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

A SHARED-COST PROJECT

2ND MEETING AT AFRC-IFR, NORWICH, UNITED KINGDOM
16 AND 17 JUNE 1993
RESEARCH AND MANAGEMENT PLANNING
TO BE DISCUSSED AT THE 2ND MEETING
AT AFRC-IFR, NORWICH, UNITED KINGDOM
ON 16 AND 17 JUNE 1993

EC-AIRI-CT92-0125
IMPROVEMENT OF THE SAFETY AND QUALITY OF
REFRIGERATED READY-TO-EAT FOODS USING
NOVEL MILD PRESERVATION TECHNIQUES
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Agenda of the 2nd Group Meeting

17 June 1993

13.30 Arrival at AFRC.IFR. Welcome and outline of the aims for the second group meeting.
14.00 Presentation ATO.DLO (Marjon Bennik & Leon Gorris)
14.30 Presentation LUW (Annette Verheul & Frans Rombouts)
15.00 Presentation INRA.AV & FRUID (Christophe Nguyen-Thé & Frederic Carlin)
15.30 Presentation CIRAD.SAR (Stéphan Guilbert)
16.00 Presentation NARF (George Nychas)
17.00 Presentation AFRC.IFR (Barbara Stringer & Mike Peck)
17.00 Presentation UL.DCLS & NBEST (Ms. C. Barry, David O’Beirne & Paddy Callaghan)
18.00 Closing of first day.
19.30 Group diner.

18 June 1993

09.00 Opening of day 2.
09.15 Demonstrations of research at AFRC.IFRN related to the project
10.15 Coffee/tea break
10.30 Open discussion. Subjects: course of the project, planning and coordination, standardisation of microbiological and product physiological assays, etc. Preparation of 1st official EC report (end of december 1993) with research and administrative details.
12.30 Lunch.
13.30 Continuation of the open discussion from the morning.
15.00 Closing of meeting.

Note: Presentations should preferably include: objectives, general approach and timescale; achievements and bottlenecks; deviations from the original plan expected (with underlying reasons). The planning of the second half of 1993 should be discussed in more detail; When appropriate, (new) links and cooperations with fellow participants and possibly other EC-projects. Also: Options for use of Travelling & Mobility Grants in AIR or generally. Dissemination policy and plans, publications etc.
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-protoctolytic Clostridium botulinum). Some mesophilic 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-protoctolytic 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:

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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 pour le Développement pour son Département 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).
AFRC.IFRN: Institute of Food Research (IFRN), Norwich, United Kingdom (participant 6).
ULMK.DFS: Department of Chemical and Life Sciences (DCLS), Universtiy 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).
A 1. (Sub-)Project title:
(a) Microbial quality and safety of mildly preserved, fresh or freshly processed vegetables
(b) Systematic investigation of the quality of fresh or freshly processed vegetables under modified atmospheres
(c) Sensory analysis of mildly preserved, vegetable products (no description given yet)
(d) Compilation of an integrated computer model (no description given yet)

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

3. Project participants and 4. Resources:
Ir. Marjon H.J. Bennik 12 months/year
Mr. Werner Voorstman 12 "
Dr. Leon G.M. Gorris 3 "
Ir. Herman W. Peppelenbos 2 "
Mrs. Ria vd Vuurst d Vries 1.5 "

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:

Modified atmosphere packaging (MAP) and refrigerated storage 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 microflora of lactic acid bacteria are 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 on 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 usefull 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 (O₂) and high in carbon dioxide (CO₂), with typical values at equilibrium of 2-3% and 5% respectively (Kadar et al., 1989). The composition of the atmosphere is the resultant of the initial gas composition, the gas exchange through the packaging material and the respiratory activity of the produce (Gorris & Peppelenbos, 1992). 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% O₂, 0.04% CO₂ and 78% N₂) but at a reduced partial gas pressure. Thus, the available amount of O₂ is about one third of the normal. As with MAP, the lower O₂ 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 witloof were used as the model products.
C. I. Overview of the work performed from 1-1-1993 to 30-6-1993

I. Population dynamics & safety of MVP system

Materials & methods

Packaging under moderate vacuum
The Moderate Vacuum Packaging system used in this study was the Food Saver®, designed by Vacu Products (Delft, the Netherlands). It consists of a rigid plastic container and a lid. The lid holds a rubber stopper through which air is evacuated as well as a thermoplastic rubber ring that provides a tightly closed container at 400 mB or below. The MVP containers were evacuated to 400 mB by use of a vacuum pump provided with a pressure gauge. Containers filled with produce were stored at 4, 7, 12 and 20°C. As a control, containers without a rubber stopper were stored in which the gas composition was air at ambient pressure.

Evaluation of microbial population dynamics
All produce used was purchased from a local grocery. Deterioration of a produce due to microbial spoilage was monitored by sampling at regular time intervals throughout the storage period and measuring the size of relevant spoilage subpopulations of the microflora (i.e. total aerobic count, enterobacteria, yeasts, lactic acid bacteria) using standard methods (Mossel & Jacobs-Reitsma, 1991).

Pathogen challenge tests
In order to evaluate the possible survival of human pathogens, mung bean sprouts were inoculated by carefully spraying a suspension of a pathogen at the start of the storage period. Tests were performed with Yersinia enterocolitica, Salmonella typhimurium and Bacillus cereus. All strains were obtained from the culture collection of the Department of Food Science of the Agricultural University of Wageningen. With Y. enterocolitica and S. typhimurium, nalidixic acid resistant strains were used as inoculum. Resistant strains were obtained by subculturing in growth medium containing nalidixic acid (up to 200 mg/l). Enumeration of Y. enterocolitica and S. typhimurium was on Yersinia Selective Agar Base or Violet Red Bile Glucose Agar, respectively, both supplemented with nalidixic acid (100 mg/l). In the case of B. cereus, cells resistant to 2-phenylethanol were used as the inoculum, whereas enumeration was on Bacillus Selective Agar, supplemented with egg yolk, polymyxine (10 mg/l) and 2-phenylethanol (2.5 ml/l).

Results and discussion

Packaging under moderate vacuum
Refrigerated storage (4-7°C) under moderate vacuum (± 400 mB), using a rigid MVP-container system, was studied as a means to prolong the shelf-life of perishable vegetables and fruits. In this MVP system, storage is initiated under a lower concentration of O₂ as compared to normal air. During the course of storage, the respiratory activity of the product may further reduce the amount of O₂ present in the storage container and give rise to an increase of the CO₂ concentration. With mung beans sprouts at 4°C, O₂ was reduced to zero in three days, whereas CO₂ rose to an equivalent of 13 vol% in 7 days (data not shown). Although these changes in the atmosphere slow down the metabolic activity of the stored product and of the spoilage.
microflora present on it, the atmospheric composition is not controlled and, since different products have different physiological demands, a situation may arise which impairs the physiological status of a product due to which physiological deterioration occurs. In addition, alterations in the gas atmosphere may counteract the "competitive exclusion" protection which the spoilage microflora normally exerts on pathogenic microorganisms. Thus, unsafe situations may enable pathogens to survive MVP-storage and possibly grow to concern levels (infectious pathogens) or produce toxins (toxigenic pathogens).

**Microbial population dynamics**
Mungo bean sprouts are highly perishable and, when bought from a grocery and stored in a home refrigerator, deteriorate within two to three days (Lipton et al., 1981). Deterioration is predominantly due to spoilage microorganisms. Figure 1 illustrates the dynamics of the relevant microbial subpopulations under MVP conditions (assessed at 4, 7, 12 and 20°C) or at ambient pressure (measured at 4°C only). From the data obtained it followed that with refrigeration alone, the total aerobic count as well as the number of enterobacteria, yeasts and lactic acid bacteria sharply increased during the course of storage period. Under moderate vacuum, however, the total aerobic count and the number of enterobacteria and yeasts did not increase significantly or even showed some reduction. With these groups of microorganisms, the MVP system establishes a certain stabilisation of the initial microbiological situation. In the case of the lactic acid bacteria, the MVP clearly appears to stimulate their growth, even at low temperatures. Since these organisms produce organic acids (lactic acid and possibly also acetic acid), the acidity of the product may increase. This could cause off-odours or a bad taste, but could also contribute to the biostatic effect of the MVP system.

**Fig. 1.** Population dynamics of relevant spoilage subpopulations on mung bean sprouts during storage under air at ambient pressure (Ambient) or 400 mB (MVP). Contours describe the range of data recorded at the temperatures indicated.
Pathogen challenge tests

Using standard procedures, challenge tests were performed with Yersinia enterocolitica, Salmonella typhimurium and Bacillus cereus on mung bean sprouts, stored under ambient pressure or under 400 mB at 7°C. None of the three pathogens survived the storage period. For B. cereus, an aerobic and mesophilic bacterium, this is illustrated in Figure 2. B. cereus did not survive storage under air at ambient pressure, but proliferated under these conditions at 10°C. Under moderate vacuum, the size of the B. cereus population inoculated initially gradually reduced to zero. Conceivably, this was due to low concentration of O₂ in the MVP system. Additionally, acids produced by the lactic acid bacteria may have affected the growth of the pathogen.

Conclusions

Investigations into refrigerated storage (4-7°C) under moderate vacuum (± 400 mB) in a technically simple MVP-system showed that this system may be useful for the storage of respiring, fresh produce since it helps to stabilise physiological or microbiological quality. However, not every produce is suitable for MVP storage and also processing may affect storability. Best results were obtained with mung bean sprouts. The sensory characteristics of this vegetable were found to be as good as fresh even after six days storage at 4°C. The MVP-system exerts a biostatic effect on several groups of spoilage organisms, whereas growth of lactic acid bacteria is promoted. Studying the potential of food-borne pathogenic bacteria to grow to concern levels in the storage system, it was found that strains of Y. enterocolitica, S. typhimurium and B. cereus did not survive during storage at 7°C. Currently, the possible survival of other relevant food-borne pathogens (i.e. Listeria monocytogenes, Aeromonas hydrophila) during refrigerated, moderate vacuum storage is investigated.
Literature cited


II. Bioconservation

Isolation of microorganisms from vegetables

Two products are used for the isolation of microorganisms: chicory and mung bean sprouts. Isolates of lactic acid bacteria (LAB), enterobacteriaceae and pseudomonads were obtained from the products before and after storage under moderate vacuum (MV) conditions for 7 days at 7°C. Selective media were used: Rogosa for Lactobacilli, Violet Red Bile Glucose agar (VRBG) for enterobacteriaceae, and Pseudomonas agar for pseudomonads. After isolation from selective media, LAB were streaked on de Mann, Rogosa and Sharp (MRS) agar plates, and enterobacteriaceae and pseudomonads were streaked on Plate Count Agar (PCA) plates.

In total 350 isolates of LAB were obtained: 50 isolates from fresh mung bean sprouts, and 150 isolates from both mung bean sprouts and chicory after storage. The number of LAB on fresh chicory was too low to allow isolation. In total 100 isolates of both enterobacteriaceae and pseudomonads were obtained: 25 isolates from fresh chicory, 25 from chicory after MV storage, 25 from fresh mung bean sprouts, and 25 after MV storage.

All isolates were examined for purity, and checked for Gram stain and catalase reaction. Enterobacteriaceae and pseudomonads were also differentiated by oxidase reaction. LAB are Gram positive and catalase negative. Both enterobacteriaceae and pseudomonads are Gram negative, and catalase positive. Enterobacteriaceae are oxidase negative, but pseudomonads are oxidase positive or negative. The pure cultures were stored at -80°C in vials containing 20% glycerol.

Screening of LAB

The mode of action of LAB in bioconservation is due to the production of antimicrobial substances. In particular we are trying to find LAB isolates capable of producing bacteriocins. Most screening methods described in literature are based upon diffusion of bacteriocin into agar containing an indicator organism, resulting in growth inhibition of that organism.

