Development of fermented sauce-based salads

assessment of safety and stability

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hoogleraar in de levensmiddelenhygiëne en -microbiologie

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Proefschrift

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in de landbouw- en milieuwetenschappen

op gezag van de rector magnificus,

dr. H.C. van der Plas,

in het openbaar te verdedigen

op dinsdag 27 oktober 1992

des namiddags te vier uur in de Aula

van de Landbouwuniversiteit te Wageningen

Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de sectie Levensmiddelenchemie en -microbiologie van de vakgroep Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen in samenwerking met Johma International Holding B.V. te Losser, met financiële steun van StiPT, Uitvoeringsorganisatie voor technologiebeleid van het Ministerie van Economische Zaken, in het kader van de subsidieregeling Programmatische Bedrijfsgerichte Technologie Stimulering. De werkwijze is beschreven in een Europese patent-aanvrage (no. 91202356.1).

STELLINGEN

1. Het gebruik van "Schutzkulturen" als indikator voor temperatuurmisbruik van levensmiddelen met een neutrale pH, welke in de koelkast behoren te worden opgeslagen, dient ernstig te worden ontraden.
(G. Cerny, Lebensmitteltechnik, **23**, 448, 450-451, 1991)

2. De observatie van Lievense *et al.* betreffende azijnzuurproduktie in de beginfase van de aktiviteitsmeting van *Lactobacillus plantarum* cellen is indicatief voor de fysiologische toestand van de geteste cellen en niet primair voor aanwezigheid van O_2 in het medium.
(L.C. Lievense, K. van 't Riet and A. Noomen, Appl. Micobiol. Biotechnol., **32**, 669-673, 1990)

3. De consumptie van tyramine bevattende levensmiddelen is niet schadelijk voor de volksgezondheid.

4. Als het om de smaak gaat valt er wel degelijk te twisten.

5. Het door Moberg beschreven 4-methylumbelliferyl-ß-D-glucuronide-medium is niet geschikt voor de detectie van pathogene *E. coli* species.
(L.J. Moberg, Appl. Environ. Microbiol., **50**, 1383-1387, 1985)

6. De mogelijke afschaffing van vakbekwaamheidseisen in het midden- en kleinbedrijf dient (in de levensmiddelensector) gepaard te gaan met een forse verhoging van de boetetarieven van de Keuringsdiensten van Waren.

7. Echt innovatieve bedrijven degraderen de uitgaven voor "Research & Development" nooit tot sluitpost op hun begroting.

8. De afwachtende houding van veel bedrijven met betrekking tot de invoering van snelle detectiemethoden is tot op zekere hoogte te begrijpen.

9. Vroegtijdige informatie en openheid naar de consument is de enig juiste methode om invoering van mogelijk als controversieel ervaren produktiewijzen te vergemakkelijken.

10. Door het tegelijkertijd laten starten van meer dan twee schaatsers bij langebaanwedstrijden wordt de aantrekkelijkheid ervan niet verhoogd.

11. Wetenschap en religie zijn beide bezig met het raadsel van het menselijk bestaan, de omringende wereld en de relatie tussen die twee. De wetenschap neemt daarin, in tegenstelling tot wat vaak gedacht wordt, geen bijzondere plaats in in die zin dat zij "objectief" zou zijn en religie "subjectief". (*naar* A. van den Beukel, De dingen hebben hun geheim, Ten Have/Baarn, 1990).

12. Met het verplicht stellen van het voeren van dimlicht overdag biedt de overheid het grote aantal slechtziende rijders de helpende hand.

M.H. Bonestroo
Development of fermented salads - assessment of safety and stability.
Wageningen, 27 oktober 1992.

Aan mijn ouders

VOORWOORD

In opdracht van saladeproducent Johma Nederland B.V. heb ik de afgelopen vier jaar mogen werken aan de ontwikkeling van een nieuwe technologie om via biotechnologische processing stabiele, goed smakende salades te bereiden. Het werken aan de ontwikkeling van nieuwe produkten, het verzamelen van kennis over het onderhavige "lastige" produkt en het introduceren van de nieuwe technologie bij genoemd bedrijf zijn zaken waaraan ik de afgelopen jaren met veel plezier heb gewerkt. Echter: voor alles wat we "goed willen" doen geldt een gulden regel: "niets voor niets". Gedurende het projekt is dan ook door velen zeer hard gewerkt. Het kunnen bewerken van de resultaten tot een proefschrift is voor mijzelf de kroon op het vele werk.

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Martin Bonestroo

ABSTRACT

Bonestroo, M.H. (1992) Development of fermented sauce-based salads - assessment of safety and stability. Doctoral thesis, Agricultural University Wageningen (135 pp., English and Dutch summaries)

Sauce-based delicatessen salads, composed of solid ingredients, such as potatoes, vegetables, fish, meat and an oil-in-water emulsion containing acidulants (acetic and lactic acids) and chemical preservatives (sorbic and benzoic acids) can be subject to microbial and chemical changes and physical deterioration. In this thesis a new method of salad preparation is presented which meets consumer demands for salads without chemical preservatives and with a less sour taste and by which the major microbiological and chemical problems of the present-day salads can be controlled. Conditions for operation and the microbiological and chemical aspects that are relevant for the process have been investigated. It is shown that preparation of salads by the new process, i.e. fermentation in their package with lactic acid bacteria at higher temperatures ($\geq$ 42°C) in a short time ($\leq$ 8 hours), followed by cooling to below 7°C, is feasible. Salads produced according to this procedure have a mildly sour taste and are microbially stable for 5-6 weeks at 7°C, i.e. yeasts and other spoilage organisms are inhibited, provided that measures are taken to assure low initial contamination with these microorganisms. Moreover, fermented salads, subjected to light exposure, are protected for lipid photo-oxidation. This protection is apparently due to the oxygen scavenging and reducing effects of the fermentation. The salads could be regarded as safe, as growth of pathogenic microorganisms and production of biogenic amines are inhibited.

CONTENTS

1

GENERAL INTRODUCTION

Sauce-based salads are composed of solid ingredients, such as potatoes, vegetables, fish, meat and an oil-in-water emulsion containing acidulants, such as acetic and lactic acids. The shelf life of these so-called delicatessen salads is determined mainly by low pH, low storage temperature and chemical preservatives, i.e. sorbic and benzoic acids (Lund *et al.*, 1984; de Wit and Rombouts, 1989). High demands on quality of raw materials, good manufacturing practices and especially the use of preservatives, have led to a microbial shelf life of these salads of 6 to 8 weeks, if stored below 7°C. Micro-organisms causing spoilage are lactic acid bacteria, yeasts, moulds and bacilli (Smittle, 1977; Kirsop and Brocklehurst, 1982; Brocklehurst *et al.*, 1983; Lund *et al.*, 1984). However, the actual shelf life of salads, especially potato salads, may be much shorter due to lipid oxidation of polyunsaturated vegetable oils. Market demands, such as the use of transparent packing materials have a negative effect on the shelf life of salads because they are usually stored under high illumination in open display refrigerators. Oxidation could be inhibited by natural and synthetic antioxidants (Pokorny, 1987; Mörsel, 1990; Mörsel and Meusel, 1990), but these are never applied. Next to these microbial and chemical changes, salads can also suffer from physical deterioration, such as changes in viscosity of the dressings, coagulation and separation.

Nowadays, there is a strong demand for salads without chemical preservatives and with a less sour taste. This trend emerges from the rising health consciousness of the consumer and his desire for quality products in which ingredients retain some of their original taste. The omission of preservatives in the present-day recipes, however, limits the shelf life of salads to 2 to 3 weeks, if stored below 7°C (Gromzik 1991). Moreover, problems with pathogenic micro-organisms may arise when manufacturers make concessions to such demands of the consumer.

It is generally accepted that the antimicrobial activity of weak acids such as acetic acid and lactic acid is due largely to the undissociated acid molecules. An increase in concentration of these molecules, independent from a decrease in pH levels, will improve the microbial stability of salads. This can be achieved by using buffer systems of weak organic

acids and their salts (Debevere, 1987). It has been suggested that an additional advantage would be that sour taste problems are avoided . However, it is known that the sour taste depends on both the hydrogen ion concentration and the molar concentration of undissociated acid molecules. Buffering of carboxylic acids with their sodium salts may not very much lower perceived sourness (Ganzevles and Kroeze, 1987). Another drawback of the method is that chemical stabilisation, i.e. the prevention of lipid oxidation, cannot be achieved. The shelf life of salads could also be extended by technological provisions, such as the production of salads under strict hygienic conditions, in closed systems with rigorous exclusion of oxygen and with the use of modified atmospheres. These methods, require, however, large capital investments. Moreover, yeasts with a fermentative metabolism and especially lactic acid bacteria will not be inhibited (Buick and Damoglou, 1989; Farber, 1991).

Lactic acid bacteria play an important role in the production of many fermented foods (Daeschel *et al.*, 1987; Gibbs, 1987; Salih and Drilleau, 1990). Lactic acid fermentation of foods generally is a low-cost method of food preservation in which spoilage and pathogenic organisms are inhibited, mostly by acid production and pH reduction but also by a lowering of the oxidation-reduction potential, competition for essential nutrients and possibly by the production of inhibitory compounds: antibiotic compounds and hydrogen peroxide. In general lactic fermented foods have desirable organoleptic properties, and losses in nutritional value are small. Many health effects, such as decreased gastrointestinal disorders, control of some types of cancer and anticholesterol activity have been attributed to the consumption of fermented foods, e.g. dairy products, although these effects are difficult to prove (Cooke *et al.*, 1987; Fernandes *et al.*, 1987; Gilliland, 1990; Lindgren and Dobrogosz, 1990).

Aim of this study

For salads, lactic acid fermentation could be an interesting and novel way of preparation. If sufficiently attractive, a certain interest may be expected from the market for these fermented salads, because of their mildly sour taste, their possible health effects and especially because the use of preservatives can be abandoned. Moreover, the reducing effect of the fermentation processes could have a protecting effect on salads towards lipid oxidation.

Traditionally vegetables are fermented by naturally occurring microorganisms at low temperatures ($\leq 25°C$): a slow process (Stamer, 1988; Salih and Drilleau, 1990). In this

study the use of starter cultures and a fermentation process at higher temperatures (> 40°C) in a short time (≤ 8 hours) were chosen. After the fermentation the product was cooled. A process was designed (Fig. 1.1.) which apart from the fermentation step closely corresponds to the existing process of preparation, cold storage and distribution of salads.

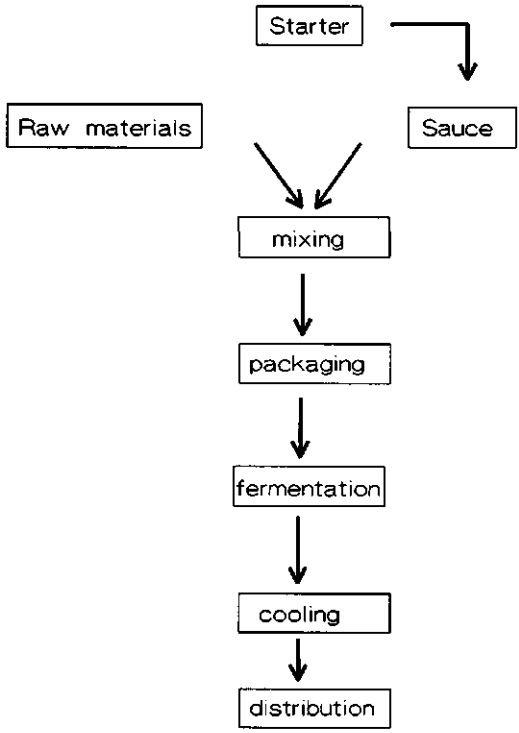

Fig. 1.1. A schematic overview of the salad fermentation process.

Essential in this process is that salads are prepared from separate ingredients and fermented in their package according to the so-called "set-yoghurt" procedure. This approach is favoured above a process in which the separate ingredients are fermented and subsequently mixed to form a salad. Main advantage is the strongly reduced risk concerning microbial contamination. Moreover, a low-oxygen environment can be created, which might prevent lipid oxidation. This concept of preparation and preservation of salads in their consumer package by means of a high temperature-short time fermentation process is totally new. Kammerlehner (1985) reported the production of yoghurt with spices, which were added after

fermentation. Other investigators, such as Abdel-Bar and Harris (1984) evaluated the addition of *Lactobacillus bulgaricus* to refrigerated tuna and potato salads to control the growth of the natural flora, but these salads were acidified chemically. Recently, Cerny (1991) reported the inhibitory effect of lactic acid bacteria (*Leuconostoc cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Lactobacillus casei*) on the growth of certain pathogens in chemically acidified mayonnaise-based meat and potato salads at room temperature.

Experimental approach

A project plan was developed which included the selection of suitable starter cultures, screening of promising strains on major performance criteria, determination of the feasibility of the designed process for preparation of salads with varying ratios of solids to sauce and assessment of optimal fermentation conditions. Furthermore, a number of tests were introduced to determine the microbiological and chemical stability, and the safety of the newly developed products.

Important criteria for the selection of potentially suitable starter cultures are, amongst others:

- rapid growth and souring at temperatures $\geq$ 40°C,
- homofermentative fermentation pattern,
- reducing capacity (oxygen scavenging activity),
- antagonistic effects on spoilage and pathogenic bacteria, yeasts and moulds.

