David O'Beirne
Ms Catherine Barry-Ryan, postgraduate student
Dr Jennifer Colford, postdoctoral research associate

Resources:
Year 1 18 man months
Year 2 18 man months
Year 3 12 man months (estimated)
Year 4 12 man months (estimated)

(A) *ACCURACY OF OXYGEN MEASUREMENT*

B. 1. ***Description of research topic or practical problem***
This project addresses accurate measurement of oxygen levels close to zero within (modified atmosphere) packages of chilled foods. Measurement problems have arisen particularly because of the co-elution of argon and oxygen in analytical procedures based on gas chromatography when these are applied to respiring produce. There are also potential inaccuracies in other analytical procedures and due to sampling methods.

2. Goal of Research

To determine the significance of inaccuracies in measurement of oxygen levels in the 0-6% range which result from the use of current analytical procedures. This will include examining the feasibility of introducing routine GC procedures which separate oxygen from argon.

3. Short description of the approach

Argon levels will be determined in packages with product modified atmospheres and in packages which are flushed with low oxygen atmospheres prior to storage. The implications of sampling procedures and resampling for accuracy will be determined. The practical use of paramagnetic and other procedures will also be evaluated. The feasibility of improved routine procedures will be addressed.

4. Experimental approach in phases

Year 1, 2

Quantification of argon in packages with product modified atmospheres and in packages with gas flushed cum product modified atmospheres. Effects of packaging materials and storage times.

Year 3, 4

(a) Significance of gas sampling procedures for accuracy and precision. Effects of resampling, interactions with gas barrier properties of packaging film.
(b) Accuracy and precision of non-GC based analytical procedures.
(c) Further development of most productive aspects. Contribution to product-package optimisation.

5. State-of-the-Art

Growth and toxin production by *Clostridium botulinum* can occur in oxygen-free packages stored at temperatures > 4°C. As a result, accurate measurement of oxygen levels close to zero is important for development and safe use of packaging systems for (respiring) ready-to-eat fruits and vegetables. In addition, accurate oxygen measurement at levels above this low range is important in research and development work on product - package optimisation.

Oxygen measurement by gas chromatography (GC) is a widely used and convenient technique because it quantifies the other major gases present (usually nitrogen and carbon dioxide) in addition to oxygen. However, argon and oxygen are not separated and oxygen levels are artificially elevated by the argon present.

Separation of argon from oxygen is possible using extremely long (8m) GC columns packed with a suitable molecular sieve and operating at sub ambient temperatures. Separation may also be possible using custom-made CTR III GC columns. Data on argon levels in ready-to-use packaged foods are not available. Levels of around 0.9% (i.e. close

to the level found in atmospheric air) are likely in packages constructed from materials with low gas barrier properties but this needs to be confirmed. Argon levels in low barrier packages which have been flushed with low-oxygen pure gas mixtures will vary depending on product storage time and gas barrier properties of the packaging used. Thus, the contribution of argon to inaccurate oxygen measurement will vary.

As a result, it is proposed to quantify argon levels in low barrier packages with product modified atmospheres and with atmospheres resulting in part from gas flushing with pure (argon free) gases. In addition, the feasibility of a routine GC procedure for oxygen measurement which includes an oxygen-argon separation will be considered.

Besides inaccuracies resulting from argon levels within packages, other sources of error will be investigated. These will include gas sampling techniques (extraction method, volume for analysis); resampling; interference from other gases; and accuracy/precision of non-GC based analytical procedures.

C. Overview of work performed 1-1-96 to 30-6-96

The main objectives of the research performed during this period were:
(a) to continue evaluation of oxygen determination using non-GC analytical procedures.
(b) to use this and other data to optimise product-package compatibility.

Materials and Methods

Plant Materials
Commercial samples of MA packaged vegetables were obtained from Nature's Best Ltd. For evaluation of non-GC procedures these were carrot discs packaged in oriented polypropylene (OPP) and in three P-Plus films with different gas permeabilities: PA60, PA120 and PA200.

Following on from previous work the following products/films were also used in the product-package campatibility work:
- Salad Bowl Mix, nitrogen flushed, packaged in oriented polypropylene (OPP) or P-Plus PA90
- Dry Coleslaw Mix, unflushed, packaged in P-Plus PA90 and PA160.

Non-GC Oxygen Analysers
TIA-111-LV Oxygen Analyzer (PBI Dansensor)

This machine has been developed for measurement of residual oxygen in gas and vacuum packaged foods and only requires a very small measuring volume (3.0 ml). The sensor is a solid-state ionic conductor with stabilized zirconium oxide electrolyte. The sensor emits an EMF signal which is logarithmic and inversely proportional with the oxygen partial pressure in the measured gas.

Results and Discussion

Tables 1-4 report the oxygen levels measured in MA packaged carrot discs in films with a range of gas permeability values. The objective was to test the accuracy of oxygen measurement in the medium (8-11%) range (Tables 1,2) in the high range (16-18%) (Table 3) and at levels close to zero. Each table contains two sets of data. The upper values compare true oxygen values based on CTRI values with the PBI Dansensor i.e. the same bags were resampled for the Dansensor values. The lower values compare oxygen values directly generated by the CTRIII column with Dansensor values.

The data are reported as mean values for 4/5 determinations with standard deviations. In the medium oxygen range (Tables 1,2) agreement between the sensor and true O_2 values was closer than previously reported, with most values within 0.5 of a percentage unit and only one value above 1 percentage point out. However, some standard deviation values were very high. In the higher O_2 range (Table 3) one value showed a difference of more than 1 percentage point. In the most important low oxygen range (Table 4), the good performance reported previously was repeated; all differences were <0.5 percentage points.

It is concluded that non-GC procedures are more reliable than simple GC procedures for accurate measurement of oxygen below 1%. For higher levels the results from non-GC are more variable.

Table 1 Atmosphere composition of carrot samples in PA 60 P-Plus Film

	Atmosphere Composition		
	Day 1	Day 3	Day 5
Oxygen (CTRI)	8.6 0.3	5.4 2.1	10.6 3.7
True Oxygen	7.6 0.3	4.4 2.1	9.6 3.7
Oxygen (TIA-111-LV)	7.0 0.2	5.2 0.5	10.3 4.1
Argon (CTR III)	0.97 0.03	0.96 0.03	0.98 .01
Oxygen (CTR III)	9.3 2.0	3.6 2.6	7.6 1.9
Oxygen (TIA-111-LV)	9.6 2.7	3.5 2.6	7.5 1.9

Table 2 Atmosphere composition of carrot samples in PA 120 P-Plus Film

	Atmosphere Composition		
	Day 1	Day 3	Day 5
Oxygen (CTRI)	9.5 0.7	10.6 3.2	11.4 2.0
True Oxygen	8.5 0.7	9.6 3.2	10.4 2.0
Oxygen (TIA-111-LV)	8.2 0.6	9.5 3.3	11.5 1.9
Argon (CTR III)	0.97 0.01	0.98 0.02	0.98 .01
Oxygen (CTR III)	10.9 0.8	11.8 0.7	11.1 2.6
Oxygen (TIA-111-LV)	10.6 0.8	11.9 0.8	10.8 2.6

Table 3 Atmosphere composition of carrot samples in PA 200 P-Plus Film

	Atmosphere Composition		
	Day 1	Day 3	Day 5
Oxygen (CTRI)	16.1 0.2	16.5 0.6	17.1 0.4
True Oxygen	15.1 0.2	15.5 0.6	16.1 0.4
Oxygen (TIA-111-LV)	16.5 0.3	16.9 0.4	17.0 0.5
Argon (CTR III)	0.97 0.01	0.97 0.02	0.97 .01
Oxygen (CTR III)	16.3 0.8	17.0 0.9	17.8 0.4
Oxygen (TIA-111-LV)	15.9 0.6	16.9 0.8	17.7 0.4

Table 4 Atmosphere composition of carrot samples in OPP Film

	Atmosphere Composition		
	Day 1	Day 3	Day 5
Oxygen (CTRI)	0.3 0.0	1.1 0.1	1.1 0.1
True Oxygen	0.0 0.0	0.2 0.1	0.1 0.1
Oxygen (TIA-111-LV)	0.3 0.0	0.3 0.1	0.4 0.3
Oxygen (TIA-111-LV)	1.0 0.6	0.7 0.5	0.7 0.6
Oxygen (CTR III)	0.6 0.3	0.5 0.3	0.5 0.2
Argon (CTR III)	1.0 0.02	0.92 0.01	0.95 .02

Figures 1-8 present the data on product-package compatibility. In the case of MA packaged carrot discs (Figures 1-4), use of OPP bags resulted in "true oxygen" values of zero/close to zero. Of the P-Plus films, PA 120 and PA 200 are clearly too permeable and PA60 is closest to the ideal.

In the case of nitrogen flushed "Salad Bowl Mix", previous data for P-Plus PA60 showed that oxygen levels started low (2.3%) but were up to 4.9% by Day 5. As a result, the less permeable PA90 was evaluated this time and OPP included as a control (Figures 5,6). OPP was too impermeable as expected, giving oxygen values of zero. P-Plus PA90, however, proved to be too permeable: while carbon dioxide values were low, it allowed oxygen to rise to 9% within one day of storage. This unexpected result is presumably due to seasonal differences in the respiration rate of raw materials and highlights the role of raw material variability in product-package compatibility studies.

In the case of dry "Coleslaw Mix", previous data had indicated that P-Plus PA160 was useful, but that oxygen levels remained high (9-10%). The work was repeated with PA160 and PA90 was included as a slightly less permeable alternative (Figures 7, 8). Use of PA160 resulted in lower oxygen levels this time and higher carbon dioxide levels. Although the carbon dioxide levels may be in the high range, PA160 performed letter in this instance than PA90. By Day 5 carbon dioxide values were at 30% in the PA90 bags.