We are now working on screening method in liquid media. Growth of indicator organisms is examined by measuring the OD (655 nm) of inoculated broths in microtiter plates. The following indicator stains are used: Enterococcus faecalis, Listeria monocytogenes Scott A, and Bacillus cereus. Lactococcus lactis supers. lactis (producing nisin) is used as a control strain.

An indicator organism (20μl ca. 10⁶ CFU/ml), with or without the addition of 50 μl supernatant from Lactococcus lactis supers. lactis, was inoculated in a suitable broth (in total 200μl per well), and incubated at 30°C. In order to exclude the effect of acids produced by LAB, dialysed supernatant was used as well.
Supernatant or dialysed supernatant was obtained in the following way. *Lactococcus lactis* super., *lactis* was grown to the late logarithmic phase / early stationary phase (18 hours 30°C, starting with a 1% inoculum). Cells were removed by centrifugation and the supernatant was filter sterilised, using a 0.2 µm filter. Sterile supernatants were dialysed against 50mM phosphate, 20mM NaCl buffer, pH 6.0, using a 3500 MWCO membrane. Finally, supernatants and dialysed supernatants were stored at -28°C.

An example of these experiments is shown in figure 1 and 2. Figure 1 shows the growth of *Listeria monocytogenes* Scott A in a suitable medium (BHI) at 30°C. Addition of supernatant from *Lactococcus lactis* resulted in very strong growth inhibition, whereas growth is not as strongly inhibited by adding dialysed supernatant, because of the absence of produced acids. Figure 2 shows the growth of T3 at 30°C. T3 is an isolate of LAB from mung bean sprouts after storage under MV for 7 days at 7°C. Up till 27 hours, growth is as strongly inhibited by supernatant, as it is by dialysed supernatant.

![Graph 3: Growth of *Listeria monocytogenes* Scott A at 30°C](image)

![Graph 4: Growth of T3 (LAB isolate from mung bean sprouts) at 30°C](image)

**Growth of microorganisms as influenced by gasphase composition**

The effect of gas conditions on growth and interaction of microorganisms (LAB versus pathogens or spoilage organisms) will be tested, using a gas flow through system. Initially *in vitro* experiments will be done, by plating organisms or mixed cultures on a suitable agar medium under various conditions (gas mixture, temperature). At different time intervals the viable organisms will be enumerated.

*AIRI-CT92-0125 Meeting #2 17 + 18 June 1993*
D. 1. Short description of work planned in the subsequent part of the project

With respect to bioconservation, work in progress focuses on the identification of potent antagonistic LABs, elucidation of their mechanism of action (acids, bacteriocins, ?) and the determination of the action spectrum. Special attention will be given to antagonism of the relevant pathogens with MAP and MVP: *L. monocytogenes* and *C. botulinum*.

The influence of the composition of the gas phase in a MAP or MVP system on the growth of microorganisms, especially LABs, and on the interaction between microorganisms will be studied with the assay designed during the first half year term. Focus will be, again, on the most relevant pathogens and LABs.

**SUB-PROJECT (B): SYSTEMATIC INVESTIGATION OF THE QUALITY OF FRESH OR FRESHLY PROCESSED VEGETABLES UNDER MODIFIED ATMOSPHERES**

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

The application of MAP as a mild preservation technique with respiring products such as vegetables is quite complex. Ideally, packaging systems should be tailor-made for each product to take into account very crucial determinative parameters such as the minimal respiratory activity of that product necessary to maintain its good physiological quality. The respiratory activity is highly dependent on (more or less) intrinsic factors of the product (e.g. type, physiological state, degree of processing) as well as on the storage conditions (temperature, RH, O<sub>2</sub> and CO<sub>2</sub> concentrations, type of packaging material) and the microbiology (spoilage, pathogens).

2. Goal of the research:
The aim is to assess optimal (equilibrium) gas-mixtures for the extension of the shelf life of selected vegetable products based on knowledge of changes in quality and the respiratory activity under specified storage conditions. The model products chosen are witloof chicory (whole and shredded), carrots (whole and graded) and mung bean sprouts (whole).

3. Experimental approach and planning

The experimental conditions will be standardized. This means a selection of temperatures (8 and 18 °C), relative humidity (99%) and O<sub>2</sub> and CO<sub>2</sub> concentrations. These set values will be controlled and recorded. The experiments with products will be carried out in an automated flow through container system. O<sub>2</sub> and CO<sub>2</sub> concentrations will be measured continuously.

Product quality comprises two mean features: consumer quality (colour, texture) and physiological quality (development stage, respiration activity). To enable the assessment of objective quality measurement data, computer image analysis will be employed if possible. Respiration activity, a key parameter of product physiology, will be measured by a special headspace technique able to determine very small differences in O<sub>2</sub> and CO<sub>2</sub> concentrations.

C. 1. Results

- Objective methods for the measurement of witloof quality are developed and will be used to evaluate the quality of this product during/after storage under controlled, modified atmospheres (incl. comparison with available literature data)
- A experimental set-up for the measurement of respiration activity of witloof chicory under different O<sub>2</sub> and CO<sub>2</sub> concentrations is in place.
- First test-runs in the automated, flow-through container system are scheduled for July.

AIRI-CT92-0125 Meeting #2

17 + 18 June 1993
2. Recent publications from ATO-DLO related to the project

Recent presentations/posters
Gorris, L.G.M., 1992. Spoilage and safety aspects of refrigerated storage of respiring produce under moderate vacuum. FLAIR Concerted Action No 7 "Food quality and safety based on the application of combined processes and Hazard Analysis Critical Control Point". Dublin, Ireland (pres.)
Gorris, L.G.M., 1993. Microbial stability of fresh foods under (semi-) vacuum. 26th FNK Conference "Potatoprocessing", Zürich, Switzerland. (pres.)
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 optimization 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 optimization 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 (carbon- and energy source) 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 optimization of mild preservation techniques.

### C. 1. Overview of the work performed from 1-1-1993 to 30-6-1993
(Part of this work is submitted for publication (Beumer et al., 1993)).

#### Introduction
The property to adapt to changes in the osmotic strength of the environment is inherent to most living cells. Cellular adaption to osmotic stress is a cardinal process, because organisms require a relatively constant cytoplasmic composition for optimal enzyme activity. The ability of *L. monocytogenes* to grow in foods with a low $a_w$ (up to 10 % NaCl, survives up to 18 %) suggests that it can adapt to the osmotic environment. Moreover, this contributes to its important role as an agent of food poisoning. Osmoregulation has been most extensively studied in the gram(-) bacteria *Escherichia coli* and *Salmonella typhimurium* (Csonka, 1989).

By accumulating compatible solutes (osmoprotectants) these organisms achieve a genetically simple and temporary flexible adaptive mechanism in the face of water stress. Through the use of osmoprotectants, adaptive changes in proteins, which are determined by changes in the DNA sequences coding these proteins may be minimized. The primary response after an osmotic upshock involves the accumulation of $K^+$ and glutamate which are replaced after or during trehalose synthesis. In the presence of externally provided proline or betaine, these solutes are taken up and preferentially used as osmoprotectants allowing growth at very high osmolarities. Through the presence of the osmoprotectants, proteins are able to work in environments with varying osmolarities and modification of large numbers of proteins is avoided. Very little is known about the mechanisms by which gram positive bacteria react to osmotic stress. *Streptomyces* species accumulate proline, glutamine and alanine resulting in high intracellular concentrations of these compounds when grown at enhanced salt concentrations (Killham and Firestone, 1984). An important role for proline and betaine has been observed in the osmoregulation of the gram positive bacteria *Staphylococcus aureus* and *Lactobacillus acidophilus* (Bae and Miller, 1992; Jewell and Kashket, 1991). Recently Patchett et al. (1992) described the effect of sodium chloride on the intracellular solute pools of *L. monocytogenes* in a rich undefined broth. At 5 % NaCl cells contained elevated concentration of potassium and betaine compared with concentrations in cells without NaCl. At 7.5 % NaCl cells contained increased concentrations of $K^+$, betaine, glycine, alanine and proline. It was showed that betaine, glycine and choline (each 1 mM) promoted growth in the presence of 4 % NaCl in a solid defined medium. It appeared that addition of 1 mM proline or 1 mM alanine did not have any effect.

Betaine (N,N,N-trimethylglycine) occurs in sugar beet and other samples of animal and plant origin (Belitz and Grosch, 1986).
In our laboratory the influence of salt concentration on the growth of *L. monocytogenes* and the effect of the osmoprotectants proline and betaine was studied in more detail.

**Methods**

Growth experiments were performed in defined minimal medium (Premaratne *et al.*, 1991) in which the osmotic strength was varied by the addition of NaCl or KCl. The growth was compared to growth in a rich medium (BHI) containing various salt concentrations. To determine the role of osmoprotectants, proline (1 or 10 mM) and betaine (1 mM) were added to the medium. After incubation (37°C for 30 hours) growth was measured turbidimetrically at 660 nm. The effect of NaCl on the lagtime and growth rate in the minimal medium with 1 mM betaine was examined by plate count experiments on TSA agar at 37 and 10°C.

**Results and Discussion**

*L. monocytogenes* is able to grow in higher NaCl concentrations in a rich medium than in the minimal medium after 30 hours of incubation at 37°C. Addition of up to 5% NaCl in BHI showed hardly any effect. In minimal medium increasing salt concentrations led to a decrease in final OD reached indicating that growth was severely reduced. Apparently, compounds present in BHI allow growth of *L. monocytogenes* at otherwise inhibitory osmotic strength. For KCl similar results were obtained, although KCl was less inhibitory to growth as compared to NaCl. This effect has also been reported for *Streptomyces* by Killham and Firestone (1984). In the OD experiments the externally added proline and betaine had a considerable protective effect on the growth in minimal medium after 30 hours of incubation. *L. monocytogenes* was able to grow at NaCl concentrations up to 5% in minimal medium in the presence of betaine. Adding 1 mM proline had no effect on the growth of *L. monocytogenes* in minimal medium with increasing NaCl concentrations. When a higher concentration of proline (10 mM) was added to the medium significant growth stimulation in the presence of NaCl was observed. Patchett *et al.* (1992) concluded previously that proline could not function as an osmoprotectant for *L. monocytogenes* at high osmotic strength. This result could be due to the low concentration of proline (1 mM) used in their experiments. In food, proline will play a minor role in osmoprotection since it is mainly present in bounded form (proteins). However, it is conceivable that the liberation of proline from proteins by proteases of other bacteria in foods could result in its availability to *Listeria* resulting in growth stimulation at low osmolality. Betaine was apparently more effective than proline since higher optical density values were reached. The concentration used in this experiment (1 mM) is comparable with the levels of betaine in foods. For KCl similar results were obtained. Adding betaine or proline to BHI did not show any stimulation indicating that osmoprotectant present in BHI are sufficient to allow growth at high osmotic strength.

There was no difference between adding betaine at the beginning of the experiment or during the exponential growth phase. This immediate stimulation of betaine following osmotic shock suggested that the system was activated rather than induced by osmotic stress. For betaine this has also been found for *L. acidobacillus acidophilus* and *Staph. aureus* (Jewell and Kashket, 1991; Porkomaillian and Booth, 1992). Betaine influenced both the lag time and the growth rate of *L. monocytogenes* in the minimal medium with salt at 37°C. The levels reached in the stationary phase for growth of *L. monocytogenes* in mineral medium with osmoprotectants were comparable with the levels reached in the mineral medium in absence of salt (about 10⁹ CFU/ml). Osmoregulation was also studied at 10°C since this might give more information on the situation in food. Externally added betaine were found to reduce the lag time and doubling time of *L. monocytogenes* cells grown at 10°C. The effect of adding osmoprotectants was at 10°C less pronounced compared with 37°C. The combined effect of low temperature and high salt concentrations is probably more difficult for the organism to overcome.
Literature cited


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

*Listeria* needs to take up fermentable carbohydrates and essential amino acids and vitamins from the environment. Transport systems involved in the accumulation of sugars, amino acids or peptides will be characterized in whole cells and membrane vesicles. Another important aspect is a study of the osmoregulation in *L. monocytogenes* (year 1).