Other criteria, based amongst others, on the fact that fermentation should not alter the flavour and texture of the salads negatively, are:

- no growth at low temperatures ($<$ 7°C),
- acid tolerant (pH $<$ 4.2),
- no extreme nutritional demands,
- fermentation of sucrose, glucose, fructose and pentoses,
- production of dextran or other extracellular polysaccharides,
- formation of flavour components which add to the natural taste of the

ingredients,

- no fermentation of organic acids,
- no amino acid decarboxylative properties,
- limited proteolytic activity,
- no polymer degrading capacities,
- genetically stable.

First, a variety of lactic acid bacteria from the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* are screened for their capacity to ferment glucose and sucrose in a model system as well as in a standardized salad. Furthermore, their production of L(+) and D(-) lactic acid and their growth properties at temperatures above 40°C is assessed (Chapter 2).

Salads, deliberately contaminated with yeasts, isolated from spoiled, industrially produced salads, are fermented with *Lactobacillus plantarum* strains to study the inhibitory effect of fermentation towards yeast growth. The microbiological stability of these salads is monitored during storage at 7°C. It is shown that ascosporogenous, facultative anaerobic yeast species like *Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Torulaspora delbrueckii* are notorious inhabitants of deteriorated salads. Growth of these spoilage yeasts in fermented salads can be inhibited by using starter cultures with high fermenting capacity, provided that measures are taken to achieve low initial contamination with yeasts (Chapter 3).

The risk that fermented sauce-based salads may cause microbial food poisoning is determined by a number of factors, namely contamination of the ingredients, growth and toxin production during the first stage of fermentation, and inactivation during fermentation or storage. In order to investigate the possible growth and persistence of spoilers and pathogens, salads are inoculated with *Klebsiella pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* and fermented with lactic acid bacteria. *Klebsiella pneumoniae*, an acid-resistant psychrotrophic organism is also used as a model organism for the behaviour of pathogenic *Enterobacteriaceae* such as *Salmonella*, *Shigella* and pathogenic *E. coli*. Lactic fermentation of sauce-based salads has been shown to cause inhibition of growth of a range of spoilage and pathogenic bacteria. Fermented salads are microbiologically safe, provided that measures are taken to attain low initial contamination with these pathogens. Also, proper hygienic care during processing should be assured

(Chapter 4).

To study the possible protecting effect of fermentation towards lipid oxidation in potato salads stored under light exposure, salads are fermented with *Lactobacillus plantarum* strains; oxidation is assessed by gas-chromatographic and sensory analyses after storage for 10 days. This 10 days period is chosen as a compromise between the time necessary for the development of detectable amounts of lipid oxidation products and the time during which interferences due to microbial spoilage can be neglected (Chapter 5).

The experiments in Chapter 5 show that fermented potato salads, subjected to light exposure for 10 days, and analyzed for lipid oxidation products with gas-chromatographic methods, differ from uninoculated, acidified control salads. Fermented salads contain lower amounts of lipid oxidation products, amongst others hexanal, indicating a lower level of lipid oxidation. During fermentation the oxygen tension in the salads decreases and at the same time there is a decrease of the oxygen tension in the headspace. To investigate the oxygen consumption by starter cultures and the effect of fermentation on the delay of lipid oxidation in further detail, potato salads are fermented with *Lactobacillus plantarum* and *Lactobacillus acidophilus* and stored under light exposure (Chapter 6).

The primary aim of the experiments in Chapter 7 is to find out whether starter bacteria can generate biogenic amines in fermented salads. The formation of biogenic amines by non-starter bacteria is studied by performing experiments in which salads are deliberately contaminated with amino acid decarboxylase positive *Enterobacteriaceae* and then fermented with an amino acid decarboxylase negative starter culture (*Lactobacillus plantarum*). To create conditions suitable for the production of biogenic amines, these salads are inoculated with free amino acids and pyridoxal phosphate, the latter compound being a cofactor in enzymic amino acid decarboxylation. It is shown that in fermented salads, the occurrence of biogenic amines can be prevented by assuring a low initial contamination, proper hygienic care during processing, and the use of amino acid decarboxylase negative starter cultures which actively suppress non-starter microorganisms.

In a general discussion (Chapter 8) the prospects and limitations of the salad fermentation process are reviewed and some suggestions for further developments are made.

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2

GLUCOSE AND SUCROSE FERMENTING CAPACITY OF HOMOFERMENTATIVE LACTIC ACID BACTERIA USED AS STARTERS IN FERMENTED SALADS

ABSTRACT

Salads, i.e. vegetables and/or meat in an oil-in-water emulsion with a low pH due to the addition of acids, are popular in western countries. The shelf life of these salads is influenced mainly by storage temperature and the use of preservatives, i.e. sorbic and benzoic acid. Lactic acid fermentation could be an interesting novel approach to salad preparation. As part of an investigation on fermented salads, lactic acid bacteria from the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* were screened for their capacity to ferment glucose and sucrose in a model system and in a standard salad. The temperature optimum of these strains was in the range of 34 - 48°C. Most of these strains produced about equal amounts of L(+) and D(-) lactic acid. To determine the specific sugar fermenting capacity (mmoles lactic acid produced per min per g dry weight) of non-growing cells of lactic acid bacteria, a semi-automated model system was used, based on monitoring the pH decrease in a phosphate buffer during conversion of sugars to lactic acid.

The strains tested showed acid production rates on glucose varying between 0.03 and 0.39 mmoles of acid produced per min per g dry weight (average 0.23). These glucose-grown cells showed varying, but lower fermentation rates on sucrose (0 to 0.25 mmol/min/g d.w., average 0.04) than sucrose-grown cells (0 to 0.33 mmol/min/g d.w., average 0.19). Results obtained with the model system were in good agreement with those of fermentation tests, carried out with a standard salad. Inhibition of spoilage bacteria in the standard salad could be achieved by using starter cultures with high fermenting capacity.

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2.1. INTRODUCTION

Lactic acid bacteria play an important role in the production of many fermented foods (Daeschel *et al.*, 1987; Gibbs, 1987; Salih and Drilleau, 1990). Lactic acid fermentation of foods generally is a low-cost method of food preservation in which spoilage and pathogenic organisms are inhibited, mostly by acid production and pH reduction but also by a lowering of the oxidation-reduction potential, competition for essential nutrients and possibly by the production of inhibitory compounds: antibiotic compounds and hydrogen peroxide. In general lactic fermented foods have desirable organoleptic properties, and losses in nutritional value are small. Many health effects, such as decreased gastrointestinal disorders, control of some types of cancer and anticholesterol activity have been attributed to the consumption of fermented foods, e.g. dairy products, although these effects are difficult to prove (Cooke *et al.*, 1987; Fernandes *et al.*, 1987; Gilliland, 1990; Lindgren and Dobrogosz, 1990).

For salads, i.e. vegetables and/or meat in an oil-in-water emulsion with a low pH (<4.8) due to the addition of acids, lactic acid fermentation could be an interesting and novel way of preparation. These traditional salads have conquered a regular place in the market of western countries. Higher demands on quality of raw materials, better preparation methods and the use of preservatives, i.e. sorbic and benzoic acid, have led to a shelf life of these salads of 6 to 8 weeks, if stored below 7°C.

A certain interest may be expected from the consumers for lactic acid fermented salads, because of their taste, their possible health effects and especially because preservatives can be avoided.

Traditionally vegetables are fermented by naturally occurring microorganisms at low temperatures ($\leq$ 25°C), which results in a prolonged fermentation. In this study the use of starter cultures and a fermentation process at higher temperatures ($>$ 40°C) in a short time ($\leq$ 8 hours) were chosen. After the fermentation the product was cooled.

Important criteria for the selection of potentially suitable starter cultures are, amongst others: rapid growth and souring, homofermentative fermentation pattern, absence of growth at low temperatures ($\leq$ 7°C) and antagonistic effects on spoilage and

pathogenic bacteria, yeasts and moulds.

The objective of this study, as part of an investigation on fermented salads, was to screen a variety of lactic acid bacteria from the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* for their capacity to ferment glucose and sucrose in a model system as well as in a standardized salad, their production of L(+) and D(-) lactic acid and their growth properties at temperatures above 40°C.

2.2. MATERIALS AND METHODS

Sources of lactic acid bacteria

Strains were obtained from our own laboratory collection and from Dr. H.M.L.J. Joosten (Netherlands Institute for Dairy Research, Ede, The Netherlands) and Ms. A.E.M. Vermunt (State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands). Several strains were collected from sugarbeet pulp using MRS agar (Merck, Darmstadt, F.R.G.) incubated at 45°C under microaerobic conditions, generated by Anaerocult™ P incubation bags (Merck). Sugarbeet pulp, remaining after sugar extraction, is frequently contaminated with lactic acid bacteria and is a reservoir for thermotrophic species, due to the fact that it leaves the factory with generally high temperatures (>40°C). Other strains were isolated from a suspension of sugarbeet pulp in MRS broth (Merck) which was incubated at 45°C for 24 hours. Strains were purified and screened for inability to produce gas from glucose and for growth at temperatures above 40°C. Pure cultures were maintained in MRS broth with 20 % glycerol (w/w) at -80°C. A laboratory stock collection was kept at -18°C on Litmusmilk (Difco, Detroit, Michigan, U.S.A.) fortified with 10 g/l glucose (Merck) and 1 g/l yeast extract (L21, Oxoid Ltd.,Basingstoke, U.K.). Calcium carbonate (Merck) was added (10 g/l) as neutralizing agent.

Activity tests with a model system

To determine the specific sugar fermenting capacity (mmoles lactic acid produced per min per g dry weight) of non-growing cells of homofermentative lactic acid bacteria, a semi-automated model system was used (Lievense *et al.*, 1990), based on monitoring the pH decrease in a phosphate buffer during conversion of sugars (i.e. sucrose and glucose) to lactic acid. The lactic acid bacteria (n=24) were cultivated for 24 hours at 30°C in a broth with the same composition as MRS broth, but containing glucose or sucrose (Merck) as carbohydrate, followed by subsequent cultivation in the same broth for 16 hours at 30°C. The cells were harvested by centrifugation at 16.000 x g for 10 min (10.000 rpm, Sorvall SB-50, GSA-rotor), cell pellets were suspended in a phosphate buffer (0.01 M K_2HPO_4/KH_2PO_4 dissolved in 0.15 M NaCl, pH 7.0), again centrifuged and resuspended in the same buffer. Dry weight of this suspension was determined by drying at 105°C until constant weight. The activity tests were performed according to Lievense *et al.* (1990) at 42°C.

Activity tests with a standard salad

The lactic acid bacteria (n=80) were cultivated as described earlier, in a broth containing glucose as carbohydrate. The standard salads, contained grated and blanched (1 min, 100°C) carrots (50 % [w/w]) and sauce (50 % [w/w]). The initial pH of the standard salads, which is influenced by the amount of acid in the starter culture, was 6.31 $\pm$ 0.27. The sauce was composed of water (52.7 %[w/w]), soybean oil (35 % [w/w]), sucrose (6 % [w/w]), salt (0.5 % [w/w]), egg yolk (3 % [w/w]) and thickening agents (2.8 % [w/w]). The salads were inoculated with 10^7 cfu per g, filled in glass jars sealed with metal Vapor-Vacuum^{tm} Twist-Off^{tm}-PT caps (White Cap International, U.S.A.) and incubated in a waterbath for 7 hours at 42°C. At time intervals jars were sacrificed for pH measurement.

Identification

The strains were identified with the API 50 CHL system (API System S.A.,

France). The reaction pattern was interpreted with the aid of a computer program (Cox and Thomsen, 1990).

Production of L(+) and/or D(-)-lactic acid

Production of L(+) and/or D(-)-lactic acid in cultures of lactic acid bacteria, grown for 24 and 16 hours at 30°C consecutively, was determined by an enzymatic assay (Boehringer Mannheim, Mannheim, F.R.G.).

Growth at different temperatures

Strains, grown twice at 30°C, first for 24 hours and then for 16 hours, were inoculated into MRS broth. Growth at different temperatures was monitored according to Zwietering *et al.* (1991) by using 20-ml tubes, each containing 10 ml of medium and inoculated with the test organism to reach a target initial concentration of $1x10^7$ organisms/ml. The test tubes were incubated statically at different temperatures from 35°C up to 48°C as follows (temperature in °C $\pm$ 0.1°C): 34.1; 35.6; 36.0; 37.0; 37.6; 39.1; 41.0; 42.4; 42.9; 44.0; 44.5; 45.6; 46.0; 46.9; 47.6; 47.8. At time intervals of approximately one hour, the incubated tubes were thorougly mixed and samples of 0.15 ml were pipetted in micro-titer plates after which optical densities at 620 nm were read (SLT Easy Reader EAR 400 FW, SLT-Labinstruments GmbH, Salzburg, Austria).

Microbiological quality of fermented salads

Mesophilic aerobic spoilage bacteria were counted using Gelysate Agar (GEL): 5 g Bacto Peptone (Difco), 5 g sodium chloride (Merck) and 14 g agar (Oxoid L13) per l, pH 7.6 $\pm$ 0.1. *Enterobacteriaceae* were counted on Violet Red Bile Glucose Agar (VRBG, Oxoid CM485). Lactic acid bacteria were selectively enumerated on MRS medium (Merck) with 12 g of agar (Oxoid L13) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l. Yeasts and moulds were counted on Oxytetracycline Glucose Yeast Agar (OGGA, Oxoid CM545).