The data will be discussed in relation to possible solutions to these product-package incompatibility problems.

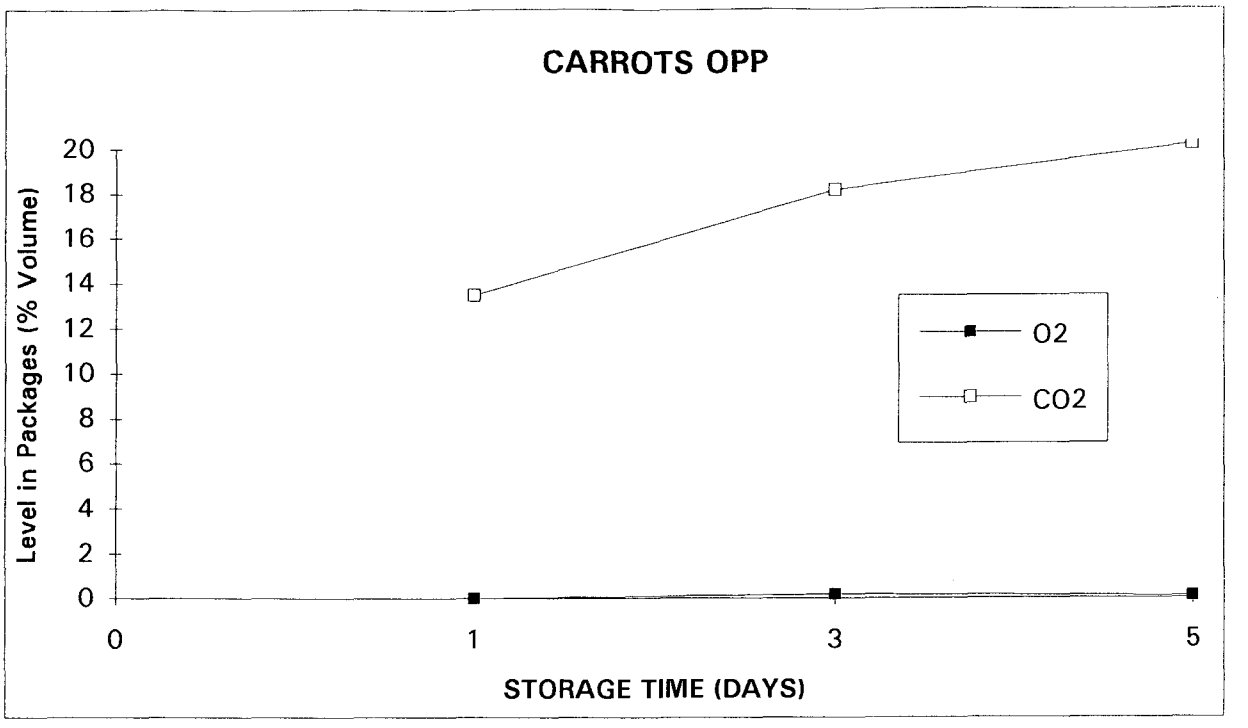


Figure 1 Changes in levels of oxygen and carbon dioxide in carrot discs packaged using oriented polypropylene (OPP)

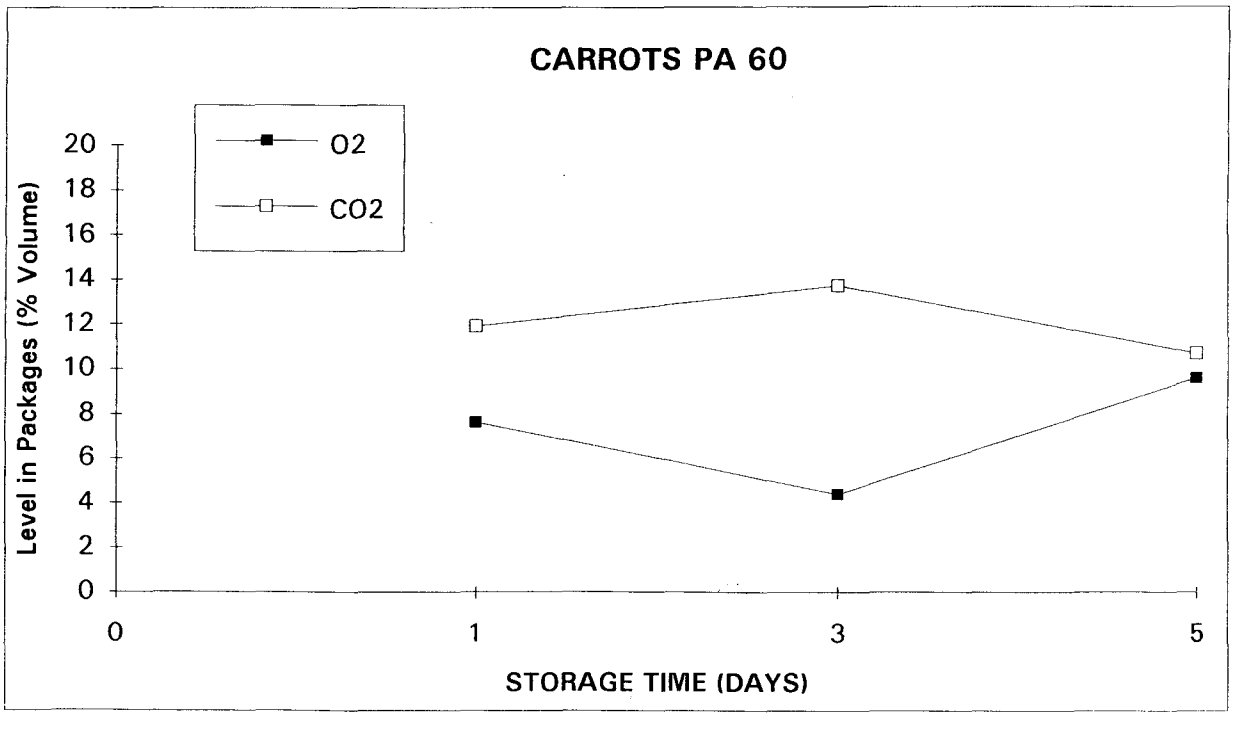


Figure 2 Changes in levels of oxygen and carbon dioxide in carrot discs packaged using P-Plus PA60

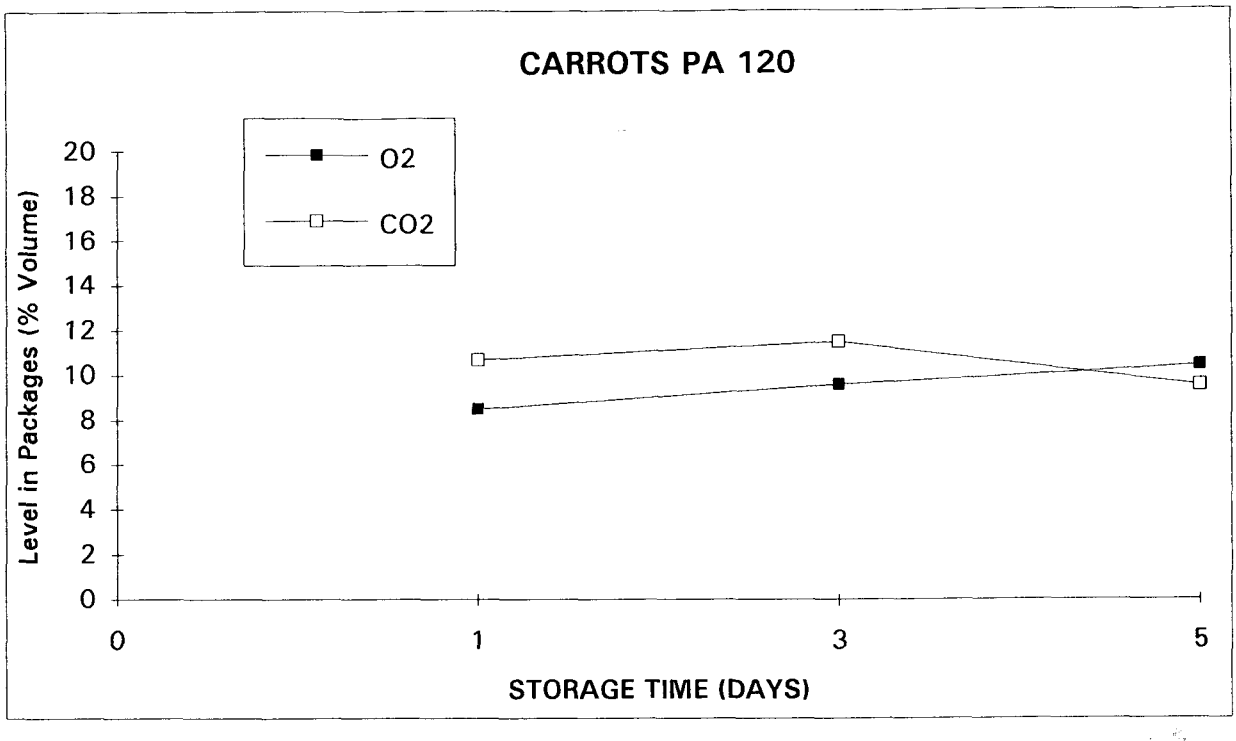


Figure 3 Changes in levels of oxygen and carbon dioxide in carrot discs packaged using P-Plus PA120