There is now substantial evidence that in a variety of bacteria the cytoplasmic pH is maintained within a relatively narrow range. The cytoplasmic pH of acidophilic bacteria ranges between 6.0-7.0, of neutrophilic bacteria between 7.5-8.0, and of alkalophilic bacteria between 8.5 and 9.0. Efficient pH homeostasis mechanisms are needed to prevent large fluctuations in the cytoplasmic pH upon changes in the external pH. Regulation of the cytoplasmic pH under various environmental conditions will be studied by measuring intracellular pH by using fluorescent pH indicators (year 2).

In the second part of this project (year 3 and 4) the above obtained results will be combined to allow predictive modelling of growth of these organisms under different environmental conditions i.e. different food products.

Furthermore, the application of bacteriocins or the producing strains in different foods as an alternative for chemical preservation will be investigated. Special attention will be paid to the antimicrobial activity of these bacteriocins under condition of modified atmosphere, low temperature and low *a*w. In addition these bacteriocins will be used in combination with acids such as lactate and citrate and the effects on spoilage bacteria and pathogens will be studied.
From the work performed in the first half of 1993, it has become clear that exogenous proline and betaine can function as osmoprotectants for the growth of *L. monocytogenes* at high and low temperature in a minimal medium at high osmolarity. In the second half, osmoregulation under modified atmosphere will be studied and transport systems involved in the accumulation of osmoprotectants will be characterized. The uptake of osmoprotectants upon hyperosmotic stress will be studied with radiolabeled compounds. Another important aspect is a study of the role of peptides as the source of nitrogen for growth of *L. monocytogenes*. *L. monocytogenes* is relatively fastidious in nutrient requirement. It needs for example six amino acids for growth (i.e. leucine, isoleucine, valine, methionine, arginine and cysteine). Growth experiments will be performed in defined mineral media in which one of the essential amino acids is provided in the form of a dipeptide. When growth is indeed supported by essential amino acids-containing peptides the involved transport systems i.e. substrate specificity and energetics, will be characterized.
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. Halna du Fretay (Hired for the project).

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

2. Goal of the research:
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, and (iii) to estimate the risk of foodborne pathogens development in already existing industrial products. In addition, the work will provide the necessary expertise to help other participants testing new, mild, preservation techniques.
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" (FRUID).

C. 1. Overview of the work performed from 1-1-1993 to 30-6-1993.

a). Behaviour of *L. monocytogenes* on salad leaves (INRA)
The first part of the work performed during this period was to measure the variability of the behaviour of *L. monocytogenes* on salad leaves so as to determine the minimum number of replicates for the next experiments. The second part was to measure the effect of biological factors and storage conditions on the behaviour of the pathogen on salad leaves. In the course of this period, temperature only was considered as storage condition. Biological factors consisted in: species of salad, age of the salad leaves, amount of epiphytic microflora on the leaves, amount of *L. monocytogenes* inoculated on the leaves, strain of *L. monocytogenes*. After storage, each salad leaf inoculated was characterized by the number of *L. monocytogenes*, the number and the nature of the epiphytic microflora and the extent of decay. Microorganisms from not inoculated commercial samples of ready-to-use chicory salads were also isolated, identified and tested for their pathogenicity on salad leaves.

Material and methods
The Scott A strain and two strains of *L. monocytogenes* isolated from the coleslaw outbreak were used in this work. Salads, belonging to various species currently processed as ready-to-use salads, were provided by Les Crudettes (FRUID). Butterhead lettuce (*Lactuca sativa*), lamb’s lettuce (*Valerianella olitoria*) and two kinds of chicory salads (*Cichorium endivia*), broad leaf chicory and curly leaf chicory were tested. In some experiments, broad leaf chicory grown in culture chamber were used. Salad leaves were sorted according to their age, colour and size; washed in distilled water; disinfected in a 10% H$_2$O$_2$ solution unless specified otherwise and rinsed in sterile distilled water; cut into peaces of approximately 25 cm$^2$ and inoculated with *L. monocytogenes*.

Inoculum consisted in two successive cultures of *L. monocytogenes* in Trypticase broth lor 4 days at 10°C. The culture was diluted appropriately and dispersed in a bath of sterile distilled water. Leaf peaces were dipped in the bath, agitated gently for 10 min and drained on filter paper. The inoculated leaf peaces were placed individually in sealed polypropylene 40 μm bags (150 cm$^2$). Control, not inoculated leaf pieces, were treated similarly. Salad leaf peaces were stored at the appropriate temperatures during one or two weeks and samples were analyzed at regular intervals.

At each sampling date, the extent of decay of each salad leaf peaces was estimated by the % of surface showing necrosis. *Listeria* and aerobic mesophilic microflora were then enumerated respectively on Oxford agar and tryptose agar. Whenever the number of listeria on leaves was lower than 10$^2$/g, the bacteria was enumerated by MPN in listeria enrichment broth (FDA procedure). Representative samples of colonies from the count plates of tryptose and Oxford agar were isolated for identification. At the end of the experiment, atmosphere composition in the control samples was measured by gas chromatography and absence of listeria was tested by enrichment in the FDA broth and plating on Oxford agar after 24 and 48h at 30°C.
**Results**

- **Control samples.** The concentration of carbon dioxide in the bags of control samples was always lower than 3%. No *Listeria* species were detected in any of the control samples.
- **The pH of leaf macerates was close to 6.0 during all the experiments.**
- **Variability.** *L. monocytogenes* (Scott A) behaviour on leaf pieces from the same batch of salads. Bacterial growth on 30 replicates of broad leaf chicory salads grown in a culture chamber was measured after 0,4 and 7 d at 10°C. Salads grown in such conditions permitted a selection of leaves very homogeneous in shape, color and age. After inoculation (t0), numbers of *L. monocytogenes* among the leaf pieces were very homogeneous, but heterogeneity increased during storage (after 7 days at 10°C, range of number of listeria on leaves could reach 2 log). Extent of spoilage and number of epiphytic bacteria were also variable. A positive correlation existed between the extent of spoilage of the leaf and the number of listeria after storage (Figure 1). Similarly, the number of epiphytic bacteria was positively correlated with the extent of spoilage. Consequently, the number of listeria was positively correlated with the number of epiphytic bacteria. In another experiment, extent of spoilage and the number of listeria was homogeneous among replicate leaf pieces.
- **Effect of storage temperature.** Growth of *L. monocytogenes* (Scott A) at 2-3°C, 6 and 10°C on leaf pieces of broad leaf chicory is presented on Figure 2. The bacteria grew at a slower rate than mesophilic microflora, although growth rates tended to be similar at 10°C.
- **Effect of inoculum level.** Growth of *L. monocytogenes* (Scott A) inoculated at different concentrations on the leaf pieces (10⁴, 10⁵, 10⁶ listeria/g) was measured at 10°C (Figure 3). The bacteria grew slightly faster at the lower inoculum concentration during the first days of storage, but the population tended toward a lower plateau. It should also be noted that inoculation at the lower concentration increased dramatically the variability of listeria counts among replicates after storage.
- **Behaviour of different strains of *L. monocytogenes.*** The three strains tested grew on chicory leaves at 10°C. One of the two coleslaw strains did not grow as well as the Scott A strain and the other coleslaw strain.
- **Effect of salad leaves.** Growth of *L. monocytogenes* (Scott A) on pieces of leaves of broad leaf chicory, curly leaf chicory, butterhead lettuce and leaves of lamb lettuce is shown in Figure 4. Leaves were not disinfected because of the different sensitivity to disinfectant of the various salad species. The highest growth rate was obtained on butter head lettuce and the lowest on lamb lettuce. The bacteria behaved similarly on the two chicory salads. Growth of aerobic mesophilic flora and spoilage development were not significantly different on leaves of the four different salads. On broad leaf chicory, *L. monocytogenes* grew faster on young, yellow leaves than on older green ones, with higher spoilage after 7 days at 10°C for young leaves than for old ones (Figure 5).
- **Effect of a reduction of background microflora.** Disinfection of leaves of broad leaf chicory with H₂O₂ (10%) reduced the number of epiphytic bacteria by 1-2 log as compared with leaves simply rinsed with water and did not caused any additional spoilage after storage at 10°C for 7 days. *L. monocytogenes* inoculated on disinfected leaves grew faster and reached higher counts after 7 days at 10°C than on leaves rinsed with water (Figure 6). The background microflora of disinfected leaves caught up with that of not disinfected ones during the first days of storage and reached similar numbers on both samples.
- **Epiphytic bacteria of chicory leaves.** Colonies of epiphytic bacteria were sampled for identification from count plates of leaf pieces with the highest and the lowest number of *Listeria* at the end of storage, to test the relation between nature of prevailing species and behaviour of listeria. Identifications and tests for pathogenicity on salad leaves are under way and results will be presented in a future report.
Discussion

Analysis of individual leaf pieces instead of pouches of commercial ready-to-use salads greatly improved the safety of experiments as it reduced the volume of inoculum suspension needed. Packaging each inoculated piece of leaf in a sealed plastic bag prevented dehydration and contamination. In the same time, as the bag had a large surface with regard to the weight of plant tissue (approximately 1 g), diffusion nearly compensated oxygen consumption and the atmosphere inside the bag was only slightly modified. The effect of atmosphere modifications will be studied separately in controlled atmosphere cabinets. The results from individual leaf pieces permit the use of the natural variability between leaves to study relations between growth of *L. monocytogenes* and other characteristics. From the results presented, 10 replicate leaf pieces for each time of storage and each treatment was determined as a minimum to compare effect of treatments.

*L. monocytogenes* grew at a slower rate on leaves than the epiphytic microflora at refrigeration temperature. Whether this is because epiphytic bacteria are more psychrotrophic than *L. monocytogenes* or because they are better adapted to the leaf environment remains to be tested. In any case, development of epiphytic bacteria on salad leaves might cause spoilage and limit the shelf life. Decreasing temperature might therefore reduce spoilage development to a lesser extent than growth of *L. monocytogenes*.

At a given temperature, behaviour of *L. monocytogenes* on leaves varied with the species or cultivar of salads and with the age of the leaves for a given salad. It sometime also varied among the different leaves of a same salad and a same class of age. Variability in spoilage development during storage, could explain part of the variability observed between replicate leaf pieces. Spoilage could also explain the better growth of *L. monocytogenes* on young leaves than on old leaves, but it does not seem to explain differences observed between the different species of salad. The absence of any development of the bacteria on lamb lettuce needs to be confirmed by other experiments.

Slight differences between the 3 strains of listeria tested were observed, but this should be confirmed by other experiments. The influence of the number of listeria inoculated on the maximum growth level is worth noting. In vitro, level of inoculum did not influence growth parameters of *L. monocytogenes* (Buchanan and Phillips 1990).

Reducing the number of epiphytic bacteria permitted a better growth of *L. monocytogenes*. If confirmed by other experiments, this result would indicate a competition between *L. monocytogenes* and epiphytic bacteria for the colonization of the leaf surface.

Conclusion

Beside storage conditions (temperature, modified atmosphere, etc), the following factors influenced the behaviour of *L. monocytogenes* and, provided that the results presented are confirmed, should be considered in the planning of the work to come: physiological age of the leaves, species of salads, number of listeria inoculated, disinfection of the leaves. Measure of the extent of spoilage proved to be a valuable information to interpret the behaviour of *L. monocytogenes*. Effect of epiphytic microflora needs further investigations.

In practice, the results obtained show that the leaves which are the more likely to carry a high load of listeria at the end of storage are leaves which are more susceptible to decay and are therefore less likely to be stored for a long period before consumption. In addition, the level of listeria naturally contaminating vegetables has always been found very low (below 50 g-1) and this presumably reduce the maximum level the bacterium could reach during storage. The effect of disinfection (if confirmed) might also have interesting implications on processing of ready-to-use salads.
b) Microbial stability of new, ready-to-use products, from Les Crudettes (cooked/raw ingredients) (INRA-FRUID)

These products are processed only in spring and summer by Les Crudettes (FRUID). The work started in April and will be presented in the next report.