Determination of the amount of sugars and organic acids

The HPLC system used consisted of a Spectra Physics model 8800 pump (Spectra Physics Inc., San Jose, C.A., U.S.A.), a model 8880 injector/autosampler, a Shodex RI-SE-61 refractive index detector (Showa Denko K.K., Japan) and a Spectra Physics model 4400 integrator, on-line coupled to an Epson PC AX2 computer with software of Spectra Physics. A Bio-rad 300 mm x 7.8 mm i.d. Aminex HPX-87-H column (Bio-rad, Richmond, C.A., U.S.A.) packed with a strong cation-exchange resin in the hydrogen form was used. The guard column was filled with Biorad AG 50 W/X4 column material. The column temperature was maintained at 30°C. The mobile phase used was H_2SO_4 (0.01 N) in Nanopure water (Barnstead, Dubuque, Iowa, U.S.A.) at a flow rate of 0.6 ml per min. Sample preparation consisted of decimal dilution of the standard salads in Nanopure water, followed by clarification with Carrez-solutions (5 ml sample or dilution, 2.5 ml Carrez-I solution containing 3.60 g potassium hexacyanoferrate-II, $K_4[Fe(CN_6)].3H_2O$/100 ml and 2.5 ml Carrez-II solution containing 7.20 g zinc sulfate, $ZnSO_4.7H_2O$/100 ml), if necessary and subsequent filtration with 0.45 μm Nylaflow filters (Gelman Sciences, U.S.A.). Samples were stored below 7°C prior to analysis (max. 5 days).

2.3. RESULTS

Table 2.3.1. shows the strain identification, along with some of the strain properties. Seventeen of the 24 strains used in the experiments with the model system were identified as *Lactobacillus plantarum*, a facultatively heterofermentative species of the genus *Lactobacillus* (group II). Two other strains were identified as *Lactobacillus casei*, belonging to the same *Lactobacillus* group. Two strains were identified as *Lactobacillus acidophilus*, an obligately homofermentative species of the genus *Lactobacillus* (group I). Two strains belonged to the homofermentative genus *Pediococcus*. All strains showed a homofermentative growth pattern in appropriate tests. However, one was identified as *Lactobacillus brevis*, an obligately heterofermentative species of the genus *Lactobacillus* (group III). We are aware that the API 50 CHL system has its limitations. Above mentioned identifications are therefore tentative. Verification of

Table 2.3.1. Identification and properties of strains used in the fermentation experiments.

Nr.	Identification	Origin	Type of acid D(-):L(+)	Optimum temp.[a] (°C)	Maximum temp.[a] (°C)
1	*Lactobacillus plantarum*	sugarbeet	1.0:1.1	43 -44	>47.6
2	*Lactobacillus brevis*	sugarbeet	1.0:0.6	39.5	45
3	*Lactobacillus acidophilus*	sugarbeet	1.0:0.4	42 -48	>47.8
4	*Lactobacillus acidophilus*	sugarbeet	1.0:0.1	45 -46	>47.1
5	*Lactobacillus plantarum*	collection	1.0:0.8	35 -37	45 -46
6	*Lactobacillus casei 1*[b]	sugarbeet	1.0:7.7	n.d.	n.d.
7	*Lactobacillus plantarum*	sour dough	1.0:0.7	36 -39	45 -46
8	*Lactobacillus plantarum*	sausage[c]	1.0:4.1	37 -39	>46
9	*Lactobacillus plantarum*	soak water[d]	1.0:0.9	36 -39	45 -46
10	*Lactobacillus plantarum*	collection	1.0:1.2	36 -37	42 -43
11	*Lactobacillus plantarum*	soak water	1.0:0.7	36 -39	46 -47
12	*Lactobacillus plantarum*	sourdough	1.0:0.3	36 -37	44
13	*Lactobacillus plantarum*	collection	1.0:0.9	35 -37	45 -46
14	*Lactobacillus plantarum*	collection	1.0:0.6	35 -37	45
15	*Pediococcus sp.*	collection	n.d.	n.d.	n.d.
16	*Lactobacillus plantarum*	sugarbeet	n.d.	41 -44	>46.9
17	*Pediococcus sp.*	collection	n.d.	n.d.	n.d.
18	*Lactobacillus plantarum*	collection	1.0:1.1	36 -38	44 -45
21	*Lactobacillus plantarum*	sausage	1.0:1.0	34 -36	41 -42
20	*Lactobacillus plantarum*	soak water	1.0:0.9	36 -39	45 -46
21	*Lactobacillus plantarum*	collection	1.0:0.7	36 -37	45
22	*Lactobacillus casei 2*[e]	soak water	1.0:2.2	36 -39	44 -45
23	*Lactobacillus plantarum*	collection	1.0:0.9	36 -39	45 -46
24	*Lactobacillus plantarum*	collection	1.0:0.9	37	45

[a] Optimum and maximum temperatures for growth in MRS broth.
[b] *Lactobacillus casei subsp. casei*
[c] Fermented sausage
[d] Soak water used in the production of tempeh
[e] *Lactobacillus casei subsp. pseudoplantarum*
n.d. not determined

phonetic classification should be based e.g. on DNA homology determination. In view of the study by Collins *et al.* (1989) this is particularly true for the *L. casei* strains.

All strains were able to grow at temperatures above 40°C (see Table 2.3.1.). However, the optimum temperature for growth was generally lower (35°C - 39°C), except for some strains, including *L. acidophilus* strains.

Most of the strains produced a mixture of about equal amounts of L(+) and D(-) lactic acid (see Table 2.3.1.), except for the *L. casei* strains and one *L. plantarum* strain (no. 8), which produced larger amounts of the L(+) isomer.

The maximum specific sugar fermenting capacity of non-growing cells in phosphate buffer was measured as velocity of pH decrease at a fixed pH value of 5.3. The acid forming rate (mmoles lactic acid produced per min per g dry weight) was calculated using this velocity and the first derivative (at pH 5.3) of an equation for the titration of phosphate buffer with lactic acid (Lievense *et al.*, 1990). The glucose-grown strains tested in the standard activity test showed acid production rates on glucose varying between 0.03 and 0.39 mmoles of acid produced per min per g dry weight (average 0.23), as expressed in Fig. 2.3.1. The *Streptococcus (thermophilus)* strains were not included in this figure, as they were poor acid producers.

Glucose-grown cells showed varying, but lower fermentation rates on sucrose (0 to 0.25 mmol/min/g d.w., average 0.04) than sucrose-grown cells (0 to 0.33 mmol/min/g d.w., average 0.19). The results obtained for a number of strains are shown in Fig. 2.3.2.

The acid producing capacity of the strains was also tested in standard salads, in which growth during fermentation and buffering capacity of ingredients also play a role. In both tests precultivation temperature (30°C) of the strains and test temperature (42°C) were the same to make comparison of results possible. A fermentation temperature of 42°C was chosen to give the starter organisms an advantage over possible spoilage organisms, i.e. lactobacilli, including *L. brevis* and *L. fructivorans*, yeasts and

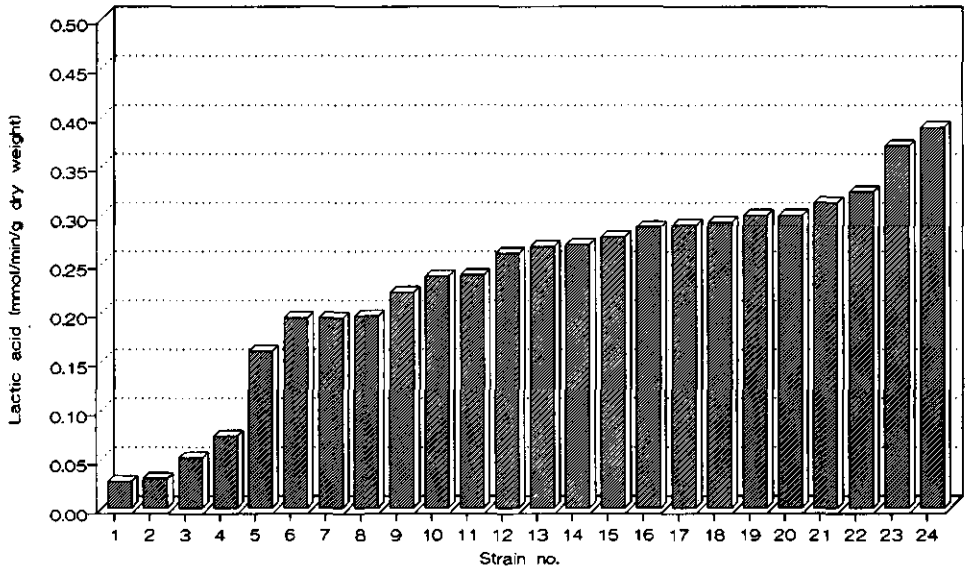
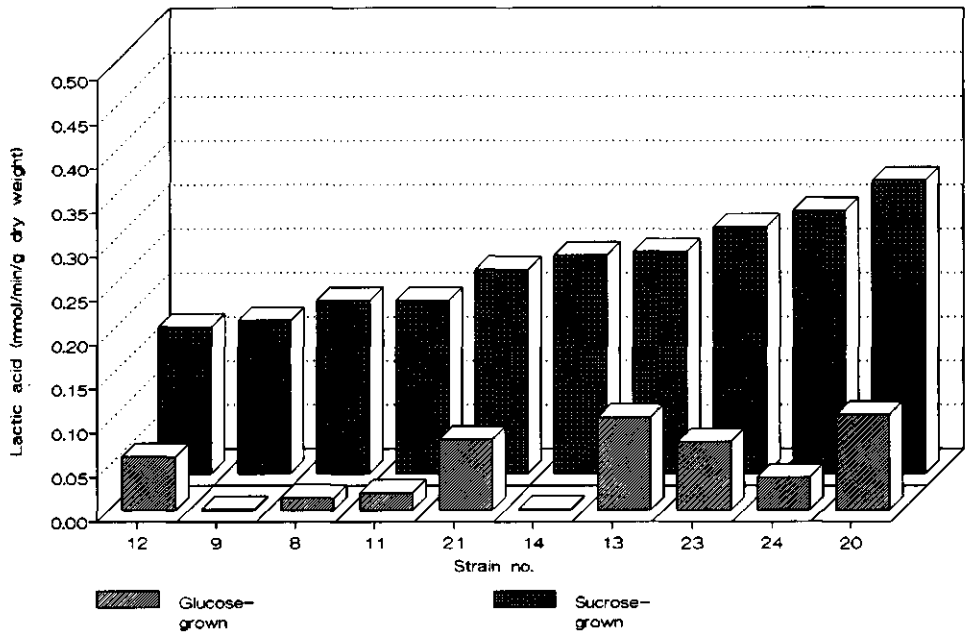


Fig. 2.3.1. Acid production rates from glucose in phosphate buffer for glucose-grown lactic acid bacteria. (For explanation of strain numbers see Table 2.3.1.).



Acid production rates from sucrose in phosphate buffer for some glucose- and sucrose-grown lactic acid bacteria. (For explanation of strain numbers see Table 2.3.1.).

Enterobacteriaceae. Also, in the activity studies with standard salads the carrots were blanched. This was done to create aseptic conditions in which any disturbance of the results by growth of spontaneous microflora, i.e. lactic acid bacteria, could be prevented. The vegetables and the sauce were stored at low temperatures prior to inoculation. The incubation of the salads was done in a waterbath rather than in air, in order to reduce the time neccessary for temperature equilibration. The 42°C in the center of the glass jars was obtained after approximately 90 min.

Results of the activity tests with the standard salads are presented in Table 2.3.2. Only three of the 80 strains used in the experiments with the standard salads were able to decrease the pH of these salads within 3.5 hours of fermentation at 42°C to the desired pH < 4.5. After 7 hours 57 % of the strains reached this level.

Table 2.3.2. Frequency distribution of relative acidifying activity of lactic acid bacteria (n=80) as determined with the standard salad.

Final pH achieved					
After 3.5 hours			After 7 hours		
≥5.0	4.5-5.0	≤4.5	≥5.0	4.5-5.0	≤4.5
70[a]	26	4	8	36	56

[a] Percentages

In most fermentation processes a rapid pH decrease, in addition to a low final pH, is necessary to achieve a microbiologically stable product. As can be seen in Table 2.3.3. a low pH (< 3.9) was reached in the salads after 7 hours of fermentation at 42°C, if strongly acidifying starters were used. The counts of bacteria (especially *Enterobacteriaceae*) were significantly reduced, compared to the uninoculated control. During storage at 7°C the pH remained low and analyses showed a good microbiological quality of the product. The salads kept their mildly acid taste and good texture. The fermented salads contained 0.35 ± 0.03 % (w/w) lactic acid after 7 hours of fermentation at 42°C, increasing to 0.46 ± 0.09 % (w/w) after subsequent storage at 7°C

Table 2.3.3. Microbiological condition and pH after fermentation and subsequent storage of standard salads.

Starter nr.	pH			CFU[d] after 7 h[b]				CFU after storage[c]			
	Initial[a]	After 7 h[b]	After storage[c]	ENT[e]	YEA	LAB	MAB	ENT	YEA	LAB	MAB
Control	6.04	5.75	4.03	5.66	<1.10	4.74	5.80	3.32	4.48	>7.70	<4.70
12	4.93	3.86	3.62	<1.10	<1.10	8.83	<3.70	<1.10	<1.10	8.56	<1.10
20	5.02	3.82	3.57	<1.10	<1.10	8.71	<3.70	<1.10	<1.10	8.42	<1.10
21	5.13	3.84	3.78	<1.10	<1.10	8.91	<3.70	<1.10	<1.10	8.27	<1.10
23	5.06	3.75	3.81	<1.10	<1.10	9.12	<3.70	<1.10	<1.10	8.99	2.04
24	5.07	3.81	3.64	<1.10	<1.10	8.86	<3.70	<1.10	<1.10	8.69	<1.10

Results are the means of determinations of duplicate samples.