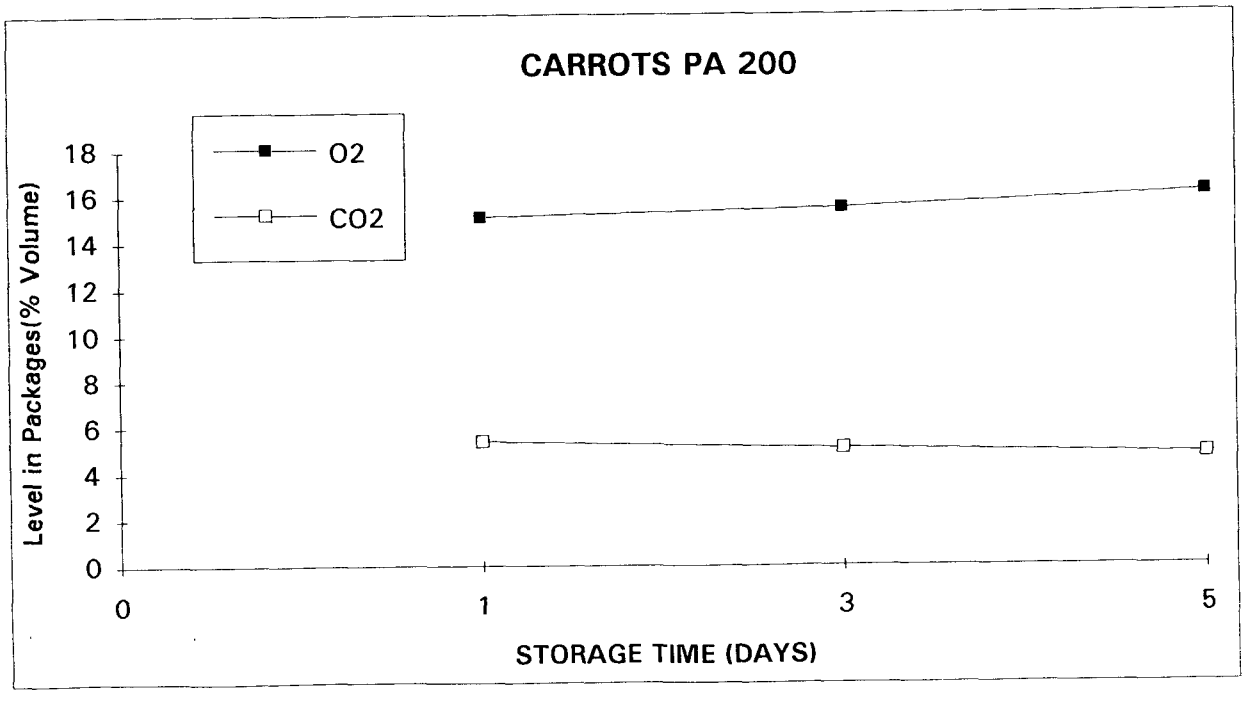


Figure 4 Changes in levels of oxygen and carbon dioxide in carrot discs packaged using using P-Plus PA200

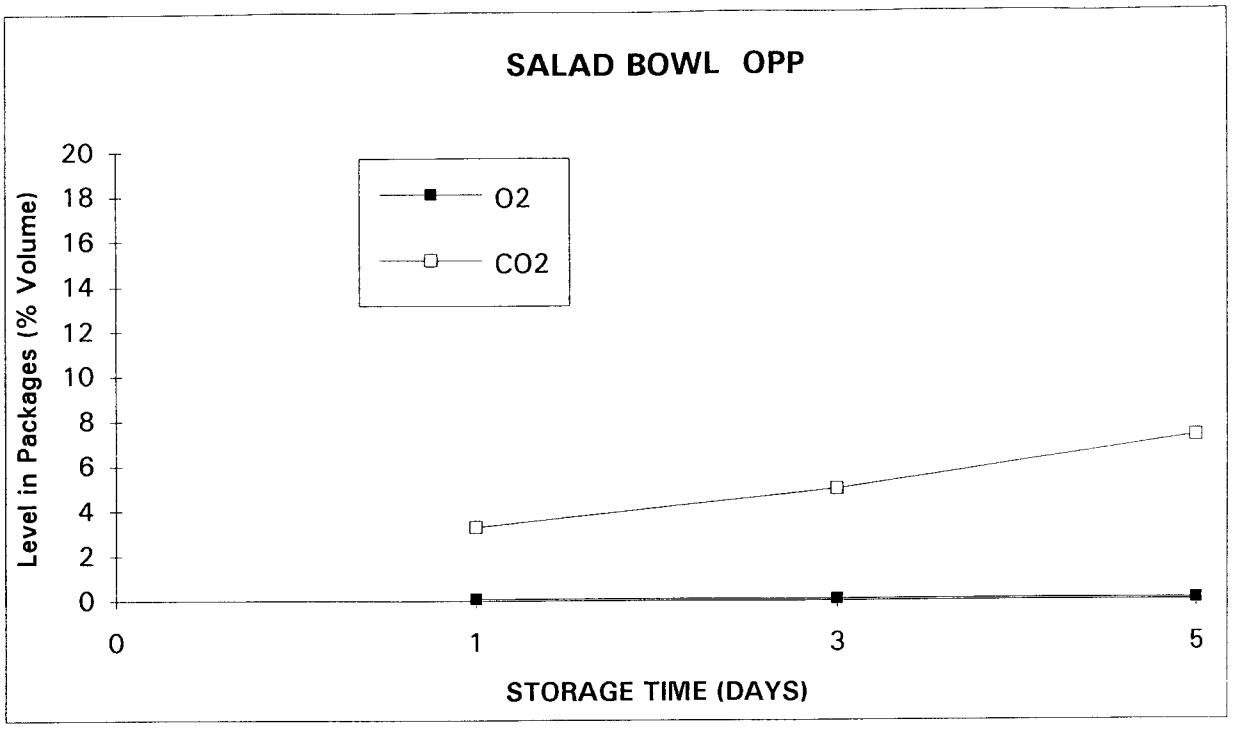


Figure 5 Changes in levels of oxygen and carbon dioxide in "Salad Bowl Mix" flushed with nitrogen and packaged using oriented polypropylene (OPP)

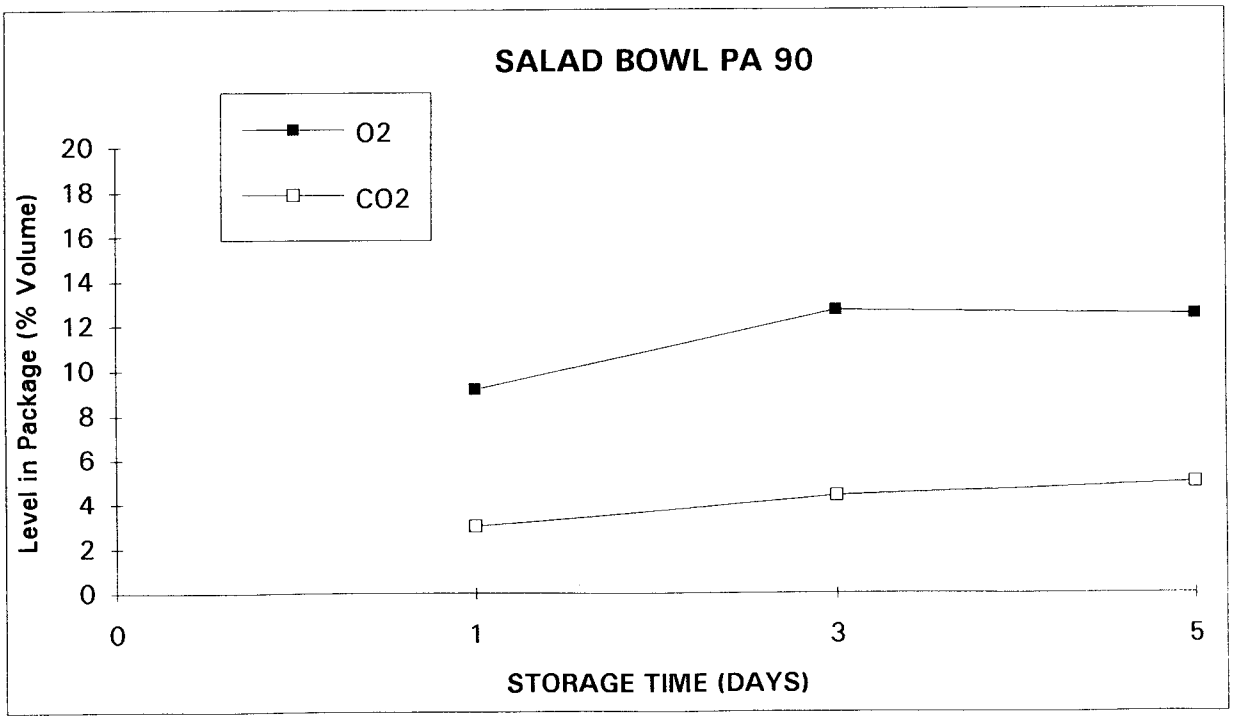


Figure 6 Changes in levels of oxygen and carbon dioxide in "Salad Bowl Mix" flushed with nitrogen and packaged using P-Plus PA90