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

a) Behaviour of *L. monocytogenes* on salad leaves

* Second half of first year
Identification of epiphytic microorganisms will be continued and results will be analyzed for possible relation between nature of epiphytic microorganisms on a piece of leaf and behaviour of *L. monocytogenes*. If any relation appears, it would be a basis for the selection of microorganisms to be challenged with *L. monocytogenes* on salad leaves. The experiments presented above will be at least duplicated to test the reproductibility of the results on salads grown at a different period of the year. The effect of atmosphere modifications on growth of *L. monocytogenes* versus growth of epiphytic microflora and spoilage development will be studied, starting with increased CO₂ concentrations.

Discussion with a mathematician (Dr. A. Lebert, INRA THEIX) are under way to decide what is the best way to present and to interpret the results, and what would be the best plan for the experiments (more analysis during storage with less replicates for each analysis or more replicates but less analysis). Fitting the data to well known growth models would permit a comparison with predictive models based on in vitro experiments, but this might not be possible because of the high variability among replicates.

* Years 2 - 4
After confirmation of the effects of the main factors on behaviour of *L. monocytogenes* of salad leaves, a more complete investigation of the effects of the most important ones might be interesting. Interactions between some of these factors will probably have to be tested. A complete experimental design between all the factors is probably not feasible in the course of this project, so a careful selection will have to be made.

Study of the interactions between epiphytic microorganisms and *L. monocytogenes*. Representative isolates will be selected on the basis of their frequency on the leaves and according to the results of the first part of the work. If any interaction is observed, an investigation of the mode of action will be launched (competition for sites of colonization, interaction during attachment on the leaf surface, production of antimicrobial compounds).

b) Microbial stability of new, ready-to-use products, from Les Crudettes (cooked/raw ingredients) (INRA-FRUID)

* Second half of first year
The first part of the work will be devoted to the physico-chemical characterization of the products, the determination of the main features of spoilage development and the investigation of microbial evolution. Analysis will be performed on the whole product, on the cooked (sweet corn and mungo spouts) and the raw (chicory salads and grated carrots) ingredients. Growth of *L. monocytogenes* on the product will be measured at two storage temperatures. Some alternatives in the processing of the cooked ingredient will be tested.
* Years 2 - 4
Growth of other foodborne pathogens as *Bacillus cereus* will be measured in the products. The application of coating to isolate cooked ingredients from raw, heavily contaminated vegetables will be tested in collaboration with the CIRAD-SAR.

c) Behaviour of *Clostridium botulinum* on cooked vegetables (AFRC-INRA)

The work will be performed in 1994 at AFRC-IFRN with a scientist from INRA-AV hosted for a year by AFRC. Growth of strains of *C. botulinum* nonproteolytic will be tested at temperatures of refrigeration on some models of cooked vegetables. Effect of sublethal heat treatments applied on spores of botulinum, of storage conditions and of saprophytic microorganisms isolated from vegetables will be tested.
Figure 1. Correlation between the rate of decay (expressed as the % of surface showing necrosis) and the number of *Listeria monocytogenes* (Scott A) on pieces of broad-leaf chicory leaves after 7 days at 10°C. Pieces of leaves were inoculated with $10^4$.g$^{-1}$ *L. monocytogenes* at the start of the experiment.
Figure 2. Growth of *L. monocytogenes* (Scott A) (——) and of epiphytic microflora (········) on pieces of broad-leaf chicory leaves at different temperatures: 2°C (□), 6°C (+) and 10°C (▲). Results are mean value from 10 replicate leaf pieces.
Figure 3. Growth of *L. monocytogenes* (Scott A) at 10°C on pieces of broad-leaf chicory leaves for different level of inoculum. For the lowest level of inoculation (□), results from all replicates (open symbol) and of the mean value (closed symbol) are presented. 

▼: value lower than limit of detection. 

↓: confidence interval at 95 %.
Figure 4. Growth at 10°C of *L. monocytogenes* (Scott A) (A) and of epiphytic microflora (B) on pieces of leaves of broad-leaf chicory (■), curly-leaf chicory (□), butterhead lettuce (*), and lamb’s lettuce (▲). Results are mean value from 10 replicate leaf pieces.
Figure 5. Number of *L. monocytogenes* (Scott A) versus rate of decay after 7 days at 10°C for pieces of young, yellow leaves (+) and of old, green leaves (■). Leaf pieces from broad-leaf chicory salads were inoculated with $10^4$-$10^5\text{ g}^{-1}$ *L. monocytogenes*. 
Figure 6. Growth at 10°C of *L. monocytogenes* (Scott A) (—) and of epiphytic microflora (.....) on leaves disinfected (▲) or not (■) before inoculation.
Depart. Systèmes Agro-Alimentaires er Ruraux
73 Rue J.F. Breton
Domaine de Lavalette
F-34000 Montpellier
France

A. 1. (Sub-)Project title:
Improvements of microbial stability of ready-to-eat, chilled, fresh fruits and vegetables by using edible protective superficial layers (EPLS), which may include antimicrobial compounds, as biodegradable packaging with selective permeability to gas.

2. Project coordinator:
Prof. Dr. Stéphan Guilbert

3. Project participants and 4. Resources:
Prof. Dr. S. Guilbert (responsible 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 pursued 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. Successful 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 EPLSs or active EPLSs (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 (EPLSs) in the form of films or coatings, possibly containing preservatives, and test their diffusion barrier properties (their water vapour, O₂ CO₂ permeability) and ability to protect ready-to-eat, chilled fruits and vegetables.

C. 1 Overview of the work performed from 1-1-93 to 30-6-93
Material and methods

After preliminaries experiments, the following procedures were choosen as the best ones.

Film preparation

a) Reticulated pectins films
Pectin (UNIPECTINE 325 NH 95, SANOFI) were mixed into distilled water at 60°C (in a water bath), eventually containing K-sorbate, to have a solution with a dried matter content of 4 % (w/w). The mixture is kept between 60 and 70°C and strongly stirred until complete solubilization of the macromolecules (during about 1 hour). Then, the mixture is maintained into the water bath to eliminate air bubbles and get cold at room temperature. The solution is put into the fridge (during about 1 hour) to make spreading easier. The film forming solution is spread with a standard applicator for fine layer chromatography, the thickness of the spread solution must be of 1,15 mm.

The film forming solution is allowed to dry at room temperature (about 20°C) for 24 h and a 3 % CaCl\textsubscript{2} solution is spread in excess onto the surface. Excess was eliminated after 20 sec. and the film was allowed to dry again. The films obtained have a thickness of 90 \textmu m.

b) Composite films
The film forming solution was prepared as previously describe. Melt Panodan (PANODAN 507 GRINDSTED) was mixed at 60-70°C with the film forming solution under strongly stirring to obtain an emulsion with 20 % of Panodan. The emulsion was spread, dried and reticulated according to previously.

c) Multilayer films
Composite films was prepared as mentionned before, with pectin and wax. After drying and reticulation, put the films in an oven at 70°C. Melt wax was applied at 70°C onto to the base film surface. The amount of deposited wax was controlled by weighing before and after applying. Then, let get cold at room temperature.

Determination of sorbic acid retention in the films

30 g of a 2 % agar solution was poured into sampling pots of 100 ml volume and 5,5 cm diameter. After cooling, acidifying every gel with 1 ml of a 2 % citric acid solution. The pots were put in an oven at the desired temperature (18°C). The films are cut such that the diameter of the disc is about 5 cm, and put onto the area of the gels. After a certain time of experiment, the films were taken out of the pots and the amount of sorbic acid was analyzed by HPLC after extraction.

Extraction : Every gel is crushed with a WARING COMMERCIAL BLENDOR to obtain an homogeneous mixture. Distilled water was added up to 100 g and stirred 10 min at room temperature. The mixture was put into a water bath at 60°C for 30 min. The cooling were made at room temperature under stirring. The solution was filtered through hold papers and SARTORIUS filters with pores of 0,45 \textmu m, before HPLC analysis.

Sorbic acid assay
All the chromatographic material were provided from BECKMAN Company. We used a C18 column ODS and an eluent made with 60% (v/v) 0.05 M dehydrogen phosphate (plus HCl 37% up to pH = 2.5) and 40% (v/v) acetonitrile, pH of the final solution equalled 3.

**Determination of sorbic acid diffusion coefficient**

**Diffusion Cell Preparation**
We used a glass cylinder of 10.5 cm in length and 3.6 cm in diameter, fitted. The cap has a circular opening which may be used to seat a piece of membrane (Fig. 1). A second washer were made from a smooth piece of rubber sheet with the same diameter as the one purchased. A piece of film is cut such that the diameter of the disc is slightly less than the out diameter of the washers.

To check for a good seal, a piece of thin polymeric film, which was impermeable to water, was cut so that its diameter is about the same as the prepared film and inserted between the two washers in place of the membrane as shown in Figure 1. The cap on the pipe was screwed tight. KMnO₄ solution or any other colour, was poured into the diffusion cell and left in a beaker partially filled with distilled water. If, after a period of 2-3 hr, the KMnO₄ leaks into the beaker, the seal is not good and improvement is necessary until there is no further leak. We tested our seal over a period of several days with no visible sign of leakage. Exactly 70 ml of distilled water was poured into the clean diffusion cell. The level of the water was located and clearly marked on the outer wall of the diffusion cell. The diffusion cell was reassembled using the prepared film and ready to be used in experiments.

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**Figure 1**: Various parts of diffusion cell: A, glass cylinder with screw thread; C, first washer; D, film; E, second washer; F, screw cap with circular opening whose diameter is 3.5 cm
Experimental Procedure
The diffusion cell were poured into a 1 liter beaker containing 800 ml distilled water. The membrane entering the water should be at an angle, otherwise air bubbles may become trapped under the membrane. 70 ml of distilled water were transferred into the diffusion cell slowly. At the same time, the cell was allowed to sink gradually into the beaker water so that the water levels inside and outside the cell are approximately the same. This procedure ensures that the net volume of water passing through the membrane was kept to the minimum. The beaker was placed into a thermostatted bath at 18°C. Clamped diffusion cell was adjusted such that the water level outside the cell was right on the calibrated mark on the outer wall of the cell. The mark is used only as an indicator for the water level inside the cell. Stirred water in the diffusion cell and in the beaker as shown in Figure 2. The setup is now left to reach thermal equilibrium. 100 ml of stock solution of K-sorbate was prepared. For sorbic acid, distilled water is replaced by a 2 % (w/w) citric acid solution (in the beaker, the diffusion cell and for the stock solution). When the water in the beaker and in the diffusion cell reaches thermal equilibrium with the bath, 10 ml of water was pipetted out of the diffusion cell. The experiments started when half of 10 ml of the diffusant solution was transferred into the diffusion cell. An aliquot was removed from the beaker with a 1 ml pipet every 1,5 and 10 min and then at 10 min intervals thereafter until a total of 90 min has elapsed. Each sample was analyzed by HPLC as mentionned before.

When a sample is removed from the beaker, it creates a very small drop in the level of the solution in the beaker; this may be compensated by dropping in small glass beads. However, since the cross-sectional area of the beaker is large, there is only a small change in the level of the solution.

Add a colour (KMnO₄ for example) in the cell diffusion at the end of the experience to verify there isn't any leak or any hole in the film.

Remove the diffusion cell and measure the volume of the solution in the beaker.

Figure 2. Experimental setup: P, water bath; Q, 1-l beaker; R, diffusion cell; S, mechanical stirrer; T, magnetic bar; U, magnetic stirrer
The following experiment had two major advantages: the apparatus is inexpensive, simple to construct and the whole experiment may be performed within a single 3-hr laboratory period. There are diverse methods to determine diffusion and coefficient (1,2); We have adopted those used by many investigators (3).