[a] Before fermentation; the pH of the standard salads is influenced by the amount of acid in the starter culture.

[b] after 7 h of fermentation at 42°C.

[c] after 7 h of fermentation at 42°C and subsequent storage at 7°C for 14 days.

[d] CFU = Colony forming units (log N/g).

[e] ENT, *Enterobacteriaceae*; YEA, Yeasts; LAB, Lactic acid bacteria; MAB, Mesophilic aerobic bacteria.

for 14 days. Little or no acetic acid could be found in the fermented salads, whilst ethanol production was absent (results not included). Minor amounts of sugars, generally less than 0.6 % (w/w), were metabolised, which again indicates a homofermentative metabolism. The uninoculated control salads contained approximately 0.18 % (w/w) lactic acid after 7 hours of fermentation at 42°C, increasing to 0.27 % (w/w) after subsequent storage at 7°C for 14 days. Acetic acid concentrations were 0.05 % (w/w) and 0.22 % (w/w), respectively.

2.4. DISCUSSION

Utilization of sugars by starter cultures

Sucrose, glucose and fructose are the primary substrates in plant material that are fermented by lactic acid bacteria. According to the literature, the strictly homofermentative lactobacilli are rarely isolated from plant fermentations (Mundt, 1970; Daeschel *et al.*, 1987). However, we found that many of the lactic acid bacteria isolated from fresh sugarbeet pulp, including the two strains used in this study, can be identified as *L. acidophilus*, a thermotrophic and obligatory homofermentative species of the genus *Lactobacillus*. Sugarbeet pulp could be a reservoir of these particular lactic acid bacteria, certain strains of which are often described as beneficial to human health.

As can be seen in Fig. 2.3.1. large differences occur in the ability to produce lactic acid from glucose as sole carbon source. It is clear from Fig. 2.3.2. that glucose-grown cells of some of the lactic acid bacteria used in the test do not possess high fermenting capacities on sucrose as sole carbon source, which may be ascribed to low constitutive levels of specific permeases and/or hydrolyzing enzymes (ß-fructofuranosidase and sucrose hydrolase) (Mital *et al.*, 1973; Kandler, 1983; Gonzalez and Kunka, 1986). These plasmid-mediated enzymes (Gonzalez and Kunka, 1986) are evidently inducible, as lactic acid production of the corresponding strains is much higher after cultivation in MRS broth containing sucrose as sole carbon source.

Some strains used in this study, belonging to our laboratory collection, include

acid-tolerant species like *L. plantarum* and *Pediococcus* sp. These strains were (originally) isolated from sugarbeet pulp, sourdough and tempe soak water. These species are adapted to their specific substrates and indeed show a rapid growth and souring when applied in salad fermentation tests. The fermenting capacities of lactic acid bacteria from the species *Streptococcus thermophilus* on sucrose as sole carbon source were generally low, even after induction. Hence, these species were not included in Table 2.3.1. Most of these strains are normally used in dairy fermentations where lactose is the major carbohydrate, which may explain low activities of specific sucrose permeases and/or sucrose splitting enzymes. Additionally, more complex nutritional requirements for these types of *Streptococcus* sp., may be a factor of importance.

In all experiments salads were fortified with sucrose, to promote a rapid growth of the starter cultures and at the same time to give these salads a pleasant taste. Sucrose was used in view of the overall economics of the fermentation process.

Configuration of lactic acid produced by starter cultures

The lactic acid formed possesses either the L(+)- or the D(-)-configuration depending on the stereospecificity of the lactate dehydrogenases. Mixtures with about equal amounts of both isomers may be formed when both dehydrogenases are present, which is the case in most of the *L. plantarum* strains and the heterofermentative *L. brevis* strain. *L. acidophilus* normally produces a mixture of about equal amounts of L(+) and D(-) lactic acid. However, the strains used in this study produce larger amounts of D(-) lactic acid. A possible explanation for this discrepancy could be that the ratio of L(+) and D(-) lactate formed may vary with growth conditions used (Garvie, 1986; Kandler and Weiss, 1986). One strain, tentatively identified as *L. plantarum* and the two *L. casei*[1] strains produced large amounts of L(+) lactic acid. This is normal for *L. casei* subsp. *casei*, but rare for *L. casei* subsp. *pseudoplantarum*, as this species normally possesses a L(+) lactic acid racemase (Kandler and Weiss, 1986). The strain tested either lacks this enzyme or the enzyme activity is very low. Starter cultures which produce large amounts

[1] Recently the name *Lactobacillus paracasei* was proposed for a large number of strains of the species *L. casei* (Collins et al., 1989).

of L(+) lactic acid are generally preferred, because of some assumed benefits to the health of the consumer (Gurr, 1987).

Fermentation characteristics

According to Buckenhüskes and Gierschner (1985) fermented vegetables are microbiologically stable if a sufficient acidity is reached, i.e. pH 4.1 for sauerkraut. In our fermentation tests with a standard salad several strains were able to reach this final pH after approximately 7 hours of incubation. However, as can be seen from Table 2.3.2. large differences exist between strains in the rate of pH decrease in the standard salad. This may be caused by differences in lag phase, in sucrose fermentation inducibility and intrinsic acidifying capacity.

Data about the acid-forming capacity, as measured according to Lievense *et al.* (1990) with washed cells in the model system, were in good agreement with results of fermentation tests with the standard salad, as can be seen from Table 2.4.1. In this Table the data of the 21 strains, used in both tests are compared.

Table 2.4.1. Comparison of acidifying activity of lactic acid bacteria (n=21) as measured in standard salad and phosphate buffer.

	Final pH achieved		
Test form	≥5.0	4.5-5.0	≤4.5
Standard salad	5[a]	10	85
Model-system[b]	5	24	71

[a] Percentages
[b] Calculated values; cells pre-grown in glucose.

For that purpose, the data from the model system were recalculated to suit the salad system. Although fermentation tests with a model system are less time consuming than tests with standard salads, the latter tests remain necessary for screening cultures as starters in fermented salads. The starter cultures create a characteristic flavour in the

product, which is a major reason for screening lactic acid bacteria with standard salads. Most of the lactic acid bacteria used gave the fermented salads a mildly sour taste and did not affect the crispy texture negatively. A characteristic difference of the fermented salads with the commercially available salads, acidified with acetic acid, was that the ingredients kept more of their own characteristic taste. Uninoculated control salads had off-flavours and showed curdling of the sauce (Table 2.3.3.).

After two weeks of storage at 7°C the control salad (Table 2.3.3.) was spoiled by yeasts and lactic acid bacteria. These organisms are the normal spoilage flora of commercial salads. Particularly important are psychrotrophic heterofermentative lactobacilli, such as *L. fructivorans* (Smittle, 1977) and *L. brevis* (Baumgart, 1977). However these do not grow at the fermentation temperature of 42°C. Gas production during subsequent storage of fermented salads at 7°C was not observed.

In all methods for testing starter cultures, standardization of inoculum preparation is a factor of major importance (Chamba and Prost, 1989). For that purpose strains were cultivated successively 24 and 16 hours to shorten and stabilize their lag phases (Zwietering *et al.*, 1991). The inoculation level in the standard salads was 1 % which corresponded with $\log N = 7.37 \pm 0.5$ cfu/g salad (2 x 21 determinations).

In subsequent papers it will be shown that spoilage (*Enterobacteriaceae*, yeasts) and pathogenic micro-organisms (*Staphylococcus aureus*, *Listeria monocytogenes*), deliberately added to salads can be inhibited by using starter cultures with high fermenting capacity.

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3

INHIBITION OF THE GROWTH OF YEASTS IN FERMENTED SALADS

ABSTRACT

Salads composed of vegetables and/or meat in an oil-in-water emulsion were prepared by fermentation for 7 hours at 42°C or 45°C with strains of *Lactobacillus* spp. Their stability towards spoilage yeasts was studied using *Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Torulaspora delbrueckii*, isolated from salads, as well as *Pichia membranaefaciens* and *Zygosaccharomyces bailii*. Salads fermented with good lactic starters usually had pH values of $\leq$ 4.2 and lactic acid concentrations of 0.28 to 0.43 % (w/w). High numbers of spoilage yeasts (and production of large volumes of CO_2) were not attained in these salads, provided the initial concentration of spoilage yeasts was sufficiently low ($\leq$ 100/g). Inhibition of spoilage yeasts in lactic fermented salads is probably due to lactic acid, the low storage temperature and the low residual oxygen concentration.

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3.1. INTRODUCTION

Salads, composed of vegetables and/or meat in an oil-in-water emulsion with a low pH (< 4.8) due to the addition of acids, are popular in western countries. The shelf life of these salads is influenced mainly by low pH, storage temperature and the use of chemical preservatives, i.e. sorbic and benzoic acids. Higher demands on quality of raw materials, better preparation methods and especially the use of preservatives, have led to a shelf life of these salads of 6 to 8 weeks, if stored below 7°C. However, consumer trends are towards salads without chemical preservatives and with a less sour taste. The omission of preservatives limits the shelf life of salads to 2 to 3 weeks, if stored below 7°C (Gromzik, 1991). Micro-organisms causing spoilage are lactic acid bacteria, yeasts, moulds and bacilli. According to literature data, the most important spoilage yeasts are *Saccharomyces exiguus* and *Saccharomyces dairensis*. *Pichia membranaefaciens*, *Debaryomyces kloeckeri*, *Geotrichum candidum*, some *Candida* spp. and *Zygosaccharomyces bailii* are also found in salads (Smittle, 1977; Baumgart, 1977: Smittle and Flowers, 1982; Brocklehurst *et al.*, 1983; Brocklehurst and Lund, 1984).

It is generally accepted that the antimicrobial activity of weak acids such as acetic acid and lactic acid is due to the undissociated acid molecules. An increase in concentration of these molecules, independent from a decrease in pH levels, will improve the microbial stability of salads. This can be achieved by using buffer systems of weak organic acids and their salts (Debevere, 1987). It has been suggested that an additional advantage would be that sour taste problems are diminished. The shelf life of salads could also be extended by technological solutions, such as the production of salads under strict hygienic conditions, in closed systems with rigorous exclusion of oxygen and the use of modified atmospheres. These methods, require, however, large capital investments. Moreover, the growth of yeasts having a fermentative ability and especially lactic acid bacteria will not be prevented (Buick and Damoglou, 1989; Farber, 1991).

Lactic acid fermentation of foods generally is a low-cost method of food preservation in which spoilage and pathogenic organisms are inhibited, mostly by acid production and pH reduction but also by lowering the oxidation-reduction potential, competition for essential

nutrients and possibly by the production of inhibitory compounds: antibiotics and hydrogen peroxide. In a previous paper (Bonestroo *et al.*, 1992) lactic acid fermentation was proposed as a novel way of preparation of salads. If sufficiently attractive, a certain interest may be expected from the market for these fermented salads, because of their mildly sour taste, their possible health effects and especially because the use of preservatives can be abandoned.

The objective of this study, as part of an investigation on lactic acid fermented salads, was to investigate the possible inhibitory effect of fermentation on the growth of yeasts. Therefore, yeasts, isolated amongst others from spoiled, industrially produced salads, were deliberately added to salads, which were then fermented. Changes in microbiological condition and chemical composition were monitored during storage at 7°C.

3.2. MATERIALS AND METHODS

Organisms and culture conditions

The lactic acid bacteria which were used in this study were obtained from our laboratory collection (Bonestroo *et al.*, 1992). Prior to use the strains of the genus *Lactobacillus* were cultivated for 24 hours at 30°C or 42°C in a broth with the same composition as MRS broth (Merck, Darmstadt, F.R.G.), but containing sucrose (Merck) as carbohydrate, instead of glucose, followed by subsequent cultivation in the same broth for 16 hours at 30°C or 42°C. *Pichia membranaefaeciens* CBS 107 and *Zygosaccharomyces bailii* QST2877 were kindly provided by Dr. W.J. Middelhoven (Department of Microbiology, Wageningen Agricultural University, The Netherlands) and Mr. P. Breeuwer (Department of Food Science, Wageningen Agricultural University, The Netherlands), respectively. Other yeast strains were isolated, using Oxytetracycline Glucose Yeast Agar (OGYA, CM545, Oxoid Ltd., Basingstoke, U.K.) incubated at 25°C, from fresh salads, sell-by date salads and spoiled salads with bulging packings, all produced without preservatives. Strains were purified and after morphological screening, preliminary characterized with the API ATB 32 C identification kit (API System S.A., France). Predominant yeast species were identified by Dr. M. Th. Smith (CBS Yeast Division, Identification Service, Delft, The Netherlands). Yeast cultures were maintained at 4°C on Malt Extract Agar (CM59, Oxoid).

Prior to use the yeast cultures were cultivated twice, at 25°C for 48 hours, consecutively, in Malt Extract Broth (CM57, Oxoid).