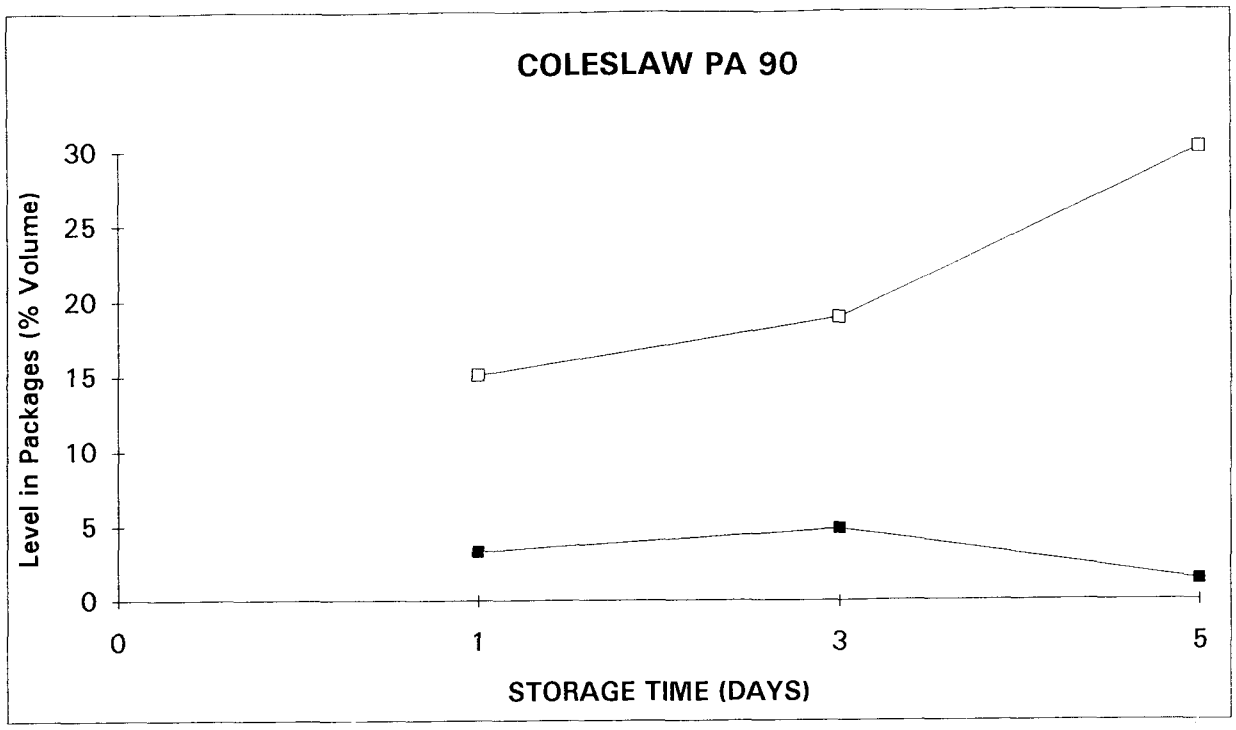


Figure 7 Changes in levels of oxygen and carbon dioxide in dry "Coleslaw Mix" packaged using P-Plus PA90

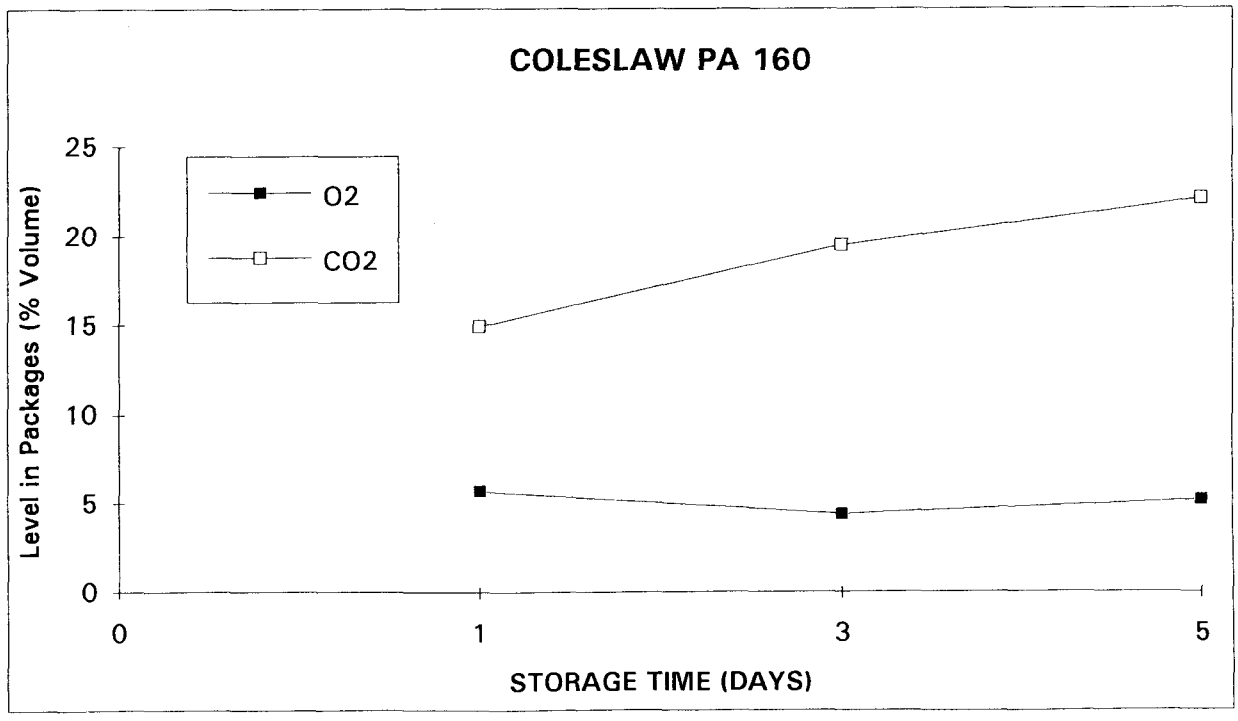


Figure 8 Changes in levels of oxygen and carbon dioxide in dry "Coleslaw Mix" packaged using P-Plus PA160

(B) EFFECTS OF RAW MATERIALS AND PREPARATION

1. Description of the research topic or practical problem

This project addresses gaps in our understanding of physiological, microbiological and nutritional consequences of minimal processing of vegetables. It will examine the effects of raw materials (cultivars and physiological age) and raw material preparation (cutting, peeling, washing/dipping and storage) on microbial growth, acceptability and nutrient retention. The data obtained will contribute to the development of a systems approach to optimise quality and storage-life of minimally processed products.

2. Goal of Research

To optimise raw material selection and processing procedures for MA packaged ready-to-eat vegetables using shredded lettuce, grated carrots and carrot discs as examples. To quantify the effects of controllable variables on respiration rate, microbial growth, changes in sensory quality and nutrient content.

3. Short description of the approach

For the product examples selected the following will be evaluated:

- the effects of cutting/peeling technology
- the effects of washing/dipping procedures
- the effects of important cultivars
- the effects of physiological age of intact raw materials
- the effect of packaging film and storage temperature

Opportunities to slow physiological ageing, microbial growth, loss of sensory quality, and nutritional value will be identified.

4. Experimental approach in phases

Year 1

Investigation of effects of cutting, peeling and washing/dipping on the deterioration (respiration, microbial growth and sensory aspects) of MAP processed vegetables (using carrot discs as a model product). Development of optimum processing procedure.

Year 2

The effects of physiological age and processing procedures (cutting) on deterioration (respiration rate, microbial growth and sensory aspects) of MAP carrot discs and shredded lettuce. Further work on the washing/dipping of shredded lettuce.

Year 3

Further work on the effects of dipping in chlorine, cutting and physiological age on the quality and storage life of the carrot discs was examined. Also the effects of cultivar type and storage temperature on deterioration (respiration rate, microbial growth and sensory aspects) were examined in an attempt to optimise raw material selection. Determination of effects of minimal processing and storage on ascorbic acid levels in shredded lettuce was monitored.

Year 4 Further examination of the effects of the peeling and cutting steps on quality deterioration, by examination of these products on a more fundamental level. Also examination of the effects of storage temperature and packaging film on the quality and storage life of MAP grated carrots.

Consolidation of work targeted for Years 1-3, and examination of these results using multivariate statistical analysis (Principal Component Analysis).

5. State-of-the-Art

There are major gaps in our understanding of the physiological, microbiological and nutritional consequences of minimal processing of vegetables. Because these products are highly perishable and the scope for use of antimicrobial chemicals is very limited, it is essential to understand the effects of variables within industry's control.

Processing of fresh vegetables causes the destruction of surface cells and the bruising of underlying tissues. Enzymatic reactions in damaged cells are responsible for off-flavour development, discolouration and loss of firmness. Respiration rate is increased and ethylene synthesis can be activated. Differences in rates of deterioration as measured by respiration rate, microbial growth and sensory scores are related to the extent of tissue damage. Differences in spoilage rates between cultivars appear to be related to differences in susceptibility to tissue damage.

Microflora responsible for the spoilage of ready-to-use vegetables include a large number of fungi, yeast and bacteria species. Among gram negative species *Pseudomonads* and *Enterobacteriaceae* are the most important. Dipping cut vegetables in solutions containing chlorine (up to 100 ppm) may retard microbial growth as may dipping in solutions of citric acid/ascorbic acid. Little data is available on the fate of native ascorbic acid in stored ready-to-use vegetables.

These considerations have led to the research approach proposed. Initially it will involve a detailed investigation of the effects of cutting and washing/dipping on product deterioration. Once an optimum process has been defined, the effects of differences due to cultivar and physiological age will be determined. In order to obtain information on the effects of minimal processing on nutrients, the levels of ascorbic acid and dehydroascorbic acid will be monitored during storage.

C. *Overview of work performed from 1-1-96 to 30-6-96*

In this period, a synthesis of the results obtained so far was performed and further work was carried out on the effects of processing on ascorbic acid levels in prepared lettuce.

Data on respiration rates was assembled and shared with other partners.