Fick's first law forms the basis of our diffusion studies and can be expressed as:

$$\frac{dn}{dt} = -DA\frac{dC}{dx}$$

(1)

where \( n \) is the amount of diffusant, \( \frac{dn}{dt} \) is the rate of transfer of diffusant through a membrane whose area is \( A \) in the direction perpendicular to the membrane cross section, \( D \) is the diffusion coefficient, \( C \) is the concentration of diffusant, \( x \) is the position coordinate in the direction normal to the membrane, and \( \frac{dC}{dx} \) is the concentration gradient at the membrane. The negative sign in (1) indicates that the flow of the diffusant is in the opposite direction to the direction of positive concentration gradient. For a finite change, eqn. (1) can be written as:

$$\frac{dn}{dt} = DA \frac{C_I}{l}$$

(2)

when \( C \) is the concentration difference across the membrane whose thickness is \( l \). If the membrane thickness is constant, it may be incorporated in the diffusion coefficient to give a permeability constant, \( P \). Thus eqn. (2) becomes:

$$\frac{dn}{dt} = PA (C_o - C_i)$$

(3)

Here, \( n \) is the amount of substance leaving the diffusion cell, \( C_o \) is the concentration of the substance inside the diffusion cell and \( C_i \) is the concentration of the substance outside the diffusion cell. If it is now realized that the amount of substance \( n \) outside the diffusion cell divided by the volume of water \( V \), is actually the concentration \( C_i \) (amount of substance per unit volume), then \( n/V = C_i \). Thus, \( n = C_i V \). Using this relation for \( n \) in eqn. (3), we have:

$$\frac{dC_i}{dt} = PA/V (C_o - C_i)$$

(4)

Next, we assume that the volume \( V \) outside the diffusion cell is constant, eqn. (4) can be integrated using the conditions that \( C_i = 0 \) at \( t = 0 \) and \( C_i = C_i \) at any time \( t \). We get:

$$\ln (C_i/(C_o - C_i)) = PA/t/V$$

or

$$\log (C_i/(C_o - C_i)) = PA/2.303V$$

(5)

Results

Sorbic acid retention in films
The experiments have been realized with films containing 0,4 et 4 % K-sorbate related to dried matter. All experiments have been conducted, and presently we are analysing results. Preliminaries results show that sorbic acid was diffusing very quickly from pectin films to agar.

Evaluation of diffusion coefficients
The results are shown in Table 1 and Figure 3, for:

A = 9,6 cm$^2$
l = 0,012 cm
V = 800 cm$^3$

Diffusion coefficients calculated

D = 3,2 $10^{-4}$ cm$^2$ min$^{-1}$ for K-sorbate
D = 6,8 $10^{-4}$ cm$^2$ min$^{-1}$ for sorbic acid

We have determined a diffusion coefficient for sorbic acid and K-sorbate at 18°C with fixed experiment parameters. Presently, more experiments are conducting to verify these results and to study:
- the influence of stirring (to determine the minimal stirring necessary)
- the influence of starting concentration (to verify that there is no influence of the starting concentration on the diffusion coefficient)
- the influence of the temperature
- the influence of pH
- the diffusion coefficient of other type of films
TABLE 1: Evolution of K-sorbate and sorbic acid concentration out of the diffusion cell

$C_t$ (mg/l)

<table>
<thead>
<tr>
<th>T (min)</th>
<th>K SORBATE $C_o = 10$ mg/ml</th>
<th>SORBIC ACID $C_o = 0.3$ mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0579</td>
<td>0.0256</td>
</tr>
<tr>
<td>5</td>
<td>0.1877</td>
<td>0.1013</td>
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<tr>
<td>10</td>
<td>0.3392</td>
<td>0.1950</td>
</tr>
<tr>
<td>20</td>
<td>0.6334</td>
<td>0.3908</td>
</tr>
<tr>
<td>30</td>
<td>0.9362</td>
<td>0.6005</td>
</tr>
<tr>
<td>40</td>
<td>1.2377</td>
<td>0.8054</td>
</tr>
<tr>
<td>50</td>
<td>1.5573</td>
<td>1.0410</td>
</tr>
<tr>
<td>60</td>
<td>1.8774</td>
<td>1.2214</td>
</tr>
<tr>
<td>70</td>
<td>2.2173</td>
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<td>80</td>
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</tr>
<tr>
<td>90</td>
<td>2.9040</td>
<td>1.8252</td>
</tr>
</tbody>
</table>

* We are limited by the sorbic acid solubility

Figure 3. Plot of $\ln\left(C_t/(C_o - C_i)\right)$ versus t; a : k-sorbate; b : sorbic acid
D. 1. Short description of work in the subsequent part of the project

Using an EPSL of high antimicrobial or antioxygen concentration may allow:
I) the use of the additive without the destruction of food integrity
II) the use of small amounts of the additive relatively to the total weight of the food. In this case, the diffusivity of the additive within the film and the food is of particular importance.

YEAR 1
1) realisation of edible films based on hydrophilic, hydrophobic or composite materials
2) test their ability to retain preservatives of different molecular weight at the surface of a model food.

YEAR 2-4
1) test the microbial stability of coated model food and coated real foods (carrots in pieces, other?)
2) realisation and selection of biodegradable films (mainly gluten or chemically modified gluten) with high CO₂/O₂ permeability ratio
3) test their ability to improve the stability of carrots in pieces (with other participants)

2. Detailed description of the work to be done in the second six months period

see last part of C
Part B: I. Description of the research topic or practical problem

The last six months ITAP was concentrated on the research topics described above. The main problem was that of inoculation with pathogens. A standard method for inoculation should be discussed in detail. In our studies we found that the attachment of the two bacteria (Staphylococcus aureus and Salmonella enteritidis) used in this part of the study, on the carrot surface varied.

2. Goal of the research
We examined the initial flora of carrots and its growth/inhibition during the storage under MAP conditions. In parallel we analysed the carrots in order to examine the indigenous factors, such as pH, low molecular weight compounds (eg. glucose, l-lactate) which may play an important role on the growth/survival of the microbial flora.
We also investigated the effect of carbon dioxide on various bacteria in order to study their physiology under these conditions (Task 1).

3. Short description of the approach
see below
During the first six months, attention was given to the different gas mixtures (Tasks 3 & 5; see Table 1) to the microbiology and physico-chemistry of the shredded carrot salad in order to establish its microbial and physicochemical characteristics. Fresh shredded carrots packaged in different mixtures of gases (vacuum pack, 100% CO$_2$; 100% N$_2$; 20%/80% CO$_2$ using packaging materials commonly used to extend the shelf life of such products, and stored at a range of temperatures (10-20°C). At suitable intervals, packs will be removed and level of microbial association (Total Viable Count; pseudomonads, yeasts, lactic acid bacteria etc.) determined. In parallel with microbiological changes of these packs, physico-chemical changes in these products will be monitored: pH, acid profile with HPLC, changes in low molecular weight compounds.

The chemical analysis of the food for the end-products of microbial activity (e.g. lactic acid, acetic acid, ethanol) were investigated as potential index of microbial activity and as (an) indicator(s) of spoilage.

Moreover carrots were inoculated with *Staph. aureus* and *S. enteritidis* in order to study the growth/survival of these two pathogens under modified atmospheres. The effect of carrot juice was studied also on *S. enteritidis* and *Lactobacillus plantarum*.

### Materials and Methods

#### A) Analysis of natural microbiological flora

**Sample preparation.** Fresh carrots obtained from a local market. The samples (fresh carrots) were transported to our laboratory within one (1) hour. On their arrival, the carrots were shredded and individual portions (80-100g) from these shredded carrots and were either vacuum-packed or modified atmosphere packed (Henkoval 1700 sealer, Holland) with 100% CO$_2$ or 100% N$_2$ or 20%/80% CO$_2$/O$_2$ when appropriate. The samples were evacuated and flushed or vacumed twice before their final treatment. In both cases semi-rigid aluminum containers were used a low oxygen permeability of polyethylene bags, which were double sealed. The containers used with each atmosphere were stored at 10 and 20°C for ± 5 days. On every sampling day two containers from each treatment removed for analysis. This experiment was done two times for each type of food pathogen. Samples (25g) from mixed bulk meat 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 quarter 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 (Table 2) for the isolation of particular groups of bacteria or yeasts:

#### B) Inoculation of pathogenic microorganisms

*Staph. aureus* S-6 (NCTC 10657) was used. It produces large amounts of staphylococcal enterotoxin B (SEB) and small amounts of enterotoxin A (SEA). The culture was maintained on Plate Count Agar (Lab M) slopes and at 4°C and subcultured regularly every week. Also, *S. enteritidis* was used which produces pink colonies surrounded by bright red medium on Brilliant Green Agar (BGA). Before the experiment, *S. enteritidis* was grown on BHI or MRS broth at 3°C. Two transfers were made at 24h intervals. With respect to sample preparation, the procedure described above for the natural flora was used with the addition that shredded carrots were inoculated with *Stap. aureus* or *S. enteritidis*. The microbiological analysis was as described above.
Table 1. Duration and target dates for each task in six month interval

<table>
<thead>
<tr>
<th>DESCRIPTION OF SUBJECT (RELATION TO TASKS DESCRIBED IN PARTS A)</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>7th</th>
<th>8th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiological &amp; Physico-chemical analysis of products (TASK 5 &amp; 6)</td>
<td>x *</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Characterization of microbial population dynamics under MAP/VP (TASK 5)</td>
<td>x *</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effect of different gas atmosphere composition under MAP/VP on spoilage bacteria (TASK 5)</td>
<td>x *</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effect of MAP/VP on food pathogens a. Staphylococcus aureus b. Salmonella spp. (TASKS 1,5)</td>
<td>*</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Study physico-chemical changes in product under MAP/VP storage (TASK 6)</td>
<td>x *</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Correlation of microflora to observed physico-chemical changes (TASKS 5 &amp; 6)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Study potential indicators (TASK 6) a. microbial b. physico-chemical</td>
<td>*</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Interaction of microflora, MAP and temperature (TASK 5 &amp; 6) a. spoilage b. physico-chemical changes c. foodborne growth foodborne toxin production d. biopreservation (TASK 4)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Modelling (TASK 10)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

x : work to be done in the specific period of the project
* : work studied so far
Table 2. Media, incubation period, temperatures and techniques used in this study.

<table>
<thead>
<tr>
<th>group or organism</th>
<th>medium</th>
<th>technique</th>
<th>incubation period</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Viable Count</td>
<td>PCA*</td>
<td>1</td>
<td>3 d</td>
<td>25° C</td>
</tr>
<tr>
<td>Pseudomonads</td>
<td>CFC*</td>
<td>1</td>
<td>3 d</td>
<td>25° C</td>
</tr>
<tr>
<td>(Mead &amp; Adams, 1977)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeasts</td>
<td>RBC*</td>
<td>1</td>
<td>5 d</td>
<td>25° C</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>MRS*</td>
<td>2</td>
<td>5 d</td>
<td>25° C</td>
</tr>
</tbody>
</table>

*: from oxoid when available, otherwise made from basic ingredients in the laboratory

C) Growth and enumeration of food-pathogens
Growth was determined by viable counts. Serial dilutions in Ringer’s buffer were prepared for each sample. Bacterial counts were made by spreading 0.1 ml of appropriate dilutions over the dried surface of duplicate Petri dishes containing Brillind Green (salmonella counts) and Baird-Parker’s (staphylococcous counts) medium with subsequent incubation at 37° C for 24h. In all cases, samples (5 ml) were removed aseptically before being frozen at -80° C for subsequent toxin and other determinations.

The effect of carrot juice on S. enteritidis and L. plantarum studied with the changes in the absorbance using a microplate reader at 620 nm. The medium used for the latter organism was MRS while for the first Nutrient broth. Three different concentrations of carrot juice were used (0.1 and 2 % v/v).