Preparation and storage of salads

Four types of salads were prepared, one containing 65 % (w/w) peeled, sliced (3 mm) and cooked (5-10 min 90-100°C) potato tubers (cultivar Bintje) and 35 % (w/w) sauce; the second containing 23 % (w/w) shredded (10x6x3 mm) leek, 23 % (w/w) shredded (15x10x3 mm) cabbage, 10 % (w/w) cooked ham and 44 % sauce; the third containing 50 % (w/w) grated (30x3x3 mm) and blanched (1 min, 100°C) carrots and 50 % (w/w) sauce; the fourth containing 50 % (w/w) shredded (15x10x3 mm) cabbage and 50 % sauce. The sauce was composed of water (35.9-52.2 % [w/w]), soybean oil (35-50 % [w/w]), sucrose (6 % [w/w]), salt (1-1.8 % [w/w]), egg yolk (3-3.5 % [w/w]) and thickening agents (2.8 % [w/w]). Occasionally small amounts of seasonings were added to the salads. The salads were inoculated with 10^6 to 10^7 cfu of lactic acid bacteria per g and filled in glass jars (100 g salad, headspace approx. 50 ml) air-tightly sealed with metal Vapor-Vacuum™ Twist-Off™ caps (White Cap International, U.S.A.). The jars were incubated in a waterbath for 7 hours at 42°C or 45°C, and then chilled in ice water to a temperature of 7°C and stored at this temperature. Uninoculated, acidified (0.4 % (w/w) lactic acid) salads were used as control.

Growth at different temperatures

Yeast strains, grown twice at 25°C for 48 hours, consecutively, were inoculated into Malt Extract Broth (CM57, Oxoid) and cultivated at different temperatures. Growth was monitored either by pipetting aliquots in micro-titer plates and reading optical densities at 620 nm or by enumerating the yeasts on Oxytetracycline Glucose Yeast Agar (CM545, Oxoid).

Evaluation of the quality of fermented salads

- *Measurement of pH.* The pH of the salads was determined with a pH-electrode (N61, Schott Geräte GmbH, Hofheim a. Ts., F.R.G.) coupled to a combined pH/mV-meter (pH522, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, F.R.G.).

- *Microbiological quality.* The vegetable salads were judged on the basis of microbiological analyses directly after fermentation and up to three weeks of storage at 7°C. Mesophilic aerobic spoilage bacteria were enumerated using Gelysate Agar (GEL): 5 g Bacto-peptone (Difco, Detroit, Michigan, U.S.A.), 5 g sodium chloride (Merck) and 14 g agar (L13, Oxoid) per l, pH 7.6 ± 0.1, incubated at 30°C for 3 days. *Enterobacteriaceae* were counted on Violet Red Bile Glucose Agar (VRBG; CM485, Oxoid) with a top layer of the same medium and incubated for 24 hours at 30°C. Lactic acid bacteria were selectively enumerated on MRS medium (Merck) with 12 g of agar (L13, Oxoid) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l. Yeasts and moulds were counted on Oxytetracycline Glucose Yeast Agar (OGGA; CM545, Oxoid), incubated at 25°C for 5 days, as recommended by Banks and Board (1987).

- *Determination of sugars, ethanol and organic acids.* Sugars, ethanol and organic acids in fermented salads were determined by means of HPLC as described earlier (Bonestroo *et al.*, 1992).

3.3. RESULTS

In most fermentation processes a rapid pH decrease, in addition to a low final pH, is necessary to achieve a microbiologically stable product. As can be seen in Table 3.3.1. a low pH (< 3.9) was reached in the salads after 7 hours of fermentation at 42°C. Although the fermented salads contained raw ingredients, i.e. cabbage, no growth of yeasts occurred. During storage at 7°C the pH remained low and the fermented salads were microbiologically stable for at least 3 weeks. The fermented salads contained 0.35 ± 0.03 % (w/w) lactic acid after 7 hours of fermentation at 42°C, increasing to 0.46 ± 0.09 % (w/w) after subsequent storage at 7°C for 3 weeks. Little or no acetic acid could be found in the fermented salads, whilst ethanol production was absent and minor amounts of sugars, generally less than 0.5 % (w/w), were used (results not shown).

Table 3.3.1. Microbiological condition and pH of fermented salads composed of equal amounts of cabbage and sauce.

Starter	pH			CFU[d] after 7 h[b] (logN/g)				CFU after storage[c] (logN/g)			
nr.[a]	Initial	After 7 h[b]	After storage[c]	ENT[e]	YEA	LAB	MAB	ENT	YEA	LAB	MAB
9	5.07	3.80	3.64	<1.00	<1.00	9.12	<3.70	<1.00	<1.00	8.99	<1.00
20	5.02	3.82	3.57	<1.00	<1.00	8.71	<3.70	<1.00	<1.00	8.42	2.04
21	4.93	3.86	3.62	<1.00	<1.00	8.83	<3.70	<1.00	<1.00	8.56	<1.00
23	5.07	3.80	3.64	<1.00	<1.00	8.86	<3.70	<1.00	<1.00	8.69	<1.00
24	5.13	3.84	3.78	<1.00	<1.00	8.91	<3.70	<1.00	<1.00	8.27	<1.00

Results are the means of determinations of duplicate samples.

[a] Numbers refer to *Lactobacillus plantarum* strain numbers described in Bonestroo *et al.* (1992).

[b] After 7 h of fermentation at 42°C.

[c] After 7 h of fermentation at 42°C and subsequent storage at 7°C for 21 days.

[d] CFU = Colony forming units

[e] ENT = *Enterobacteriaceae*
YEA = Yeasts
LAB = Lactic acid bacteria
MAB = Mesophilic aerobic spoilage bacteria

Table 3.3.2. shows the identification, along with some temperature characteristics of various yeast strains isolated from fresh salads, sell-by date salads and spoiled salads with bulging packings, all industrially produced without preservatives. All strains failed to grow in Malt Extract Broth at 3 to 3.5°C. They showed good growth at higher temperatures, except for *Saccharomyces exiguus* and *Torulaspora delbrueckii*, of which no growth was observed at 30°C. None of these strains showed growth at 42°C. At a temperature of 7°C, all strains reached maximum cell numbers within 7 to 14 days. Most strains possess a fermentative metabolism and are thus able to grow in the absence of oxygen, except *Trichosporon beigelii*, which possesses an oxidative metabolism.

The tolerance of the various yeast species to acids is shown in Table 3.3.3. *Trichosporon beigelii*, isolated from fresh salads, failed to grow in Malt Extract Broth at pH 4.5 and pH 4.0 at 7°C in the presence of 0.5 % acetic acid. Other strains showed moderate to good growth under these conditions, with the exception of *Saccharomyces cerevisiae*, which could tolerate 1 % acetic acid at pH 4.5, but not at pH 4.0. It is clear that acetic acid is more inhibitory to the yeasts than lactic acid. Increase of the lactic acid concentration in the acid mixture to 1 % did not change the growth patterns as shown for the acid mixtures in Table 3.3.3.

Various salads, deliberately inoculated with a mixture of acid tolerant yeasts (approximately 100 cfu/g salad), fermented with *Lactobacillus* spp. for 7 hours at 42°C and stored at 7°C, remained microbiologically stable for at least 3 weeks. Growth of yeasts was obviously suppressed. However, with higher inoculation levels (> 1000 cfu/g salad), growth of yeasts within 2 weeks generally could not be prevented (data not included).

Other experiments, in which carrot salads were inoculated with single cultures of different acid tolerant yeasts (250 - 2350 cfu/g salad) and the yeasts *Pichia membranaefaciens* and *Zygosaccharomyces bailii*, fermented for 7 hours at 42°C with different *Lactobacillus plantarum* strains and subsequently stored at 7°C, gave comparable results (Table 3.3.4.). Strains of *Torulaspora delbrueckii*, *Pichia membranaefaciens* and especially *Saccharomyces cerevisiae* showed abundant growth in the fermented salads after 10 days of storage at 7°C, while growth of other strains was more or less inhibited.

Table 3.3.2. Temperature characteristics of yeast strains isolated from industrially produced salads.

Species	Growth at					Metabolism
	3-3.5°C	4-4.5°C	7°C	20°C	30°C	
Saccharomyces exiguus	—[a]	+++	+++	+++	—	fermentative
Saccharomyces cerevisiae	—	+++	+++	+++	+++	fermentative
Trichosporon beigelii	—	+++	+++	+++	+++	oxidative
Torulaspora delbrueckii	—	+++	+++	+++	—	fermentative

[a] — = no growth observed within 30 days.
+++ = dense growth within 7 days.

Table 3.3.3. Tolerance to acids of yeasts isolated from industrially produced salads.

Species	Growth at 7°C and pH 4.5 in the presence of				Growth at 7°C and pH 4.0 in the presence of			
	acetic acid		acid mixtures		acetic acid		acid mixtures	
	0.5 %	1.0 %	1[a]	2[b]	0.5 %	1.0 %	1[a]	2[b]
Saccharomyces exiguus	+++[c]	+++	+++	+++	+++	++	+++	+++
Saccharomyces cerevisiae	+++	+	++	++	++	—	+	+
Trichosporon beigelii	—	—	—	—	—	—	—	—
Torulaspora delbrueckii	+++	+++	+++	+++	+++	++	+++	+++

[a] mixture of 0.5 % acetic acid + 0.5 % lactic acid.
[b] mixture of 0.5 % acetic acid + 1.0 % lactic acid.
[c] — no growth observed within 21 days,
+ dense growth after 15 to 21 days,
++ dense growth after 10 to 15 days,
+++ dense growth within 10 days.

After 20 days storage at 7°C, most salads were spoiled by yeasts, except for the salads inoculated with *Saccharomyces exiguus* 2 and *Zygosaccharomyces bailii*, and the salad inoculated with *Trichosporon beigelii* fermented with *Lactobacillus plantarum* nr. 20. The fermented salads contained 0.28 ± 0.02 % (w/w) lactic acid after 10 days storage at 7°C, increasing to 0.35 ± 0.07 % (w/w) after 20 days. Little or no acetic acid could be found in the fermented salads with little or no yeast growth (≤ 0.09 ± 0.02 % [w/w]), whilst ethanol production in these salads was nearly absent (≤ 0.02 ± 0.01 % [w/w]). Minor amounts of sugars, generally less than 0.5 % (w/w), were used. However, in the salads deliberately contaminated with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* larger amounts of acetic acid and ethanol were found, with corresponding larger sugar consumption.

Growth of *Saccharomyces cerevisiae* was further studied in potato salads and salads containing leek, cabbage and ham (Table 3.3.5.). When inoculated with ≤ 100 cfu/g salad, no growth of yeasts occurred within 4 weeks at 7°C. Potato and leek-cabbage-ham salads contained 0.41 ± 0.01 % (w/w) and 0.43 ±0.02 % (w/w) lactic acid after fermentation for 7 hours at 45°C, respectively. After 42 days storage at 7°C the lactic acid concentrations were 0.72 ± 0.08 % and 0.46 ± 0.07 % (w/w), respectively. Little or no acetic acid could be found in the fermented salads (≤ 0.03 % [w/w]), whilst ethanol production was nearly absent (≤ 0.05 % [w/w]). Minor amounts of sugars, generally less than 0.5 % (w/w), were used. The fermented salads had a mildly sour taste and good texture. A striking difference of the fermented salads with commercially available salads, acidified with acetic acid, was that the ingredients kept more of their own characteristic taste. The addition of small amounts of seasonings further contributed to this taste.

3.4. DISCUSSION

It is frequently reported that raw vegetables can contain high numbers of yeasts (Hartog and Jansen, 1986; King Jr. *et al.*, 1991, Török and King, 1991). Most of the yeast species isolated from different plant surfaces are asporogenous, non-fermentative, and most commonly represent the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and, in some cases, *Torulopsis*. *Candida* species are usually in the minority (Middelhoven and van Baalen, 1988; Phaff and Starmer, 1990).

Table 3.3.4. pH and number of yeasts in fermented carrot salads inoculated with ≥ 250 spoilage yeasts per g.

Species	Starter nr.[a]	pH at different times[b]				CFU[c] at different times		
		A	B	C	D	A	C	D
Saccharomyces exiguus 1[d]	9	6.51	4.52	4.02	3.81	2.48	<2.00	4.64
	20	6.51	4.39	4.07	3.85	2.48	<2.00	2.78
Saccharomyces exiguus 2	9	6.48	4.38	3.92	3.73	2.40	<2.00	<2.00
	20	6.48	4.47	4.00	3.73	2.40	2.52	<2.00
Saccharomyces cerevisiae	9	6.51	4.47	4.00	3.69	2.77	>6.00	>6.00
	20	6.51	4.33	3.94	3.69	2.77	>6.00	>6.00
Trichosporon beigelii	9	6.45	4.23	3.84	3.72	3.31	<2.00	4.78
	20	6.51	4.32	4.04	3.81	2.65	<2.00	<2.00
Torulaspora delbrueckii	9	6.48	4.22	4.06	3.77	2.42	4.16	>6.00
	20	6.48	4.22	4.12	3.73	2.42	3.81	>6.00
Pichia membranaefaciens	9	6.45	4.30	3.90	3.75	2.60	4.61	>6.00
	20	6.45	4.30	3.82	3.77	2.60	4.86	>6.00
Zygosaccharomyces bailii	9	6.45	4.44	4.01	3.78	3.37	3.65	3.73
	20	6.45	4.33	4.01	3.83	3.37	3.31	3.34

[a] Numbers refer to *Lactobacillus plantarum* strain numbers described in Bonestroo *et al.* (1992).
[b] A = before fermentation; B = after 7 hours fermentation at 42°C; C = after fermentation and subsequent storage at 7°C for 10 days; D = after fermentation and subsequent storage at 7°C for 20 days.
[c] Colony Forming Units of yeasts (log N/g), average of duplicate determinations.
[d] *Saccharomyces exiguus* 1 was isolated from salad, *Saccharomyces exiguus* 2 was isolated from equipment.