Materials and Methods

Prepared iceberg lettuce was dipped in 100ppm chlorine and packaged in OPP bags with or without nitrogen flushing as described previously. In this case three cutting treatments were used:

(a) leaves were manually torn to approx 2.5cm square pieces
(b) leaves were shredded using a sharp knife (6mm shreds)
(c) leaves were machine shredded (Sammic CA300, 6mm shreds).

Ascorbic acid levels were determined as before using 2,6 dichlorophenol-indophenol. Dehydroascorbic acid was determined after reduction using homocysteine. Ascorbic acid and dehydroascorbic acid levels were also determined using an enzymatic kit (Boehringer Mannheim)

Results and Discussion

The main conclusions from the synthesis were:

1. There was an effect of cultivar used on quality. Carrot discs prepared using the cultivar Nairobi were of a higher quality and had longer shelf-lives than those prepared using the cultivar Narbonne. It is unclear precisely what this was due to,difference in respiration rates were recorded but no significant differences inmicrobial loads were observed. Further work is required to compare the intrinsic perishability of the different cultivars.

2. Carrot discs produced from unstored carrots were of higher quality and more acceptable from Day 1 and throughout storage, than those prepared using the stored vegetables. This appears to be due to the higher microbial loads, exudate production and cell permeability in products prepared from the stored vegetables.

3. Abrasion peeling caused a lot of cellular disruption, which was evident from the higher respiration rates, and higher levels of carbon dioxide within the packs, and from the microscopic evidence. Microbial loads were also higher on abrasion peeled products, and proliferated at higher rates leading to increased rates of off-odour development. In addition, there was a greater decline in the appearance of abrasion peeled products over storage. This can be attributed to the drying out and suberisation caused by this severe processing method.

4. In relation to commercial practice, this work shows that abrasion peeling can be improved by the use of a fine carborundum plate, which results in lower respiration rates and a reduction in some microbial loads.

5. The magnitude of these effects are dependent on the physiological age of the raw material used. Hand peeling carrots greatly enhanced the quality of MA packaged carrot discs and extended the shelf-life by two days over coarse abrasion and one day over fine abrasion peeling, when the unstored vegetables were used. This extension of storage life was also found when stored carrots were used, hand peeling increasing it by two days over both fine and coarse abrasion peeled products. This was due to the effects of physiological age being greater than those caused by the abrasion plate type.

6. The addition of chlorine (100 pm) to the wash water had some beneficial effects on during storage. For the products prepared from stored carrots, chlorine reduced microbial loads slightly and the effect lasted throughout storage.

7. Overall the beneficial effects of adding chlorine to the washing solution were small when examined in the laboratory and, under commercial practice, the effects may be even smaller.

8. For carrot discs, hand slicing carrots greatly enhanced the quality of these products. The manual slicing step caused less cellular disruption, which was evident from the lower respiration rate, and hence lower levels of carbon dioxide within the packs, and also from the microscopic evidence. Microbial loads were also lower on the hand sliced products, and did not proliferate at the rates found within the packs containing machine sliced carrots. This higher level of microflora increased the rate of off-the machine sliced products over storage, can be attributed to the drying out and suberisation caused by the severe processing step.

9. The magnitude of these effects were dependent on the physiological age of the raw material used. When unstored carrots were examined a difference between the two machine blades could be detected, the sharp blade produced higher quality products. When the stored carrots were used as raw material, the effect of physiological age was greater than those caused by the machine blade type, and as a result these effects were lost.

10. For shredded iceberg lettuce it was found that rotation of the blade had a greater effect on quality than the blade condition, i.e sharp versus blunt.

11. Of the films examined for packaging grated carrots the film with a permeability of 25K lO_2/m/day seems the most suitable followed by oriented polypropylene. (OPP)

12. Ascorbic acid levels in unpacked lettuce decreased at a faster rate than for OPP packaged lettuce. Nitrogen flushing before sealing enhanced retention.

13. Packs of shredded lettuce stored at 3°C retained much higher levels of ascorbic acid than those stored at 8°C.

14. At low temperatures the physiological activity and microbial growth of carrot tissue is slowed down and thus packs stored at 3°C received significant higher scores than those stored at 8°C.

In this period, both dehydroascorbic acid and ascorbic acid were measured in MA packaged prepared iceberg lettuce. Previous data showed ascorbic acid loss to be very rapid during storage. However, degradation of ascorbic acid proceeds via the production dehydroascorbic acid and this reaction is reversible. As a result, the nutritional significance of storage of MA-packaged lettuce may be over estimated if only ascorbic acid is measured. This is illustrated in Table 5.

Table 5 Effects of temperature and storage time on levels of ascorbic acid and dehydroascorbic acid in MA packaged shredded iceberg lettuce flushed with N_2.

		DAY 1	DAY 3	DAY 6	DAY 8	DAY 10
3°C	Ascorbic acid	18.0	14.8	10.7	7.8	7.0
	Dehydroascorbic acid	4.5	5.4	7.0	3.7	6.3
8°C	Ascorbic acid	16.1	11.8	8.1	7.0	6.5
	Dehydroascorbic acid	5.0	6.3	7.5	7.0	5.0

means for 3 determinations

Figures 9-13 report the effects of cutting methods and package atmosphere on changes in levels of ascorbic acid and dehydroascorbic acid levels. In general, rises in dehydroascorbic acid levels paralleled losses in ascorbic acid, though they are smaller in magnitude. Relatively high levels of dehydroascorbic acid developed in unsealed packages. As before there were major effects of gas atmosphere, storage temperature and cutting method on ascorbic acid retention.

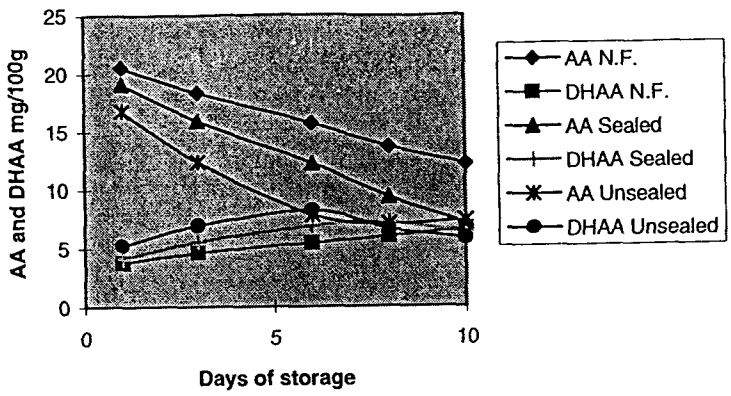


Figure 9 Effects of packaging atmosphere on ascorbic and dehydroascorbic acid levels in prepared, iceberg lettuce stored at 3°C (lettuce torn to 2.5cm squares).

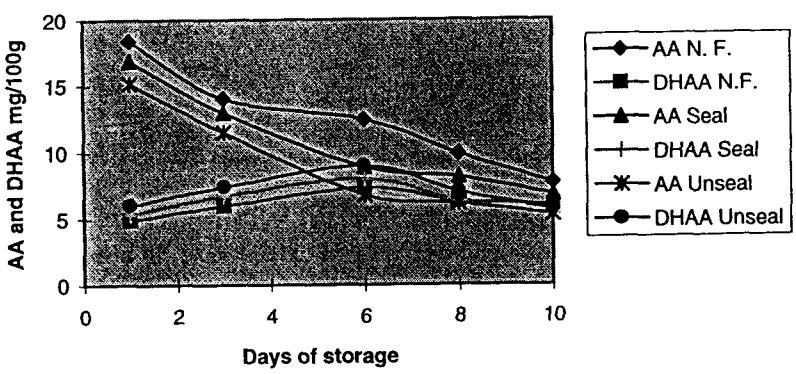


Figure 10 Effects of packaging atmosphere on ascorbic acid and dehydroascorbic acid levels in prepared iceberg lettuce stored at 8°C (lettuce torn to 2.5cm squares).

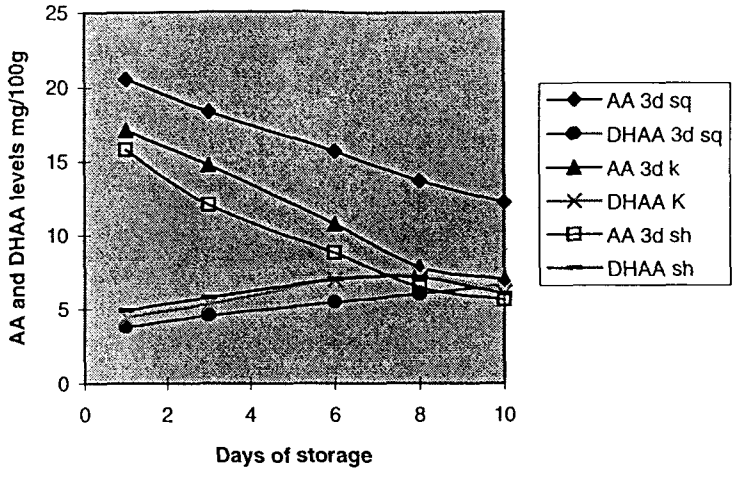


Figure 11 Effects of cutting method on ascorbic acid and dehydroascorbic acid levels in prepared iceberg lettuce (3°C, nitrogen flushed).