D) Enterotoxin assay
In this study the Reversed Passive Latex Agglutination (RPLA) kit (Oxoid Ltd) for the estimation of enterotoxin B was used. A portion of the extract was centrifuged at 10,000 x g (2 min) and the supernatant liquid filtered (0.45 μm Millipore). The filtrate was added to latex beads coated with antienterotoxin B serum in wells in microtiter plates according to the manufacturer’s instruction. Agglutination of the latex spheres was recorded as a positive reaction. Purified enterotoxin B (kindly supplied by H.S Tranter) was used as control as well as that included in the kit. The amount of toxin in samples was calculated with the equation:

SEB/ml of original solution= detection limit x 2^n, where the detection limit is 0.5 ng (Oxoid Ltd) and n is the number of the well in which the end point of a given sample was found (Salomon and Tew 1968). In the first well for the titration of the enterotoxin B the extract was undiluted (Salomon and Tew 1968). According to Park and Szabo (1986) the kit shows a high specificity and sensitivity with a detection limit of 0.75ng enterotoxin/g food. However Tranter and Brehm (1990) have reported that this test is sufficiently sensitive to detect 1-2ng/ml. Moreover the SET-RPLA kit did not show a non specific reaction in either phosphate-buffered saline or extracts from carrots. In our study the latter was confirmed as well as that of raw chicken extract from lower leg, thigh and breast.

E) Physico-chemical analysis
Immediately after microbiological sampling, the pH of the diluted mixed carrots sample was measured.
F) Chemical tests.
After the microbiological examination a liquid portion (20ml) was filtered and the clear filtrate, stored at -80°C and then used for physicochemical analysis
1. The concentration of glucose in the supernatant was assayed using the GOD-PERID kit (Boehringer, Mannheim GmBH) as well as with Sigma Kit method (Sigma diagnostic Glucose Kit Cta.510)
2. Acetate was measured using the acetic acid test kit (Boehringer, Manheim GmBH), without further treatment of the clear filtrate
3. L-Lactate by the method of Gutmann and Wahlefeld (1974) without further treatment of the clear filtrate described above.

Note: for comparison reasons the acetic and lactic acid determination was performed also as follows. To deproteinise samples, a portion of carrots 2-3 g was added to 5ml of ice cold 1N perchloric acid shaken vigorously and centrifuged (15 min, 4000 x g) The supernantant - after centrifugation was adjusted to a pH between 8-9 by addition potassium hydroxide, followed by filtration. Samples were assayed at 340nm as described above.
4. Ammonia was determined with the method of Chakey & Marbach (1962) or enzymatically (Boehringer, Manheim GmBH)
5. The content of soluble proteins (MW> 3000 daltons) was determined by the method of Sedmak and Grossberg (1977) using bovine serum albumin as a standard.

G) HPLC analysis of l-lactic and acetic acid.
The profile of water soluble compounds (treated with TFA) of uninoculated and inoculated carrots 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 column. The compounds were separated isocratically with buffer (0.009N H₂SO₄ 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.

1. spreading technique ; 0.1 ml x 2plates.
2. pouring technique; 1ml mixed with 15 ml medium followed by 5 ml overlayed.

Results & Discussion
The results obtained from the first six months are shown in Tables 3 to 12 and in Figures 1 to 5
Fig. 3

Carrot
10°C

0 days/N₂

2 days/N₂

5 days/N₂
Part D: Short description of the work planned for the subsequent part of the project

1. There will be a continuation of the subjects study in the first period in order to evaluate and to establish our findings.

   In particular the preservation of carrots will be done with packaging in modified atmospheres (100% CO$_2$ or in different portions of CO$_2$/N$_2$ in order to establish the effect of such storage conditions on the dynamic population (indigenous microbial flora). The microbial flora which will be developed (mainly lactic acid bacteria) will be used, together with other indigenous antimicrobial substances derived from these products, in the final stage of this project to keep the quality and the safety of these products. Measurement of safety and quality will be a main concern of this study.

   Fresh carrots shredded or cubes (inoculated or not with different levels of specific bacteria e.g. pseudomonads, staphylococci) will be packaged in different mixtures of gases (100% CO$_2$, vacuum pack, 100% N$_2$, 20% CO$_2$/80% O$_2$) commonly used to extend the shelf life of such products, and stored at a 10-20°C. At suitable intervals packs will be removed and the level of various relevant microbial association will be determined.

   In parallel with the microbiological changes of these packs, physico-chemical changes in the products will be monitored in an attempt to correlate these changes with the microbial associations developing in packs. Indeed so far we have evidence that various physico-chemical parameters such as pH, acid profile with HPLC, changes in low molecular weight compounds could likely used as potential indicators of the spoilage.

2. Emphasis was given to basic characterisation of lactic acid bacteria in order to use them in the later phase of this project as biopreservatives (Task 3)

3. Special emphasis will be given to the way of inoculation of vegetables with various bacteria in order to study the effect of MAP/VP
Table 3. Changes in the microbial flora of carrot inoculated with Staphylococcus aureus and stored at 10°C (initial inoculum log$_{10}$ 6.2 cfu/ml)

<table>
<thead>
<tr>
<th>Packaging</th>
<th>Microorganisms</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>TVC</td>
<td>5.85</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.37</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td>Lactic bacteria</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>VP</td>
<td>TVC</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph. aureus</em></td>
<td>0</td>
</tr>
<tr>
<td>100% CO$_2$</td>
<td>TVC</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph. aureus</em></td>
<td>0</td>
</tr>
<tr>
<td>100% N$_2$</td>
<td>TVC</td>
<td>6.03</td>
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<tr>
<td></td>
<td>Yeasts</td>
<td>4.80</td>
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<tr>
<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph. aureus</em></td>
<td>0</td>
</tr>
</tbody>
</table>

Control uninoculated samples stored under vacuum
100% CO$_2$ samples inoculated with *Staph. aureus* and stored in this gaseous combination
100% N$_2$ samples inoculated with *Staph. aureus* and stored in this gaseous combination
TVC counts of Total Viable Count
*Staph. aureus* counts of *Staph. aureus*
Lactic bacteria counts of lactic acid bacteria
Pseudomonads counts of pseudomonads
Yeasts counts of yeasts
Table 4. Changes in the microbial flora of carrot inoculated with *Staph.aureus* and stored at 20°C (initial inoculum log₁₀ 6.2 cfu/ml)

<table>
<thead>
<tr>
<th>Packaging</th>
<th>Microorganisms</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Control</td>
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<tr>
<td></td>
<td>Lactic bacteria</td>
<td>3.95</td>
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<tr>
<td></td>
<td><em>Staph.aureus</em></td>
<td>0</td>
</tr>
<tr>
<td>VP</td>
<td>TVC</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.45</td>
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<tr>
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<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph.aureus</em></td>
<td>0</td>
</tr>
<tr>
<td>100% CO₂</td>
<td>TVC</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph.aureus</em></td>
<td>0</td>
</tr>
<tr>
<td>100% N₂</td>
<td>TVC</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>Pseudomonads</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>Lactic bacteria</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>Staph.aureus</em></td>
<td>0</td>
</tr>
</tbody>
</table>

Control: uninoculated samples stored under vacuum
100% CO₂ samples inoculated with *Staph.aureus* and stored in this gaseous combination
100% N₂ samples inoculated with *Staph.aureus* and stored in this gaseous combination
TVC counts of Total Viable Count

*Staph. aureus* counts of *Staph. aureus*
Lactic bacteria counts of lactic acid bacteria
Pseudomonads counts of pseudomonads
Yeasts counts of yeasts
Table 5. Changes in the microbial flora of carrot inoculated with *Salmonella enteritidis* (initial inoculum log\(_{10}\) 7.7 cfu/ml)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Packaging</th>
<th>Microorganism</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>10°C</td>
<td>20% CO(_2)</td>
<td><em>S. enteritidis</em></td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td>80% O(_2)</td>
<td>TVC</td>
<td>5.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>100% CO(_2)</td>
<td><em>S. enteritidis</em></td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>5.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.95</td>
</tr>
<tr>
<td>20°C</td>
<td>20% CO(_2)</td>
<td><em>S. enteritidis</em></td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td>80% O(_2)</td>
<td>TVC</td>
<td>5.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>100% CO(_2)</td>
<td><em>S. enteritidis</em></td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>5.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.95</td>
</tr>
</tbody>
</table>

Control

20% CO\(_2\) / 80% O\(_2\) samples inoculated with *S. enteritidis* and stored in this gaseous combination

100% CO\(_2\) samples inoculated with *S. enteritidis* and stored in this gaseous combination

TVC counts of Total Viable Count

*S. enteritidis* counts of *S. enteritidis*

Lactic bacteria counts of lactic acid bacteria
Table 6 Changes in the microbial flora of carrot inoculated with *Salmonella enteritidis* (initial inoculum log<sub>10</sub> 7.7 cfu/ml)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Packaging</th>
<th>Microorganism</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>10°C</td>
<td>Control</td>
<td><em>S. enteritidis</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>20% CO₂</td>
<td><em>S. enteritidis</em></td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td>80% O₂</td>
<td>TVC</td>
<td>5.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>100% CO₂</td>
<td><em>S. enteritidis</em></td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>5.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.82</td>
</tr>
<tr>
<td>25°C</td>
<td>Control</td>
<td><em>S. enteritidis</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>20% CO₂</td>
<td><em>S. enteritidis</em></td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td>80% O₂</td>
<td>TVC</td>
<td>5.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>100% CO₂</td>
<td><em>S. enteritidis</em></td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TVC</td>
<td>5.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactic bacteria</td>
<td>2.82</td>
</tr>
</tbody>
</table>

Control un inoculated samples stored under vacuum
20%:80% CO₂/O₂ samples inoculated with *S.enteritidis* and stored in this gaseous combination
100% CO₂ samples inoculated with *S.enteritidis* and stored in this gaseous combination
TVC counts of Total Viable Count
*S. enteritidis* counts of *S. enteritidis*
Lactic bacteria counts of lactic acid bacteria
Table 7 Effect of carbon dioxide on the growth of different organisms.

<table>
<thead>
<tr>
<th>Time (h) of sampling</th>
<th>Lactobacillus 241 in MRS</th>
<th>Pseud. fragi in NB</th>
<th>S. enteritidis in SPYE broth</th>
<th>Staph. aureus in BHI broth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂</td>
<td>Air</td>
<td>CO₂</td>
<td>Air</td>
</tr>
<tr>
<td>0</td>
<td>0.170</td>
<td>0.170</td>
<td>0.139</td>
<td>0.139</td>
</tr>
<tr>
<td>24</td>
<td>0.188</td>
<td>0.171</td>
<td>0.246</td>
<td>0.638</td>
</tr>
<tr>
<td>48</td>
<td>0.929</td>
<td>1.542</td>
<td>0.337</td>
<td>0.815</td>
</tr>
</tbody>
</table>
Table 6  Microbiological changes in shredded carrot samples dipped in a Staphylococcus aureus S-6 broth culture, for different period of time, and then stored under different gaseous conditions at 10° C

<table>
<thead>
<tr>
<th>Packing</th>
<th>organism</th>
<th>dipped time</th>
<th>day of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>vp</td>
<td>pseudomonads</td>
<td>30s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>30s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>St.aureus</td>
<td>30s</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>Staphylococci</td>
<td>30s</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>2.60</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td>100% CO₂</td>
<td>pseudomonads</td>
<td>30s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>30s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>St.aureus</td>
<td>30s</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>Staphylococci</td>
<td>30s</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120s</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300s</td>
<td>2.60</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Control</td>
<td>pseudomonads</td>
<td>30s</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>30s</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>St.aureus</td>
<td>30s</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Staphylococci</td>
<td>30s</td>
<td>2.63</td>
</tr>
</tbody>
</table>

VP; samples inoculated with St. aureus and stored in vacuum pack
100 % CO₂: samples inoculated with St.aureus and stored in 100% carbon dioxide
Control: uninoculated samples
Table 9  Detection time of microbial activity (malthus Instr) in shredded carrot samples dipped in a *Staphylococcus aureus* S-6 broth culture, for different period of time, and then stored under different gaseous conditions at 10° C