Table 3.3.5. pH and number of yeasts in salads inoculated with 70 - 90 cfu of *Saccharomyces cerevisiae* per g salad.

Salad type	Starter nr.[a]	pH at different times[b]					CFU[c] at different times			
		A	B	C	D	E	A	C	D	E
I[d]	4	5.66	3.99	3.91	3.91	3.92	1.86	<1.70	<1.70	>3.00
	control[e]	4.00	4.00	3.94	3.86	3.88	1.86	4.15	>5.00	>5.00
	control[f]	4.05	3.88	4.02	3.93	3.98	1.86	5.25	4.65	>3.00
II	25	5.69	4.04	4.35	4.13	4.07	1.96	<1.70	<1.70	>2.80
	control[e]	3.52	4.06	4.18	4.18	4.22	1.96	<1.70	>4.00	>4.00
	control[f]	3.57	4.11	4.19	4.24	4.10	1.96	<1.70	>4.00	>6.00

[a] *Lactobacillus acidophilus* strains
[b] A = before fermentation; B = after 7 hours fermentation at 42°C; C = after fermentation and subsequent storage at 7°C for 14 days; D = after fermentation and subsequent storage at 7°C for 28 days; E = after fermentation and subsequent storage at 7°C for 42 days.
[c] Colony Forming Units of yeasts (logN/g), average of duplicate determinations.
[d] Salad type I, potato salad; salad type II, salad containing leek, cabbage and ham.
[e] not inoculated, acidified with 0.4 % lactic acid.
[f] not inoculated, acidified with 0.4 % lactic acid, subjected to a temperature of 45°C during 7 hours.

However, in fermentation experiments with salads containing raw ingredients, i.e. cabbage, no growth of yeasts occurred (Table 3.3.1.), indicating that most of the yeasts, naturally occurring on fresh vegetables, do not cause spoilage of fermented salads.

To study yeast spoilage in further detail, more acid tolerant yeasts, isolated from industrially produced salads, were deliberately added to various salads, which were then fermented. As can be seen in Table 3.3.4., some strains, inoculated at a level of more than 250 cfu/g of salad, were inhibited for at least 20 days at 7°C (*Saccharomyces exiguus* 2, *Zygosaccharomyces bailii* and *Trichosporon beigelii*), while others (*Torulaspora delbrueckii*, *Pichia membranaefaciens* and especially *Saccharomyces cerevisiae*) already showed abundant growth after 10 days of storage at 7°C. When salads were inoculated with *Saccharomyces cerevisiae* at a lower level (< 100 cfu/g of salad), and subsequently fermented with 10^6-10^7 cfu/g of lactic acid bacteria, growth at 7°C was absent for at least 4 weeks (Table 3.3.5.). Although the relative low numbers of yeasts, compared to the lactic acid bacteria, may account in part for this phenomenon, other factors, among which the amount of acid, the low storage temperature and the oxygen concentration in the salads, have to be considered.

Regarding the inhibitory effect of acids on the growth of micro-organisms, not only the amount of acid is important, but also the degree of dissociation of the acids which is influenced by pH. It is generally accepted that the antimicrobial activity of weak acids is due largely to undissociated acid molecules. In their studies concerning the spoilage of mayonnaise-based salads, Kirsop and Brocklehurst (1982), showed that yeasts were to be found in the aqueous phase of the mayonnaise emulsion and were not to any substantial degree associated with the surface or the internal parts of the vegetable tissue present in the mayonnaise. Therefore, it seems reasonable to calculate the concentration of undissociated acids in the aqueous phase of the salad. Calculated values for the concentration of undissociated lactic acid in the aqueous phase for carrot, leek-cabbage-ham and potato salads as presented in tables 3.3.4. and 3.3.5. are 0.27 $\pm$ 0.06 % (w/w), 0.34 $\pm$ 0.01 % (w/w) and 0.24 $\pm$ 0.03 % (w/w), respectively. The concentrations of undissociated acetic acid in the aqueous phase of the above mentioned salads generally were $\leq$ 0.08 % (w/w). These values are significantly lower than the concentrations of undissociated acids in Malt Extract Broth which can be calculated from Table 3.3.3. Regarding the level of acid in the control salads

and the extreme levels of acid tolerance, inhibition of yeasts in fermented salads is not solely due to the amount of acid.

We are aware that acetic acid generally is more inhibitory to yeasts than lactic acid. According to several researchers a synergistic antimicrobial effect of combinations of these acids exists (Moon, 1983; Adams *et al.*, 1989). However, in our experiments synergistic effects of the two acids were not observed (Table 3.3.3.). These findings were confirmed by various experiments done in our laboratory in which sauces were inoculated with *Saccharomyces cerevisiae*, which showed good growth at 7°C and pH 4.5 in the presence of 0.78 % (w/w) acetic and 1.17 % (w/w) lactic acid (values calculated on the aqueous phase).

In our opinion the effect of a storage temperature of 7°C on the growth of spoilage yeasts in the salads is also limited. Growth of spoilage yeasts is inhibited at storage temperatures below 3.5°C (Table 3.3.2.). In broth cultures all yeast strains tested reached maximum cell numbers within 7 to 14 days at 7°C. At fermentation temperatures (>40°C), however, growth of these yeasts in broth was retarded or completely inhibited, possibly due to their phychrotrophic nature (Table 3.3.2.). These effects were intensified by the inclusion of acids in the broth cultures. However, in control salads, acidified with 0.4 % (w/w) lactic acid, and subjected to a temperature of 45°C during 7 hours, growth of *Saccharomyces cerevisiae* was abundant (Table 3.3.5.). Problems with growth of thermotrophic yeast species, such as *Kluyveromyces* spp., during fermentation or storage were never encountered.

In view of the considerable amount of sucrose added to the sauces used, i.e. generally 6 % (w/w), the amounts of monosaccharides commonly found in vegetables, and the limited conversion of sugars to acids (< 0.5 % [w/w]), competition for fermentable sugars does not contribute to the observed inhibition of yeasts in fermented salads.

Oxygen is absent in fermented dairy products after culturing (Langeveld and Bolle, 1989). One might assume that the reducing effect of the fermentation processes could have an inhibitory effect on the growth of yeasts. This is certainly true for oxidative yeasts, i.e. *Trichosporon beigelii*. However, most yeasts isolated from spoiled salads possess a fermentative metabolism and are able to grow in the absence of oxygen, although at a lower rate. It is generally assumed that out of a low initial contamination massive growth of yeasts can occur under aerobic conditions, due to a higher energy efficiency. Upon depletion of

oxygen this large population of yeasts may be able to ferment the residual sugars, with attendant massive production of CO_2, resulting in bulging of packings (Scheffers, 1987). As stated earlier, during fermentation above a temperature of 40°C growth of yeasts is retarded or inhibited. Simultaneously, the concentration of oxygen in the salads decreases with approximately 85 %[1]. Therefore, high numbers of yeasts, necessary for the production of large volumes of CO_2 will probably not be attained in fermented salads, provided the initial yeast contamination is sufficiently low ($\leq$ 100 cfu/g of salad).

The consumption of oxygen by lactic acid bacteria could result in a simultaneous production of hydrogen peroxide (Condon, 1987), a compound which might be inhibitory to yeasts. However, in broth cultures of various aerobically grown lactic acid bacteria, production of hydrogen peroxide could not be detected (data not included).

In conclusion, inhibition of spoilage yeasts in fermented salads can be achieved by using starter cultures with high fermenting capacity, provided that measures are taken to attain low initial contamination ($\leq$ 100 cfu/g salad) with these spoilage yeasts. The inhibitory effect of lactic fermentation on the growth of yeasts in salads is probably due to several factors, including the amount of lactic acid, the low storage temperature and the low residual oxygen concentration.

In a subsequent paper it will be shown that spoilage (*Enterobacteriaceae*) and pathogenic bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*), deliberately added to salads can be inhibited by using starter cultures with high fermenting capacity.

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4

THE FATE OF SPOILAGE AND PATHOGENIC BACTERIA IN FERMENTED SAUCE-BASED SALADS

ABSTRACT

The risk that fermented sauce-based salads may cause microbial food poisoning is determined by a number of factors, namely contamination of the ingredients, growth and toxin production during the first stage of fermentation, and inactivation during fermentation or storage. In order to investigate the possible growth and persistence of spoilers and pathogens, salads were inoculated with *Klebsiella pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* and fermented with lactic acid bacteria. A rapid decrease of pH, in addition to a low final pH (<4.2) is necessary to inhibit the growth and survival of *Klebsiella pneumoniae*. Growth or survival of *B. cereus* in cabbage salads fermented with different starters could not be detected. In salads, such as leek-cabbage-ham salads, which were inoculated with *S. aureus* (420 cfu/g) and fermented at 45°C with *Lactobacillus* spp., *S. aureus* increased with 5 to 6 generations, after which the numbers declined. In other salads, composed of ingredients with lower buffering capacities, less generations occurred. Populations of 10^6 cfu/g, required to produce amounts of enterotoxin which could invoke clinical symptoms, were never attained. A rapid decrease of pH and a low final pH seem to be adequate to control the possible growth of *S. aureus* during fermentation and subsequent storage. When *L. monocytogenes* Scott A was inoculated into cabbage salads, which were then fermented with different strains of *Lactobacillus* spp., 2 to 3 generations of growth occurred during fermentation, followed by a rapid decline to below detectable levels. Lactic fermentation of sauce-based salads has been shown to cause inhibition of growth of a range of spoilage and pathogenic bacteria due largely to the production of organic acids and a decrease in pH. Primarily, however, measures should be taken to attain absence or low initial contamination with these pathogens. Also, proper hygienic care during processing should be assured.

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4.1. INTRODUCTION

The shelf life of salads, composed of vegetables and/or meat in an oil-in-water emulsion with a low pH (< 4.8) due to the addition of acids, is determined mainly by low pH, storage temperature and chemical preservatives, such as sorbic and benzoic acids. The shelf life of these salads varies from 6 to 8 weeks, if stored below 7°C. Micro-organisms causing spoilage are lactic acid bacteria, yeasts, moulds and bacilli.

Mainly through the acid content in the aqueous phase, the low pH and the low temperature of storage, salads do not normally permit growth of pathogenic micro-organisms. Some pathogens, when inoculated into the salad, can survive some time, but they cannot multiply (Holtzapffel and Mossel 1968; Fowler and Clark 1975; Smittle 1977). However, consumer trends are towards salads without chemical preservatives and with a less sour taste. The omission of preservatives limits the shelf life of salads to 2 to 3 weeks, if stored below 7°C (Gromzik 1991). Moreover, problems with pathogenic micro-organisms may arise when manufacturers make concessions to such demands of the consumer.

Lactic acid fermentation of foods generally is a low-cost method of food preservation in which spoilage and pathogenic organisms are inhibited, mostly by acid production and pH reduction but also by lowering the oxidation-reduction potential, competition for essential nutrients and possibly by the production of inhibitory compounds: antibiotics and hydrogen peroxide. In a previous paper (Bonestroo *et al.*, 1992a) lactic acid fermentation was proposed as a novel way of preparation of salads. A process was developed through which salads could be prepared with a mildly sour taste, while the use of preservatives can be abandoned.

Given the relatively long storage life of refrigerated ready-to-eat foods, it is felt that pathogenic microorganisms which can grow at refrigeration temperatures may be a particular problem (Farber, 1991). In fermented salads, conditions should be attained to ensure adequate limitation of proliferation and metabolism of these pathogens. Primarily, however, measures should be taken to attain absence or low initial contamination with pathogens.

The risk that fermented salads may cause food poisoning is determined by a number

of factors, namely contamination of the ingredients (amongst others meat, vegetables, egg yolk, spices), outgrowth during the first stage of fermentation, and inactivation during fermentation or storage. To draw conclusions on the safety of fermented salads with respect to pathogens, information on the contamination level of the raw materials should exist. In our fermented salads, ingredients such as meats and spices usually are treated, i.e. cooked or pasteurized to reduce the microbial load. Furthermore, manual handling which could be involved in making these products, is drastically minimized. Main contamination is brought about by the use of fresh vegetables. Contamination of these vegetables can occur in the field or during post harvest handling, and the presence of *Escherichia coli* and *Salmonella* spp. has been reported for salad vegetables offered for retail sale (Tamminga *et al.*, 1976). The avoidance of such contamination depends primarily on the use of good agricultural practices in growing crops and good hygienic practices during harvesting, packing, transporting and processing. In their survey of the microbiological quality of raw-vegetable salads and ready-to-eat mixtures of vegetables Hartog and Jansen (1986) showed high contamination levels with *Enterobacteriaceae*: 41 % of the fresh samples (n = 149) possessed counts exceeding 10^5 cfu/g. The extent of contamination with *S. aureus* and *B. cereus* was small: only 1 % of the samples (n = 320) possessed *S. aureus* counts $\geq$ 500 cfu/g., 2 % showed counts of *B. cereus* $\geq 10^3$ cfu/g. However, Hildebrandt *et al.*, (1989) were unable to detect any pathogens in a total of 113 samples of raw vegetables for salad preparation. According to Brackett (1987), pathogens which could be found in fresh fruits and vegetables are psychrotrophs, especially *L. monocytogenes* and *Aeromonas hydrophila.*

In order to investigate the possible growth and persistence of spoilage and pathogenic bacteria, salads were inoculated with *Klebsiella pneumoniae* subsp. *pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* and fermented with lactic acid bacteria. *Klebsiella pneumoniae*, an acid-resistant psychrotrophic organism was also used as a model organism for the behaviour of pathogenic *Enterobacteriaceae* such as *Salmonella*, *Shigella* and enteropathogenic *E. coli*. Changes in microbiological condition and chemical composition were monitored during fermentation at temperatures > 40°C and subsequent storage at 7°C.