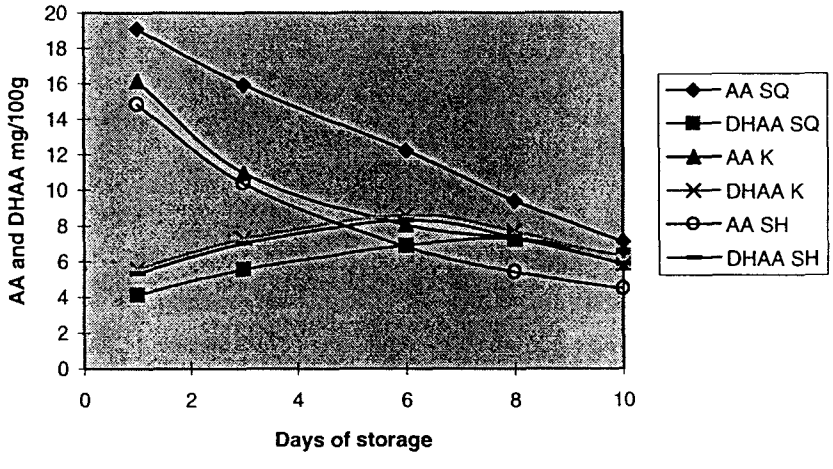


Figure 12 Effects of cutting method on ascorbic acid and dehydroascorbic acid levels in prepared iceberg lettuce (3°C, product modified atmosphere).

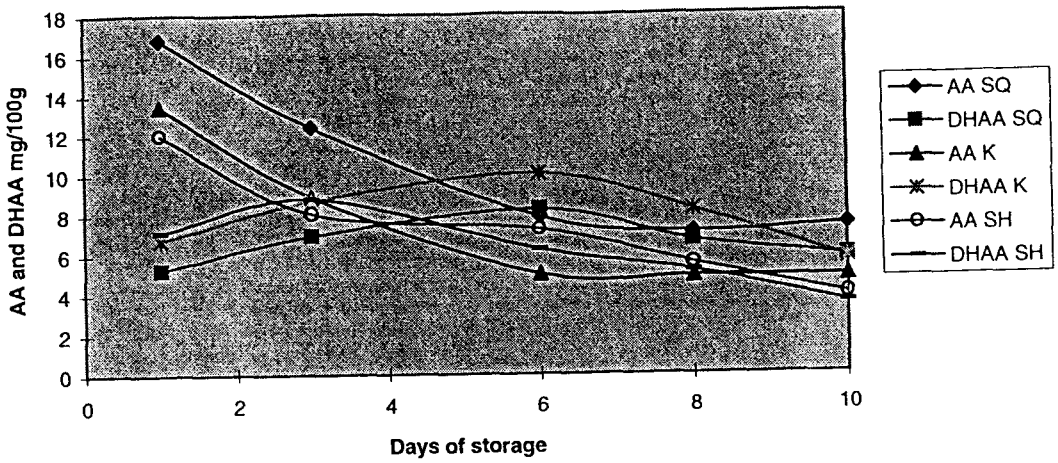


Figure 13 Effects of cutting method on ascorbic acid and dehydroascorbic acid levels in prepared iceberg lettuce stored in air (3°C).

D. 1. Description of work planned for the subsequent part of the project

1-7-96 to 31-12-96

Collaboration on effects of packaging and coatings on quality

Publications/Disseminations

Barry-Ryan, C., L. Doyle and D. O'Beirne, 1995. Ascorbic acid retention in minimally processed lettuce. Irish Journal of Food Science and Technology 34(2), 225.

Francis, G. A. and D. O'Beirne, 1995. Survival and growth of pathogens on modified atmosphere packaged ready-to-use lettuce. Irish Journal of Food Science and Technology 32(2), 221.

Lynch, M. and D. O'Beirne, 1995. Effects of modified atmosphere packaging of whole potatoes on reducing sugar levels and non-enzymatic browning in potato chips. Irish Journal of Food Science and Technology 34(2), 223.

O'Beirne, D. 1996. Modified atmosphere packaging of ready-to-use vegetables. Proceedings "Showcase for modified atmosphere packaging of foods (University of Limerick. May, 1996): 1-16.

O'Beirne, D., 1996. True oxygen concentrations in modified atmosphere packages. VR Interpack 96 Special: E40.

Participant 9: Nature's Best Limited
Carnes West
Duleek
Co Meath

A.1 ***(sub) - Project Title***

Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild preservation techniques.

2. ***Project Co-ordinator***
Paddy Callaghan

3. ***Project participants and 4. Resources***
Anne-Marie Kierans
Marloes Bos
Wypke Ero

B.1 ***Description of Research Topic of Practical Problem***

Providing production level microbiological analysis data of ready-to-eat food products subjected to mild preservation techniques. The main preservation technique employed by Nature's Best is chilled storage and distribution and therefore chill chain maintenance throughout the product life cycle i.e. from raw material intake, through storage, processing and distribution to retail outlet.

2. ***Goal of the research***

To build up a database of information and deduce optimum production methods, packaging materials and codes of practice resulting in safer high quality products with extended shelf-life.

3. ***Short description of approach***

Samples of three products:-

(a) Prepared Iceberg Lettuce packed in standard OPP film with nitrogen gas flushing.

(b) Spinach packed in PA200 films (pillow packs)

(c) Dry Coleslaw Mix (shredded white cabbage and carrot mixed in the ratio of 88:12) packed in stretch film.

were taken from production lines and stored at 4^0C. Samples of these three products were also taken from supermarkets two days before the end of shelf-life and returned to Nature's Best kept at the same temperature as the samples from the production line and both sample types were analysed at the end of the shelf-life.

Table 1 - Analysis Conducted Over Life:

Day	Microbiological	Sensory	Oxygen Level
P	*	*	*
P + 1			
P + 2			
P + 3			
P + 4	*	*	*
P + 5			
P + 6	*	*	*

(Note: P = Day of Production, P + 1 = One day after production etc.)

* Lettuce and Spinach produced by Nature's Best is washed using chlorine/pH controlled washing equipment. According to this system an improved lettuce can be attained in terms of general appearance, microbiological levels and consumer appeal. Product discoloration is retarded by packing in nitrogen gas flushed packs, where an oxygen level < 3% is sought.

* To show the importance of the chill chain Iceberg Lettuce was followed from raw material intake, through the wash system, packing distribution and chilled storage.

* Gas Flushing of beansprouts using 21% CO_2, 20% O_2 and 59% N_2 was also tried using two different film types.

4. *Technical data*

Table 2 shows the technical data for the films used.

	Stretch Film	PA200	PA120	Standard OPP
Film Gauge	14.5 um	35 um	35 um	35 um
O_2 Permeability ($cc/m^2/day/bar$)	9,600	54,000	17,000	1500

Table 3 - Dimensions of Packaging

Product	Pack Size (cm)
Prepared Lettuce (200g)	20 x 20
Spinach (200g)	35 x 20
Dry Coleslaw Mix (300g) Stretch Wrapped Tray	22 x 17 x 4.5

C.1 *Overview of Work Carried Out Before 1.7.95 and 1.12.95*

In the period from 1.1.96 to 30.5.96, Nature's Best have produced a variety of production level microbiological, oxygen and sensory results from analysis on a range of prepared vegetable products. These include products packaged in orientated OPP via an Ishida multi-head weigher and Ilapak vertical form fill seal packaging machine and products in trays stretch wrapped with PVC stretch film via an Omori Stretch Wrapper

Tables 4.1 to 4.4 and figures 1.1 to 1.4 show Oxygen levels obtained over life on selected products. Tables 5.1 to 5.4 show sensory analysis results for selected lines during shelf life. Tables 6.1. to 6.4 and figures 2.1 to 2.4 show results for microbiological analysis conducted on selected products.

Table 4.1 Percentage Oxygen in Beansprouts Packed in PA120 and PA160 films with and without Gas Flushing

	% Oxygen Level				
	P	**P+1**	**P+3**	**P+7**	**P+8**
PA120GF	20	16.7	14.3	14.2	14.0
PA120NGF	20.2	15.3	14	13.8	13.3
PA160GF	20.0	16.2	15.7	15.5	13.2
PA160NGF	20.2	16.0	14.4	14.4	14.0

GF = Gas Flushed
NGF = Not Gas Flushed

Table 4.2 Percentage Oxygen in Iceberg Lettuce Packed in Standard OPP

	% Oxygen Level			
	P	**P+1**	**P+4**	**P+6**
In-house Packs	2.7	2.7	1.8	1.6
Retail Packs	2.7	2.5	1.9	1.7

Table 4.3 Percentage Oxygen in Spinach Packed in PA200

	% Oxygen Level			
	P	**P+1**	**P+4**	**P+6**
In-house Packs	20.2	20.1	19.1	19.0
Retail Packs	20.2	20.1	20.0	20.2

Table 4.4 Percentage Oxygen in Dry Coleslaw Mix Stretch Wrapped

	% Oxygen Level			
	P	**P+1**	**P+4**	**P+6**
In-house Packs	20.2	20.1	19.8	17.7
Retail Packs	20.2	20.1	20.0	19.8

Table 5.1 Sensory Results on Beansprouts Packed in PA120 and PA160 with and without Gas Flushing

1=Poor 5=Excellent

	Visual					Aroma				
	P	**P+1**	**P+3**	**P+7**	**P+8**	**P**	**P+1**	**P+3**	**P+7**	**P+8**
PA120GF	5	5	5	3.5	3	5	5	5	2.5	1.5
PA120NGF	5	5	5	4	3	5	5	4.5	3.5	2
PA160GF	5	5	5	3.5	3	5	5	4.5	3	2
PA160NGF	5	5	5	3.5	3.5	5	5	4.5	3	1

GF = Gas flushed with 21% CO_2, 20% O_2, 59% N_2
NGF = Not gas flushed

Table 5.2 Sensory Results on Iceberg Lettuce Packed in Gas Flushed Standard OPP

	P	**P+4**	**P+6**
In-house Packs	5	5	4
Retail Packs	5	5	4

Table 5.3 Sensory Results on Spinach Packed in PA200

	Visual			Aroma		
	P	**P+4**	**P+6**	**P**	**P+4**	**P+6**
In-house Packs	5	4	4	5	5	5
Retail Packs	5	4	4	5	5	5