<table>
<thead>
<tr>
<th>Packing</th>
<th>dipped time</th>
<th>day of sampling</th>
<th>Detection time of microbial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>vp</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30s</td>
<td></td>
<td>8.3</td>
<td>4.6</td>
</tr>
<tr>
<td>120s</td>
<td></td>
<td>9.2</td>
<td>5.5</td>
</tr>
<tr>
<td>300s</td>
<td></td>
<td>9.2</td>
<td>5.4</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td>8.3</td>
<td>7.1</td>
</tr>
<tr>
<td>CO₂</td>
<td>30s</td>
<td>9.2</td>
<td>7.6</td>
</tr>
<tr>
<td>120s</td>
<td></td>
<td>9.2</td>
<td>7.5</td>
</tr>
<tr>
<td>300s</td>
<td></td>
<td>9.2</td>
<td>7.5</td>
</tr>
<tr>
<td>control</td>
<td>30s</td>
<td>8.9</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Table 10  pH of the shredded carrot samples dipped in a *Staphylococcus aureus* S-6 broth culture, for different period of time, and then stored under different gaseous conditions at 10° C

<table>
<thead>
<tr>
<th>Packing</th>
<th>dipped time</th>
<th>day of sampling</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>vp</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30s</td>
<td></td>
<td>5.77</td>
<td>5.65</td>
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<tr>
<td>120s</td>
<td></td>
<td>5.72</td>
<td>5.68</td>
</tr>
<tr>
<td>300s</td>
<td></td>
<td>5.75</td>
<td>5.77</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td>5.77</td>
<td>5.72</td>
</tr>
<tr>
<td>CO₂</td>
<td>30s</td>
<td>5.72</td>
<td>5.80</td>
</tr>
<tr>
<td>120s</td>
<td></td>
<td>5.75</td>
<td>6.00</td>
</tr>
<tr>
<td>300s</td>
<td></td>
<td>5.75</td>
<td>6.00</td>
</tr>
<tr>
<td>control</td>
<td>30s</td>
<td>5.70</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Table A  Organoleptic characteristics developed during the storage of these samples stored under different gaseous conditions

1. Control samples (stored in vacuum; uninoculated)
   sour, diacetyl, acetic acid, ropy, discoloration
2. samples in vacuum dipped for 30s in broth with *St.aureus*
   ropy, diacetyl,
3. samples in 100% carbon dioxide
   good color, without ropy, diacetyl, sour
Table 12. Survival/growth of *Lactobacillus plantarum* and *Salmonella enteritidis* in broths, in which carrot juice has been added in various concentrations and then stored at 10, 25 and 37° C.

<table>
<thead>
<tr>
<th>organism</th>
<th>carrot (%)</th>
<th>time of sampling</th>
<th>0</th>
<th>8</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>juice added</td>
<td>10° C</td>
<td>25° C</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>.134</td>
<td>.134</td>
<td>.234</td>
<td>.230</td>
<td>.230</td>
<td>.230</td>
</tr>
<tr>
<td>1</td>
<td>.134</td>
<td>.134</td>
<td>.520</td>
<td>.520</td>
<td>.520</td>
<td>.520</td>
</tr>
<tr>
<td>2</td>
<td>.128</td>
<td>.128</td>
<td>.830</td>
<td>.830</td>
<td>.830</td>
<td>.830</td>
</tr>
<tr>
<td><em>S. enteritidis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>.071</td>
<td>.071</td>
<td>.190</td>
<td>.190</td>
<td>.190</td>
<td>.190</td>
</tr>
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<td>1</td>
<td>.074</td>
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<td>.720</td>
<td>.720</td>
<td>.720</td>
<td>.720</td>
</tr>
<tr>
<td>2</td>
<td>.068</td>
<td>.068</td>
<td>.830</td>
<td>.830</td>
<td>.830</td>
<td>.830</td>
</tr>
</tbody>
</table>

AIR1-CT92-0125 Meeting #2
17 + 18 June 1993
**Participant 6: AFRC-Institute of Food Research (AFRC.IFRN)**
Norwich Laboratory
Norwich Research Park
Colney
Norwich, NR4 7UA
United Kingdom

A. 1. (Sub-)Project 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.

2. Project coordinator:
Dr. Michael W. Peck

3. Project participants and 4. Resources:
Dr. Michael W. Peck (9.6 man-months)
Sandra C. Stringer (48 man-months).

B. 1. Description of the research topic or practical problem:
Non-proteolytic strains of *Clostridium botulinum* produce heat-resistant spores, 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).

2. 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 *C. botulinum* in refrigerated ready-to-eat foods.

3. Short description of the approach.
   
   **Studies in laboratory media.** Initial studies will be carried out in laboratory media in order to identify, more rapidly, suitable mild preservative factors that inhibit growth from spores of non-proteolytic *C. botulinum* at refrigerated temperatures. The effect of gas atmosphere and other mild preservative factors on growth from heat-damaged and unheated spores of non-proteolytic *C. botulinum* will be determined. Spores will be heated in saline or an anaerobic broth, with recovery on a defined medium in the presence of mild preservative factors.

   **Studies in foods.** The suitability of the gas atmosphere and other mild preservative factors to inhibit growth from spores of non-proteolytic *C. 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 *C. 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 *C. botulinum* will be determined in relation to the normal epiphytic flora of salad vegetables.
C. 1. Short overview of research performed January to June 1993

Spores have been produced of the non-proteolytic \textit{C. botulinum} type B strain 17B. The heat-resistance of these spores has been determined at 75°C (recovery in absence of lysozyme) and 90°C (recovery in the presence of 105g lysozyme/ml), and shown to be similar to that of other spores of non-proteolytic \textit{C. botulinum} produced at IFR Norwich. Recovery conditions were 30°C/pH 6.8/0.1% NaCl.

In order to determine the effect of NaCl (a mild preservative factor) on growth from heat-damaged and unheated spores of non-proteolytic \textit{C. botulinum}, spores of strain 17B have been heated for five time periods at 75°C and 90°C (recovery in the presence/absence lysozyme - as above), and recovered in medium containing 0, 1.5%, 3.0% and 4.5% NaCl. The medium is optimal apart from the salt concentration. Growth from heat-damaged and unheated spores of non-proteolytic \textit{C. botulinum} is being followed for 90 days.

Further experiments have been planned to determine the effect of temperature and pH (mild preservative factors) on growth from heat-damaged and unheated spores of non-proteolytic \textit{C. botulinum}. Spores of strain 17B will be heated at 75°C and 90°C (recovery in the presence/absence lysozyme - as above), and recovered at different temperatures (5, 10, 30°C), or different pH values (5.2, 5.5, 6.0, 7.0). Recovery conditions are optimal apart from the temperature or pH. Growth from heat-damaged and unheated spores of non-proteolytic \textit{C. botulinum} will be followed for 90 days.

2. Recent publications from AFRC-IFRN related to the project


Recent presentations/posters:


Peck, M.W., 1992. A summary of current research on non-proteolytic \textit{Clostridium botulinum} at
AFRC-Institute of Food Research at Norwich

IFR Norwich. Oral Presentation at ERRC, USDA. Philadelphia, USA.


D. 1. 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 C. 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 C. 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 C. botulinum in a model, vegetable/egg-containing, food.
5. Behaviour of non-proteolytic C. botulinum in relation to gas atmosphere and the epiphytic flora of salad vegetables.
6. Evaluation of the safety, with respect to non-proteolytic C. botulinum, of novel preservation systems developed by other labs.

(ii) Experimental plan for next six months (7-93 to 12-93)
Determine effect of mild preservative factors on growth from heat-damaged and unheated spores of non-proteolytic C. botulinum. This may include the effect of gas atmospheres.

An experiment determining the effect of NaCl concentration (0, 1.5%, 3.0% and 4.5%) on growth from heat-damaged and unheated spores of non-proteolytic C. botulinum will be completed. Further experiments that determine the effect of temperature (5, 10, 30°C) and pH (5.2, 5.5, 6.0, 7.0) on growth from heat-damaged and unheated spores of non-proteolytic C. botulinum will also be completed.

Further experiments will be started in which the effect of mild preservative factors on growth from heat-damaged and unheated spores of non-proteolytic C. botulinum will be examined. This may include:
(i) a more detailed examination of the effect of temperature, pH or salt concentration
(ii) an examination of the effect of other preservative factors (e.g. acidulents, gas atmosphere)
(iii) a study of combinations of mild preservative factors.

This research will identify mild preservative factors that reduce the risk of growth from spores of non-proteolytic C. botulinum, and in subsequent studies the effect of these mild preservative factors will be examined further.
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 (no description given yet)

2. Project coordinator:
   Prof. Dr. David O’Beirne

3. Project participants and 4. Resources:
   Prof David O’Beirne
   Ms Sarah Fitzgerald (postgraduate student)
   Ms Catherine Barry (postgraduate student)

   Resources:
   Year 1  24 man months
   Year 2  24 man months
   Year 3  12 man months (estimated)
   Year 4  6 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
(a) 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.
(b) Significance of gas sampling procedures for accuracy and precision. Effects of resampling, interactions with gas barrier properties of packaging film.
(c) Accuracy and precision of non-GC based analytical procedures.

Years 2-4

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 from 1-1-1993 to 30-6-1993

The first 6 months have been used to set up gas analyses, to assemble the packaging and other materials required, and to develop collaboration with Nature’s Best and instrument manufacturers. A routine GC procedure for separation of $O_2$, $Ar$, $N_2$, and $CO_2$ using a CTR1 column (Alltech, USA) is in place. This procedure does not separate oxygen from argon. Some preliminary work has been done on apparent oxygen and carbon dioxide levels in commercial packages from Nature’s Best Ltd. and this will be presented at the Norwich meeting. The objective is to identify product types which might serve as models in subsequent work on the accuracy of low oxygen measurements and in optimisation of product-packaging compatibility.

A second GC procedure designed to separate oxygen from argon using a CTR3 column (Alltech, USA) has also been set up. Evaluation of the capability of this system is currently being carried out.

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

Second half of Year 1

(a) Set up GC procedure to quantify argon.
(b) Quantify argon in commercial and laboratory prepared packages which have product modified atmospheres. Determine the affects of packaging film and storage time.
(c) Quantify argon levels in laboratory-prepared gas flushed packages. Investigate effects of packaging films and storage time.

These experiments will provide information not currently available on limitations of GC techniques for low oxygen determination in packages of respiring produce. Its significance will be discussed and applied in subsequent development of novel product-package optimisation work.

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, physiological age) and raw material preparation (cutting - washing - dipping) 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 cut lettuce cut carrots and potato strips 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 technology
- the effects of washing/dipping procedures
- the effects of important cultivars
- the effects of physiological age of intact raw materials
Opportunities to slow physiological ageing, microbial growth, loss of sensory quality, and nutritional value will be identified.

4. Experimental approach in phases

Year 1
Detailed investigation of effects of cutting and washing/dipping on deterioration (respiration, microbial growth, sensory aspects). Development of optimum processing procedure.
Generation of data on model products (cut lettuce and carrot, potato strips).

Years 2-4
Consolidation of data from Year 1. Investigation of cultivar and physiological age effects.
Determination of effects of minimal processing and storage on ascorbic acid/dehydroascorbic acid levels.

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, discoloration 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 enterobacteria are the most important. Dipping cut vegetables in solutions containing chlorine (up to 100ppm) 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 1-1-1993 to 30-6-1993

Work in the first six months has been preparatory in nature. The literature has been thoroughly searched and detailed plans for peeling, cutting, and dipping experiments have been finalised. Apparatus to measure respiration rate is being assembled and tested.

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

Second half of Year 1

(a) Effects of cutting methods on physiological and microbiological aspects of tissue deterioration in the model products chosen.

(b) Effects of washing alone or washing combined with dipping in chlorine or ascorbic acid/citric acid solutions on tissue deterioration.

Data will form the basis of optimised processing procedure and be used for subsequent phases of the work.
Participant 9: Nature’s Best Ltd.
   Carnes West
   Duleek, Co. Meath
   Ireland

A. 1. (Sub-)Project title:
   Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild
   preservation techniques.

2. Project coordinator:
   Mr. Paddy Callaghan

3. Project participants and 4. Resources:
   Mr. Tom Hopkins 5 man-months
   Mrs. T. Power 2 man-months
   Mrs. B. Brannigan 2 man-months

B. 1. Description of the research topic or practical problem:
   Providing production level microbiological analyses 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 to the consumer.