4.2. MATERIALS AND METHODS

Organisms and culture conditions

The lactic acid bacteria which were used in this study were obtained from our laboratory collection (Bonestroo *et al.*,, 1992a). Prior to use the strains of the genus *Lactobacillus* were cultivated for 24 hours at 30°C or 42°C in a broth with the same composition as MRS broth (Merck, Darmstadt, F.R.G.), but containing sucrose (Merck) as carbohydrate, instead of glucose, followed by subsequent cultivation in the same broth for 16 hours at 30°C or 42°C. Strains of *Bacillus cereus*, *Klebsiella pneumoniae*, *Listeria monocytogenes* Scott A and *Staphylococcus aureus* were obtained from our laboratory collection. *Klebsiella pneumoniae* (subsp. *pneumoniae*), an acid-resistant psychrotrophic representative of the *Enterobacteriaceae* was isolated from spoiled salads. Prior to use the pathogenic strains were cultivated twice at 30°C or 37°C, first for 24 hours and then for 16 hours, in Brain Heart Infusion Broth (Gibco Ltd., Paisley, Scotland, UK).

Preparation and storage of salads

Four types of salads were prepared, one containing 65 % (w/w) peeled, sliced (3 mm) and cooked (5-10 min 90-100°C) potato tubers (cultivar Bintje) and 35 % (w/w) sauce; the second containing 23 % (w/w) shredded (10x6x3 mm) leek, 23 % (w/w) shredded (15x10x3 mm) cabbage, 10 % (w/w) cooked ham and 44 % sauce; the third containing 50 % (w/w) grated (30x3x3 mm) and blanched (1 min, 100°C) carrots and 50 % (w/w) sauce and the fourth containing 50 % (w/w) shredded (15x10x3 mm) cabbage and 50 % (w/w) sauce. The sauce was composed of water (35.9-52.2 % [w/w]), soybean oil (35-50 % [w/w]), sucrose (6 % [w/w]), salt (1-1.8 % [w/w]), egg yolk (3-3.5 % [w/w]) and thickening agents (2.8 % [w/w]). Occasionally small amounts of seasonings were added to the salads. The salads were inoculated with 10^6 to 10^7 cfu of lactic acid bacteria per g and filled in glass jars (100 g salad, headspace approx. 50 ml) and air-tightly sealed with metal Vapor-Vacuum™ Twist-Off™ caps (White Cap International, U.S.A.). The jars were incubated in a waterbath for 7 hours at 42°C or 45°C, and then chilled in ice water to a temperature of 7°C and stored at this temperature. Uninoculated, acidified (0.4 % (w/w) lactic acid) salads were used as

control. Unless stated otherwise, these control salads were not heated for 7 hours at either 42°C or 45°C.

Evaluation of the quality of fermented salads

- *Measurement of pH.* The pH of the salads was determined with a pH-electrode (N61, Schott Geräte GmbH, Hofheim a. Ts., F.R.G.) coupled to a combined pH/mV-meter (pH522, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, F.R.G.).

- *Microbiological quality.* The vegetable salads were judged on the basis of microbiological analyses during fermentation, directly after fermentation and after varying periods of storage at 7°C for up to six weeks. Mesophilic aerobic spoilage bacteria were enumerated using pour-plates of Gelysate Agar (GEL): 5 g Bacto-peptone (Difco, Detroit, Michigan, U.S.A.), 5 g sodium chloride (Merck) and 14 g agar (L13, Oxoid) per l, pH 7.6 $\pm$ 0.1, incubated at 30°C for 3 days. *Enterobacteriaceae* were counted on pour-plates of Violet Red Bile Glucose Agar (VRBG; CM485, Oxoid) with a top layer of the same medium and incubated for 24 hours at 30°C. In some enumerations of *Enterobacteriaceae*, resuscitation was carried out by spreading 0.1 ml of the appropriate sample dilution on thin Tryptone Soya Agar (CM131, Oxoid) plates and incubating for 2 hours at 30°C. Subsequently, a thick overlay of Violet Red Bile Glucose Agar was applied, followed by incubation at 30°C for 24 hours. Lactic acid bacteria were selectively enumerated on pour-plates of MRS medium (Merck) with 12 g of agar (L13, Oxoid) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l, incubated under anaerobic conditions (Anaerocult system, Merck) at 30°C for 3 days. Enumeration of yeasts and filamentous fungi was in pour-plates of Oxytetracycline Glucose Yeast Agar (CM545, Oxoid), incubated at 25°C for 5 days. *B. cereus* was enumerated on Cereus Selective Agar Base (Merck), supplemented with sterile egg yolk emulsion (100 ml/l; 40 g egg yolk in 160 ml physiological salt solution [0.9 % NaCl], sterilized by gamma irradiation [10 kGray]) and polymixin B sulfate (0.01 g/l, Pfizer B.V., Rotterdam, The Netherlands), incubated at 30°C for 24 hours. *L. monocytogenes* was selectively enumerated on LPM agar (Becton Dickinson and Co., Cockeysville, USA), supplemented with Moxalactam (20 mg/l; Moxalactam diammonium, Eli Lilly and Co., IN, USA), incubated at 30°C for 3 days. *S. aureus* was enumerated on Baird Parker Agar Base (Gibco),

supplemented with sterile egg yolk emulsion (100 ml/l) and filter sterilizedpotassium tellurite (0.1 g/l, Merck), incubated at 37°C for 2 days.

- *Determination of sugars, ethanol and organic acids.* Sugars, ethanol and organic acids in fermented salads were determined by means of HPLC as described earlier (Bonestroo *et al.*, 1992a).

4.3. RESULTS

Carrot salads, inoculated with acid-resistant cultures of *K. pneumoniae* were fermented with either 1 x 10^6 or 5 x 10^6 of a *L. plantarum* starter culture. The development of *K. pneumoniae* and the decrease of the pH in both salads is shown in Table 4.3.1. In this case the amount of starter culture added appears to be critical: when more starter is added there is an earlier decrease of the pH, a lower pH is attained after 7 hours of fermentation and the development of *K. pneumoniae* is completely inhibited. Resuscitation carried out with appropriate sample dilutions did not reveal any *K. pneumoniae*.

Table 4.3.1. Inhibition of the growth of *Klebsiella pneumoniae* in carrot salads fermented with *Lactobacillus plantarum* no. 9, at two inoculation levels.

	Inoculation level 1 x 10^6		Inoculation level 5 x 10^6	
Time	pH	CFU[a]	pH	CFU[a]
A[b]	6.20	3.16	5.53	2.98
B	5.24	n.d.[c]	4.24	n.d.
C	4.16	>5.47	3.67	<1.70
D	3.98	>5.47	3.56	<1.70
E	3.62	6.15	3.56	<1.70

[a] Colony Forming Units of *Klebsiella pneumoniae* (logN/g), average of duplicate determinations.

[b] A = before fermentation; B = after 3.5 hours fermentation at 42°C; C = after 7 hours fermentation at 42°C; D = after fermentation and subsequent storage at 7°C for 24 hours; E = after fermentation and subsequent storage at 7°C for 5 days.

[c] n.d. = not determined.

As in most fermentation processes, this rapid pH decrease, in addition to a low final pH, is necessary to achieve a microbiologically stable product. The rate of pH decrease is not only influenced by inoculum percentage, activity and type of starter, but also by the temperature of fermentation and by the buffering capacity of the ingredients in the salads. This was demonstrated in an experiment with leek-cabbage-ham salad, inoculated with *K. pneumoniae* and fermented with different cultures of *Lactobacillus acidophilus* at 42°C or 45°C. Table 4.3.2. shows the development of *K. pneumoniae* and the decrease of the pH in the salads. The final pH, reached after 7 hours of fermentation, showed minor variations for salads fermented at 45°C and 42°C. The decrease of pH, however, was more rapidly at 45°C, compared to 42°C. In most salads *K. pneumoniae* showed no growth during the fermentation. After 7 hours *K. pneumoniae* could not be detected in most salads fermented at 45°C. In salads fermented at 42°C, however, *K. pneumoniae* survived for longer periods. Although salads fermented with *L. acidophilus* strain no. 48 showed a low pH after 7 hours of fermentation at 42°C and 45°C, *K. pneumoniae* survived for longer periods. This phenomenon is probably caused by the slower rate of pH decrease during fermentation by this starter. All starters produced more lactic acid at 45°C than at 42°C in 7 hours. In all salads, little or no acetic acid could be found, whilst ethanol production was absent. Minor amounts of sugars, generally less than 0.5 % (w/w), were used.

To determine the effect of fermentation on the possible growth and persistence of pathogens, cabbage salads were inoculated with several pathogens and fermented with lactic acid bacteria with high (no. 20), moderate (no. 9) and low (no. P4) fermenting capacities (numbers refer to Bonestroo *et al*, 1992a). Changes in microbiological condition and chemical composition of the salads were monitored during fermentation at temperatures of 42°C and subsequent storage at 7°C. Fig. 4.3.1. shows the production of lactic acid and the decrease of pH in the cabbage salads fermented with these starters. After 7 hours of fermentation at 42°C the pH of the salads fermented with *Lactobacillus plantarum* no. 9 and no. 20 was approximately 4.0, while the pH of the salad fermented with *L. plantarum* no. P4 was 4.4. During storage of all salads, a further decrease in pH occurred. The cabbage salads fermented with strains numbers 20, 9 and P4 contained approximately 0.39 % (w/w), 0.32 % (w/w) and 0.23 % (w/w) lactic acid, respectively, after fermentation for 7 hours at 42°C.

Table 4.3.2. pH, numbers of *Klebsiella pneumoniae* and concentration of lactic acid in leek-cabbage-ham salads inoculated with *K. pneumoniae* and fermented with *Lactobacillus acidophilus* starters.

Starter nr.[a]	Fermentation temperature (°C)	pH ± s.d. at different times[b]				*K. pneumoniae* (CFU)[c]				Lactic acid concentration (g/kg salad) after fermentation
		A	B	C	D	A	B	C	D	
4	42	5.57±0.04	4.60±0.04	3.93±0.01	4.29±0.00	3.27	3.50	3.36	2.96	2.06
	45		4.34±0.08	3.90±0.02	4.14±0.04		3.16	<1.70	<1.00	2.72
39	42	5.58±0.09	4.44±0.01	3.95±0.07	4.18±0.02	3.38	3.58	3.02	2.58	2.53
	45		4.26±0.02	3.84±0.04	4.10±0.03		3.06	<1.70	<1.00	3.45
45	42	5.90±0.02	4.54±0.02	3.93±0.06	4.24±0.00	3.39	3.59	3.13	3.46	2.61
	45		4.23±0.06	3.86±0.02	4.06±0.01		2.93	<1.70	<1.00	3.33
48	42	5.79±0.03	5.65±0.11	4.01±0.10	4.62±0.02	3.36	3.71	>3.47	>4.48	1.45
	45		5.60±0.03	4.07±0.04	4.31±0.03		3.15	3.76	<2.43	2.27

[a] All starter cultures were identified as *Lactobacillus acidophilus*, inoculation level was 10^6-10^7 cfu/g of salad.

[b] triplicate determinations; A = before fermentation; B = after 3.5 hours of fermentation; C = after 7 hours of fermentation; D = after fermentation and subsequent storage at 7°C for 24 hours.

[c] Colony Forming Units of *Klebsiella pneumoniae* (log N/g), average of duplicate determinations.

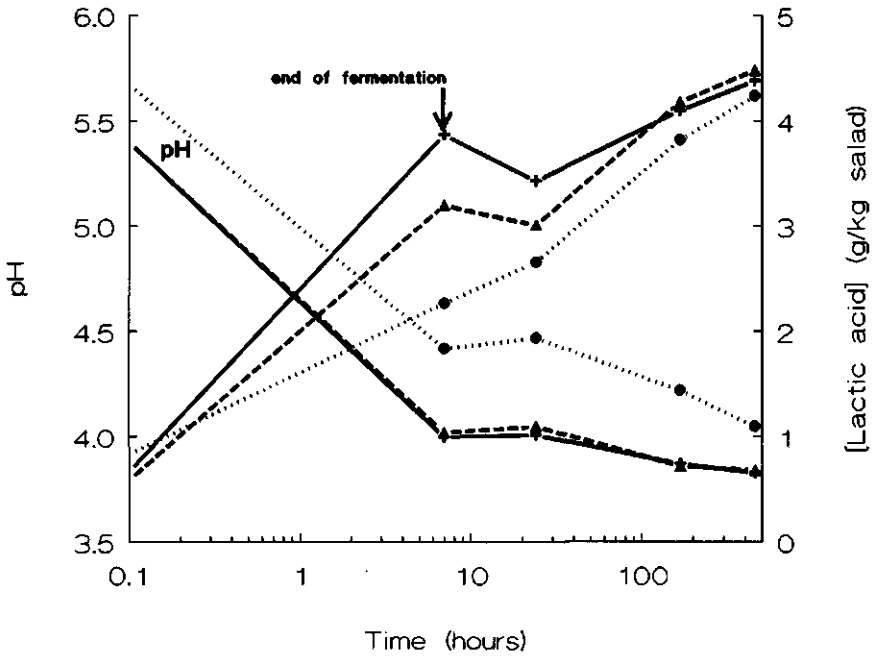

Fig. 4.3.1. pH and lactic acid production in cabbage salads, fermented with *Lactobacillus plantarum* nrs. P4 (●), 9 (▲) and 20 (+) for 7 hours at 42°C and stored at 7°C for 14 days.