Table 5.4 Sensory Results on Dry Coleslaw Mix Packed in Stretch Film

	Visual			Aroma		
	P	**P+4**	**P+6**	**P**	**P+4**	**P+6**
In-house Packs	5	4.5	4	5	4	3.5
Retail Packs	5	4	3.5	5	4	4

Table 6.1 Microbiological Results of Beansprouts Packed in PA120 and PA160 with and without gas flushing

Day		PA120GF	PA120NGF	PA160GF	PA160NGF
P	TVC log cfu/g	7.08	7.08	7.08	7.08
	Yeast & Mould log cfu/g	3.99	3.99	3.99	3.99
P+6	TVC log cfu/g	7.11	7.15	7.18	7.15
	Yeast & Mould log cfu/g	4.11	4.43	4.30	4.43
P+8	TVC log cfu/g	7.40	8.15	7.60	8.34
	Yeast & Mould log cfu/g	6.20	8.23	7.26	4.15

GF = Gash Flushed
NGF = Not Gas Flushed

Table 6.2 Microbiological Results of Iceberg Packed in Standard OPP and Nitrogen Gas Flushed

Day	Temperature ^{0}C	TVC log cfu/g	Yeast & Mould log cfu/g
Delivery	4.7	2.48	1.30
Day after delivery	4.7	4.84	1.30
Cut	-	5.62	1.90
Washed	-	3.70	0*
Rinsed	-	2.74	1.30
Production	-	3.70	2.15
P+4 In-house Packs	-	4.25	2.90
P+4 Retail Packs	-	5.09	3.00
P+6 In-house Packs	-	4.95	3.00
P+6 Retail Packs	-	5.39	3.25

* No growth observed on 10^{-1} dilution.

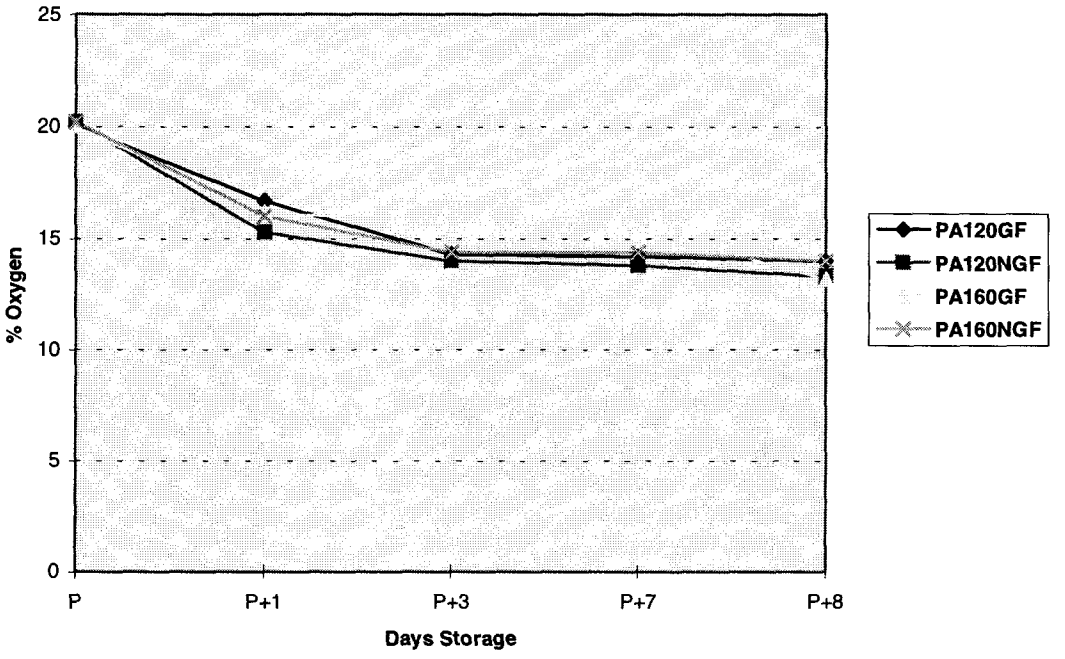
Table 6.3 Microbiological Results of Spinach Packed in PA200

	In-House Packs			Retail Packs		
	P	**P+4**	**P+6**	**P**	**P+4**	**P+6**
TVC log cfu/g	3.25	4.28	5.08	3.25	4.3	5.60
Yeast & Mould log cfu/g	2.78	3.71	3.78	2.78	3.3	4.33

Table 6.4 Microbiological Results of Dry Coleslaw Mix Packed in Stretch Wrapped Film

	In-House Packs			Retail Packs		
	P	**P+4**	**P+6**	**P**	**P+4**	**P+6**
TVC log cfu/g	4.50	5.84	6.90	5.81	6.02	6.98
Yeast & Mould log cfu/g	3.47	4.07	5.23	3.47	3.84	5.90

Fig. 1.1 Percentage Oxygen in Beansprouts Packed in PA120 & PA160 Films with and without Gas Flushing