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
   Nature’s Best will undertake to provide microbiological counts on aerobic mesophiles, lactic
   acid bacteria and yeasts and moulds from samples taken during production. Bacteriological
   counts and proliferation rates under various packaging materials e.g. shrink wrapping, stretch
   wrapping and pillow packaging. Nature’s Best will also liaise with ULMK. DCLS in order to
   ascertain gas levels in various packs in our portfolio to arrive at optimum
   product/packaging/preservation combination.

C. 1. Overview of work carried out between 1-1-93 and 30-6-93
   In the period from 1-1-93 to 30-6-93, Nature’s Best have produced a variety of production
   level microbiological analyses of products within our portfolio. These have included products
   packaged in orientated polypropylene via an Ishida Multihead weigher and an Illapak form
   filling packaging machine and product in trays stretch wrapped with poly ethylene via a Digi
AW2600 stretch wrapper. In the formulation of a meaningful HACCP plan for our beansprout production system, it was necessary to sample product prior to washing, post washing and in production.

Beansprouts are washed in Nature’s Best in a 100ppm chlorine solution which is monitored half-hourly and corrected if necessary during the washing process. Swabs are also taken to quantify the efficacy of the cleaning regime as process hygiene is the most crucial aspect of the product lifecycle.

The type of analysis performed includes TVC, enumeration of coliforms, *E. coli*, *S. aureus*, Faecal Streptococci and yeasts and moulds. The above tests on Beansprouts are carried out at the following frequencies:

<table>
<thead>
<tr>
<th>Analysis Type</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVC</td>
<td>Each production</td>
</tr>
<tr>
<td>COLIFORMS</td>
<td>Each production</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Each production</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Monthly</td>
</tr>
<tr>
<td>Faecal Streps</td>
<td>Monthly</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Monthly</td>
</tr>
<tr>
<td><em>Listeria</em></td>
<td>Weekly</td>
</tr>
<tr>
<td>YEASTS/MOULDSD</td>
<td>Weekly</td>
</tr>
</tbody>
</table>

Each analysis is carried out in accordance with ICMSF recommended methods for microbiological analysis in foods. Nature’s Best aims to have beansprouts and beansprout based products within the following specifications which have been supplied to us by Tesco Supermarkets, UK. Included also is an average value for the following tests for Nature’s Best beansprouts over the past six months.

<table>
<thead>
<tr>
<th>Analysis Type</th>
<th>Target</th>
<th>Avg Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVC</td>
<td>&lt;10⁶</td>
<td>8.3x10⁵</td>
</tr>
<tr>
<td>COLIFORMS</td>
<td>&lt;10⁴</td>
<td>3.9x10⁵</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>&lt;10</td>
<td>&lt;10/g</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&lt;20</td>
<td>absent</td>
</tr>
<tr>
<td>Faecal Streps</td>
<td>&lt;10⁵</td>
<td>absent</td>
</tr>
<tr>
<td><em>Listeria</em></td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>YEASTS/MOULDSD</td>
<td>&lt;10⁵</td>
<td>4.4x10⁵/g</td>
</tr>
</tbody>
</table>
D. 1. Work planned in the subsequent part of the project

In the next phase Nature's Best will undertake to provide microbiological data on spoilage organisms in our salad lines and continue our contribution with our beansprout testing data. We will endeavour to liaise with ULMK.DCLS with regard to product preparation and dipping and observing the effects of various dips on pertinent spoilage organisms and the subsequent effect on the shelf life of our products. We will undertake to supply to ULMK.DCLS with commercial product from our lines for the purpose of gas analysis and microbiological analysis, in an attempt to elucidate an ideal product/packaging/dipping combination. We will undertake to enumerate lactobacilli which up until now had not found a necessity to test for.
Proposal for standardized microbiological assays of (MAP) vegetables

R.R. Beumer, Dept. Food Science, Wageningen Agricultural University

Introduction
To improve the reproducability of the results, for the preparation of diluents and culture media only dehydrated basic components or complete dehydrated media should be used and the manufacturer's instructions strictly followed. Chemicals used for these media must be of recognized quality.

Use of distilled water is preferred. If not available deionized water free from antimicrobial substances may be used. Be sure that the conductivity is 3-5 μS.

Measure for every batch the pH (referred to 25°C). Adjustment is done either with hydrochloric acid (1 mol/l) or sodium hydroxide (1 mol/l).

If prepared culture media are not used immediately, store them in the dark at low temperature. Broths and diluents between 0 and 5°C, poured plates preferably between 10-12°C. Be sure evaporation of water is restricted to a minimum (< 5%). The storage time should not exceed 1 month for general media (no elective or selective agents). Follow the manufacturer's instructions for the storage of media with elective and/or selective agents. Be sure you only investigate the effect of MAP, temperature and storage time on the microbial growth. So, do not use vegetables that have treated with chlorinated water.

Preparing dilutions
Take under aseptic conditions 25-40 g sample and macerate this with the diluant (1:9) in a sterile plastic bag (1-2 minutes) in a stomacher. Make further serial dilutions with sterile pipets. Be sure that for each sample appropriate dilutions are within ½ hour in contact with incubation media. Use as diluant physiological saline solution (0.85% sodium chloride and 0.1% bacteriological peptone).

Total Plate Count
Standard Plate Count Agar; Incubation 3 days at 30°C
For counts of psychrotrophic microorganisms: use surface plates and incubate 7-10 days at 10°C

Enterobacteriaceae ISO/DIS 7402
Violet Red Bile Glucose Agar. Incubation: 24 h 37°C.
Confirmation: presence of oxydase (-) and fermentation of glucose (+)

Lactic acid bacteria
Rogosa Agar or MRS Agar (plates with double layer or under gas atmosphere with: 85% nitrogen, 10% carbon dioxide and 5% hydrogen). Incubation 3-5 days, 30°C.
Confirmation: Gram stain or alternatives (aminopeptidase test, KOH test): Gram positive rods or cocci and test for presence of catalase (3% hydrogen peroxide): negative.
To inhibit development of yeasts Delvocid may added (0.1-0.2%).
Recommended microbiological assays

**Yeast and moulds ISO 7954**
Oxytetracycline Yeast Extract Glucose Agar. Incubation 3-5 days 25°C.
Confirmation: water prepareate.

**Bacillus cereus ISO/DIS 7932**
MYP Agar (with egg yolk and polymyxin B sulphate). Incubation 1 day, 30°C.
Confirmation of characteristic colonies with glucose (+), VP (+) and Nitrate (+, but sometimes -). Sometimes the egg yolk reaction can only be seen just underneath colonies.

**Listeria monocytogenes**
In case of enrichment (not necessary for our experiments, in which growth should be measured) the most recent FDA procedure is recommended. Enrichment of 25 g (1:9) in Listeria Enrichment Broth at 30°C. In case of decrease of the pH to values < 5.5 an appropriate buffer should be incorporated.
Confirmation of characteristic colonies: streaking on Tryptone Soya Agar with 0.6% Yeast Extract added (1 day 30°C), listeria colonies show a blue color under Henry illumination (Olympus stereo microscope with dark field illumination). Further biochemical confirmation with API-Listeria, Micro-ID-Listeria (or the corresponding tests in culture tubes) and hemolysis.
Direct counts can be made on Palcam Agar or (perhaps) on EHA (a medium developed in our laboratory). Use of Oxford Agar should be discouraged. Confirmation as described earlier.

**Salmonella**
Use the latest ISO version (ISO/DIS 6579; 1991); Use preferably Rambach Agar as second medium.

**Counting of colonies/ Expression of results**
After the specified incubation time, count and record the number of colonies on the plates. For calculation of the colony count use preferably plates with 15-150 colonies. Determine the arithmetic mean of the counts from two plates of the same dilution. If the highest count exceeds the lower count of the same dilution with 30% or more regard the results as less reliable.
If there are fewer than 15 colonies, count the actual number on each plate and record the arithmetic mean of the counts from the two plates.
In case of selective media: select 5-10 suspected colonies and confirm these as described. If 80% or more of the selected characteristic colonies are confirmed, adjustment of the count is not necessary. In all other cases, the number shall be calculated on the percentage of the count of characteristic colonies that were confirmed.

Retain only two significant figures for the expression of results:
Mean counts < 100 : round it to the nearest multiple of 2
Mean counts > 100 : round it to the nearest multiple of 10 does not end in 5
Mean counts > 100 : round it to the nearest multiple of 20 and ends in 5
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.

<table>
<thead>
<tr>
<th>Provisional date</th>
<th>Host</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-1993</td>
<td>ATO.DLO</td>
<td>Wageningen, the Netherlands</td>
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<tr>
<td>16-6-1993</td>
<td>AFRC.IFRN</td>
<td>Norwich, U.K.</td>
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<td>16-12-1993</td>
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<td>16-6-1994</td>
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<tr>
<td>15-12-1994</td>
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<td>1-6-1995</td>
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<td>1-6-1996</td>
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<td>Cavaillon (FR) or Duleek (IR)</td>
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<tr>
<td>1-12-1996</td>
<td>ATO.DLO</td>
<td>Wageningen, the Netherlands</td>
</tr>
</tbody>
</table>
Acronym of institute / contactperson / address / communication facilities

AFRC.IFRN
Dr. Mike Peck
AFRC-Institute of Food Research
Norwich Science Park
Colney
Norwich, Norfolk NR4 7UA
U.K.
Tel: +44 6035 6122
Fax: +44 6035 07723
E-mail in"PECKM@FRIN.AFRC.AC.UK"

ATO.DLO
Dr. L.G.M. Gorris
Agrotechnological Research Institute
Haagsteeg 6
P.O. Box 17
NL-6700 AA Wageningen
The Netherlands
Tel: +31-8370-75000/-75126
Fax: +31-8370-12260
E-mail: in"L.G.M.GORRIS@ATO.AGRO.NL"

CIRAD.SAR
Prof. Dr. S. Guilbert
Dpt. Systèmes Agro-Alimentaires et Ruraux
73 Rue J.F. Breton
Domaine de Lavalette
F-34000 Montpellier
France
Tel: +33 67 615700/615759
Fax: +33 67 611223

INRA.AV
Dr. C. Nguyen-The
Lab. Technol. Biochémie Appliquée
Inst. National Rech. Agronomique
Domaine Saint-Paul
BP 91
F-84143 Montfavet Cedex
France
Tel: +33 90 316000/316160
Fax: +33 90 316258

FRUID
Mdm S. Le Hesran
Les Crudeites
Fruidor S.A.
Avenue Che. Delaye
F-84300 Cavaillon
France
Tel: +33 90 783029
Fax: +33 90 713615

NARF
Dr. G.J.E. Nychas
Inst. Techn. Agricult. Products
National Agricult. Res. Foundation
Sof. Venizelou 1
Lyvovrisi 14123
Athens - Greece
Tel: +30 1 2845940/942
Fax: +30 1 2840740/6846700

NBEST
Mr P. Callaghan
Nature's Best Ltd.
Carnes West
Duleek, Co Meath
Ireland
Tel: +353 41 23546
Fax: +353 41 23574

ULMK.DCLS
Prof. Dr. David O’Beirne
Dept. Chemistry & Life Sciences
University of Limerick
Plassey Technology Park
Limerick - Ireland
Tel: +353 61 333644
Fax: +353 61 330316
E-mail: in"O’BEIRNE@UL.IE"

WAU.DEF
Prof. Dr. F.M. Rombouts
Levensmiddelenchemie & -microbiologie
Gebouw Biotechno L.U.W
Bomenweg 2
Postbus 8129
6700 EV WAGENINGEN
Tel: +31 8370 82888/82233
Fax: +31 8370 84893

A1RI-CT92-0125 Meeting #2
17 + 18 June 1993