GF = Gas Flushed with 21% CO_2, 20% O_2 and 59% N_2
NGF = Not Gas Flushed

Fig. 1.2 Percentage Oxygen in Iceberg Lettuce Packed in Standard OPP

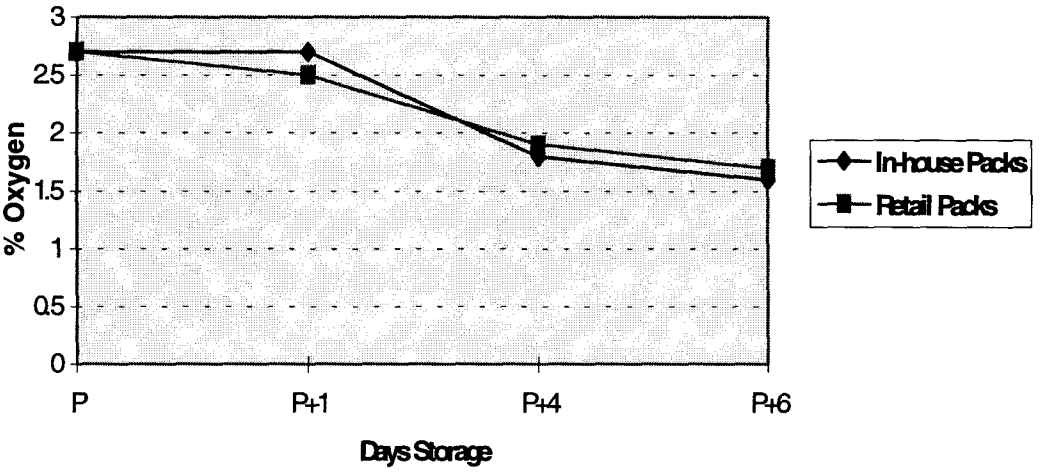


Fig. 1.3 Percentage Oxygen in Spinach Packed in PA200

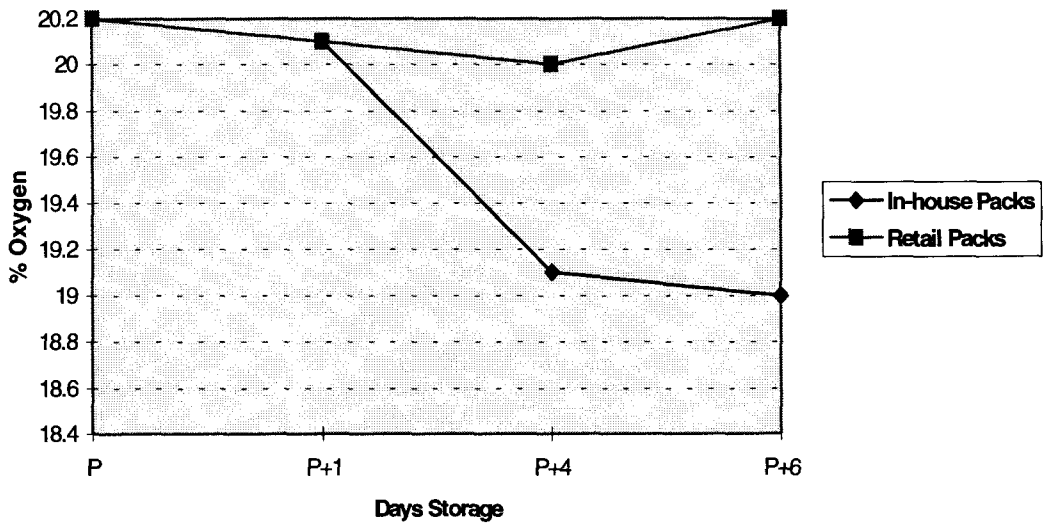


Fig. 1.4 Percentage Oxygen in Dry Coleslaw Mix Stretch Wrapped

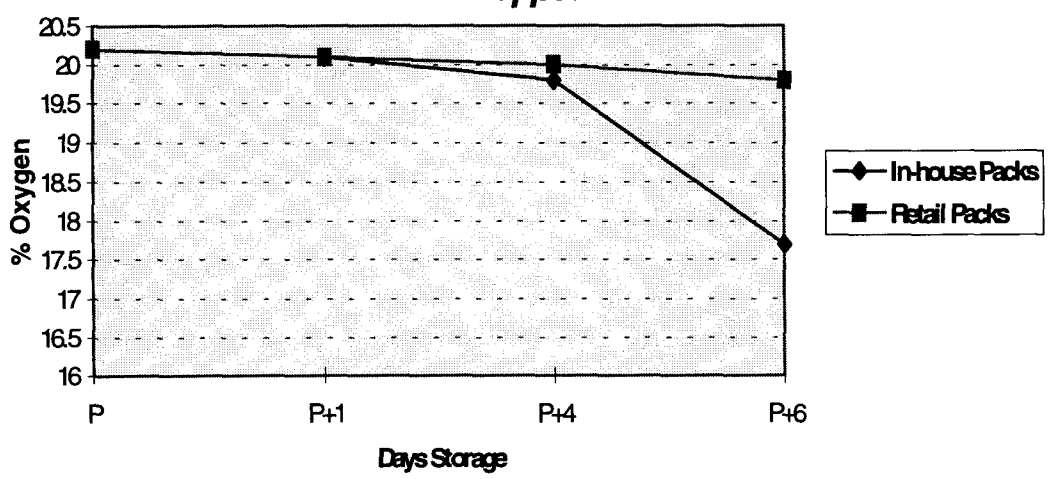
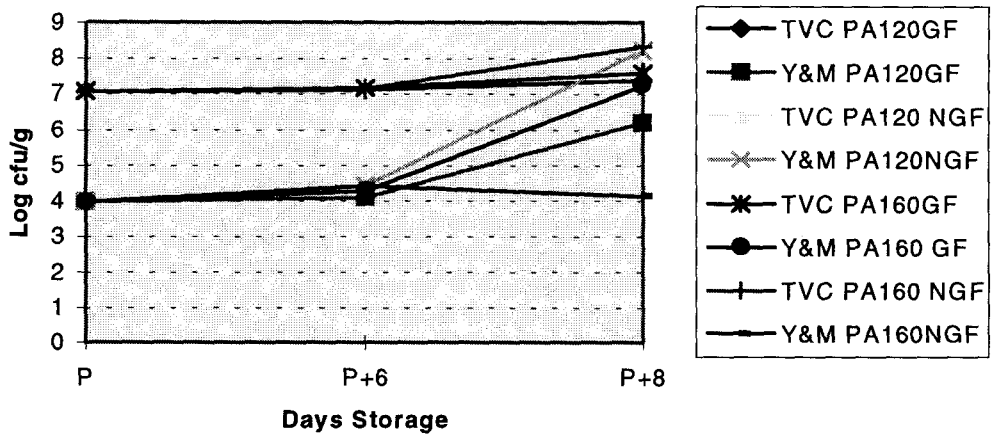


Fig. 2.1 Microbiological Results of Beansprouts Packed in PA120 & PA160 with and without Gas Flushing



GF = Gas Flushed with 20% 0_2, 21% $C0_2$ and 59% N_2
NGF = No Gas Flushing

Fig. 2.2 Microbiological Results of Iceberg Lettuce Packed in Standard OPP with Nitrogen Gas Flushing

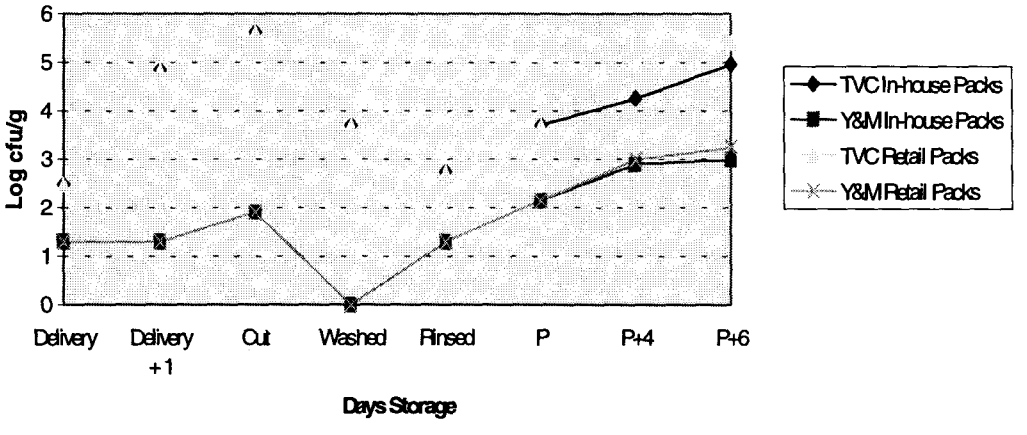


Fig 2.3 Microbiological Results of Spinach Packed in PA200

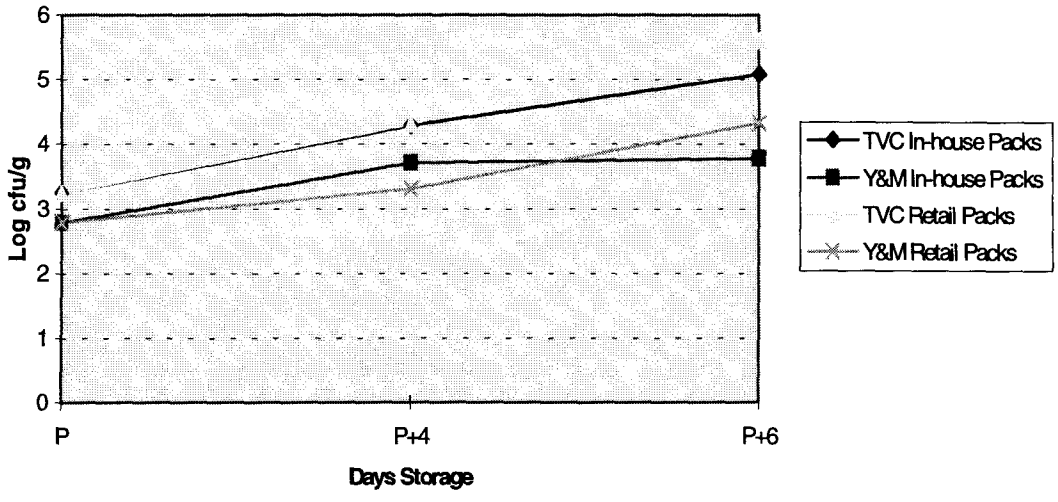
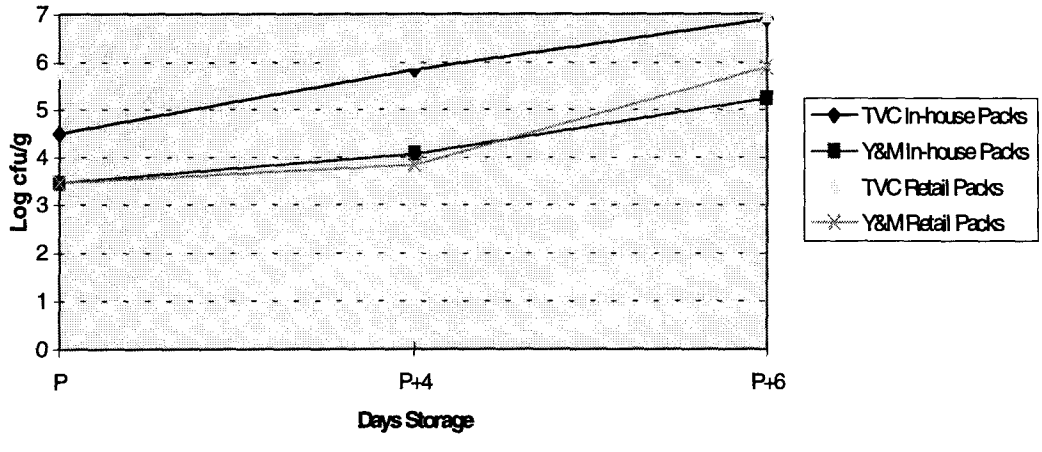


Fig 2.4 Microbiological Results in Dry Coleslaw Mix Packed in Stretch Wrap Film



Conclusion:

Beansprouts:

Gas flushing of beansprouts trial was discontinued after 8 days due to the poor sensory analysis results obtained. PA120 without gas flushing and PA160 with gas flushing gave the best results with regard to this attribute. Microbiological analysis results did not improve under these modified conditions.

Prepared Iceberg Lettuce:

There were no differences in the results for all analysis conducted on this product. There was no obvious break in the chill chain which may account for the similarity in the results.

Spinach:

There were no differences in the results for all analysis conducted on this product. There was no obvious break in the chill chain which may account for the similarity in the results.

Dry Coleslaw Mix:

This product gave different scores for the sensory analysis in retail and in-house packs at the end of life. It is not thought that conditions under which the product was stored are responsible for this but rather that this is a difficult product to pack since it requires a high permeability for good microbiological results, however this will give poor visual appearance (slight oxidation). For good visual appearance low permeability is best but this results in poor microbiological results. Seasonal material variations also contribute to the Quality Parameters.

D1. *Work Planned for the Period 1.7.96 to 1.12.96*

Nature's Best will:

a) Continue to provide microbiological data on products.
b) Continue to investigate the importance of the chill chain.
c) Investigate, at the request, of other participants the feasibility of their research in a production environment

Proposed schedule of meetings

During the course of the four year project, the group will meet twice a year for approximately 1½ day. The following table gives tentative dates and locations of these meetings.

Provisional date	Host	Location
* 7/8 - 1 - 1993	ATO.DLO	Wageningen, the Netherlands
* 17/18 - 6 - 1993	AFRC.IFRN	Norwich, U.K.
* 16/17 - 12- 1993	INRA.AV	Montfavet, France
* 16/17 - 6 - 1994	ULMK.DCLS	Limerick, Ireland
* 15/16 - 12 - 1994	WAU.DFS	Wageningen, the Netherlands
* 15/16 - 6 - 1995	NARF	Athens, Greece
* 4/5 - 1 - 1996	CIRAD.SAR	Montpellier, France
* 13/14 - 6 - 1996	INRA.AV	Le Muy, France
19/20 - 12 - 1996	ATO.DLO	Wageningen, the Netherlands

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