After 5 days storage at 7°C the lactic acid concentrations were 0.41 % (w/w), 0.42 % (w/w) and 0.38 % (w/w), respectively. Directly after fermentation little acetic acid could be found in the fermented salads (0.08 % [w/w], 0.06 % [w/w] and 0.02 % [w/w], respectively), increasing to 0.10 % [w/w], 0.09 % [w/w] and 0.02 % [w/w], respectively, after 5 days storage. Ethanol production was nearly absent ($\leq$ 0.04 % [w/w]). Minor amounts of sugars, generally less than 0.5 % (w/w), were used.

Fig. 4.3.2. shows the fate of *S. aureus* in cabbage salad fermented with the three *L. plantarum* strains. During fermentation 3 to 4 generations of growth occurred, followed by a rapid decline in numbers in salads fermented with *L. plantarum* strain numbers 20 and 9. *S. aureus* could survive for a longer time in cabbage salad fermented with *L. plantarum* strain no. P4. In salads, such as leek-cabbage-ham salads, containing ingredients with high buffering capacities, which were inoculated with *S. aureus* and fermented at 45°C with *L. acidophilus* (no. 4), *S. aureus* increased with 5 to 6 generations, after which the numbers

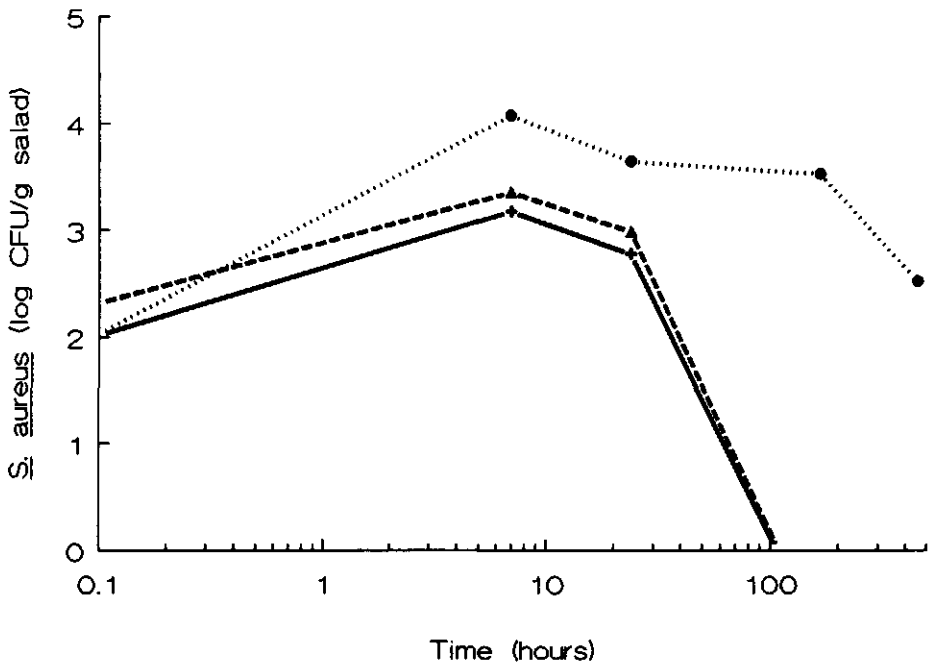

Fig. 4.3.2. The fate of *Staphylococcus aureus* inoculated into cabbage salads, fermented with *Lactobacillus plantarum* nrs. P4 (●), 9 (▲) and 20 (+) for 7 hours at 42°C and stored at 7°C for 14 days.

declined slowly (Table 4.3.3.). In control salads, acidified with approximately 0.4 % (w/w) lactic acid, *S. aureus* died rather quickly. Although in leek-cabbage-ham salads fermented with *L. plantarum* (strain no. 5) a significant lower amount of lactic acid was produced, *S. aureus* increased with only 2 to 3 generations. After 14 days storage the numbers of *S. aureus* declined below the detection level. These phenomena might be due to the production of significantly higher amounts of acetic acid in salads fermented with *L. plantarum* (strain no. 5), compared to salads fermented with *L. acidophilus* no. 4. *Enterobacteriaceae*, originally present in the raw ingredients, disappeared quickly in all salads (Table 4.3.3.).

In potato salads, which were inoculated with *S. aureus* and fermented at 45°C with *L. acidophilus* no. 4, a low pH (<4.0) was reached and *S. aureus* increased with only 3 generations. Within 6 days storage at 7°C, the numbers declined below detectable levels

(Table 4.3.4.). In control salads, acidified with approximately 0.4 % (w/w) lactic acid, *S. aureus* died more quickly.

Fig. 4.3.3. shows the destruction curves of *Listeria monocytogenes* in cabbage salad fermented with the different strains of *L. plantarum*. During fermentation *L. monocytogenes* increased with 2 to 3 generations, which was followed by a rapid decline in numbers. Growth or survival of *B. cereus* in cabbage salads fermented with different starters could not be detected. *B. cereus* was inoculated into these salads at approximately 250 cfu/g salad.

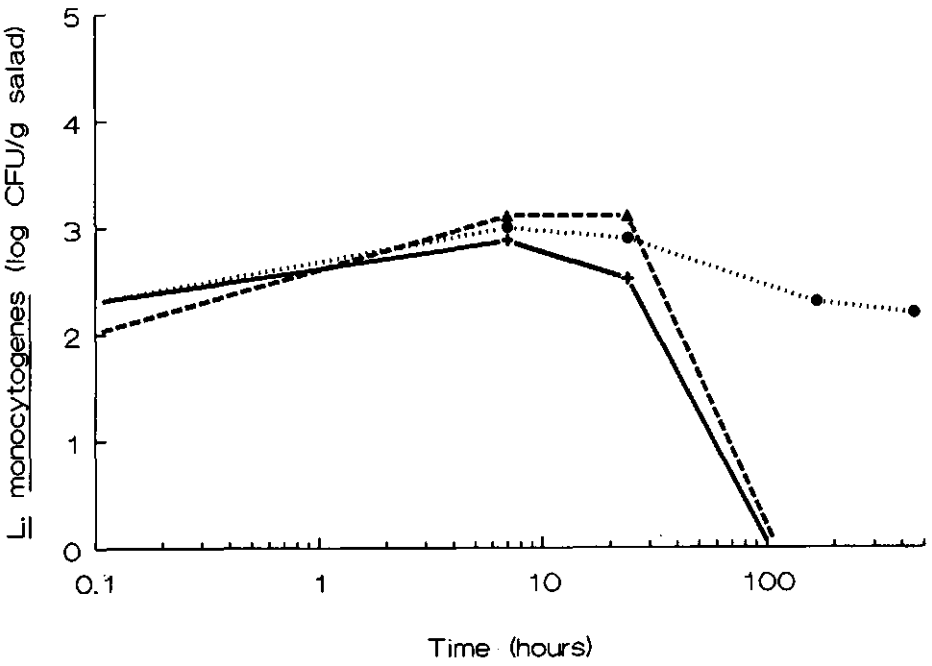

Fig. 4.3.3. The fate of *Listeria monocytogenes* inoculated into cabbage salads, fermented with *Lactobacillus plantarum* nrs. P4 (●), 9 (▲) and 20 (+) for 7 hours at 42°C and stored at 7°C for 14 days.

Table 4.3.3. pH, numbers of bacteria and concentration of lactic and acetic acids in leek-cabbage-ham salads inoculated with *Staphylococcus aureus*.

Starter nr.[a]	Time[b]	pH	*Staphylococcus aureus* (CFU)[c]	*Enterobacteriaceae* (CFU)[c]	Lactic acid (g/kg salad)	Acetic acid (g/kg salad)
4	A	5.63	2.62[d]	3.55	1.1	0.2
	B	4.34	5.10	<1.70	4.1	0.2
	C	4.38	5.35	<1.70	4.1	0.3
	D	4.32	3.90	<1.70	4.3	0.3
5	A	5.66	2.62[d]	3.25	0.8	0.2
	B	4.47	3.45	<1.70	3.2	1.2
	C	4.35	3.40	<1.70	3.8	1.5
	D	4.33	<2.70	<1.70	4.1	1.7
control[e]	A	3.67	2.62[d]	<1.70	4.8	0.2
	B	4.10	<2.70	<1.70	4.3	0.2
	C	4.18	<2.70	<1.70	4.3	0.2
	D	4.11	<2.70	<1.70	4.3	0.2

[a] No. 4, *Lactobacillus acidophilus*; no. 5, *Lactobacillus plantarum*; inoculation level was 10^6-10^7 cfu/g of salad.
[b] A = before fermentation; B = after 7 hours fermentation at 42°C; C = after fermentation and subsequent storage at 7°C for 5 days; D = after fermentation and subsequent storage at 7°C for 12 days.
[c] Colony Forming Units (log N/g), average of duplicate determinations.
[d] Number determined in the broth culture used to inoculate the salads.
[e] not inoculated, acidified with lactic acid.

Table 4.3.4. pH, numbers of bacteria and concentration of lactic and acetic acids in potato salads inoculated with *Staphylococcus aureus*.

Starter nr.[a]	Time[b]	pH	CFU[c] of *Staphylococcus aureus*	CFU[c] of *Enterobacteriaceae*	Lactic acid (g/kg salad)	Acetic acid (g/kg salad)
4	A	5.60	2.56[d]	<1.70	0.4	0.2
	B	3.90	3.40	<1.70	3.9	0.3
	C	3.97	3.40	<1.70	3.8	0.3
	D	3.97	<2.70	<1.70	3.9	0.3
control[e]	A	3.90	2.56d[d]	<1.70	3.3	0.2
	B	n.d.	<2.70	<1.70	n.d.	0.2
	C	4.01	<2.70	<1.70	3.2	0.2
	D	3.90	<2.70	<1.70	3.5	0.2

[a] No. 4, *Lactobacillus acidophilus*.
[b] A = before fermentation; B = after 7 hours fermentation at 42°C; C = after fermentation and subsequent storage at 7°C for 2 days; D = after fermentation and subsequent storage at 7°C for 6 days.
[c] Colony Forming Units (log N/g), duplicate determinations.
[d] Number determined in the broth culture used to inoculate the salads.
[e] not inoculated, acidified with lactic acid.
n.d. not determined

4.4. DISCUSSION

Fermented sauce-based salads were produced using processed and raw ingredients and various starters and by carrying out a relatively short fermentation at elevated temperatures (7 hours at 42 or 45°C). The products were subsequently stored at 7°C. The aim of this study was to establish if these products can be produced and stored with sufficient safeguards against survival and growth of pathogenic bacteria.

The fate of spoilage and pathogenic bacteria in fermented sauce-based salads is mainly determined by factors such as contamination of the ingredients, outgrowth during the first stage of fermentation, and inactivation during fermentation or storage. Starter cultures should be used which are physiologically active and which produce lactic acid at a high rate and in sufficient amounts, i.e. pH after fermentation should be lower than 4.2. These conditions are feasible, as can be concluded from the data in tables 4.3.1. and 4.3.2.2. Nevertheless, measures should be taken to guarantee absence, or low initial contamination levels of spoilage and pathogenic microorganisms in raw materials and ingredients.

In our study concerning the fate of pathogenic and non-pathogenic *Enterobacteriaceae* in fermented salads an acid-resistant psychrotrophic strain of *Klebsiella pneumoniae*, isolated from spoiled salads, was taken as model organism. According to Brocklehurst *et al.* (1987) this microorganism, and the closely related species *Enterobacter agglomerans*, can be frequently isolated from vegetable salads. *Klebsiella*, however, is generally more resistant to heat than other *Enterobacteriaceae*, and is therefore a better indicator of failure of processes that use minimal heat, such as blanching of vegetables. As can be concluded from the data in tables 4.3.1. and 4.3.2. growth and survival of *Klebsiella pneumoniae* is inhibited by a rapid decrease of pH and a low final pH. This is in agreement with findings of Rutzinski and Marth (1980), who concluded that an increase in inoculum of lactic acid bacteria into skim milk and, through that, a higher rate of pH decrease, was accompanied by an increase in inhibition of *Enterobacter* spp. and *Hafnia* spp. The rate of pH decrease in the various salads is not only influenced by differences in characteristics, activity and amounts of the starter cultures used, also the buffering capacity of ingredients plays a role. In cabbage-salads, fermented at 42°C, a pH of 4.0 is reached within 3 hours, while in leek-

cabbage-ham salads under the same conditions 4 to 5 hours are needed to reach this pH. Further improvements can be attained by using more active starter cultures and by adjusting fermentation conditions.

In our experiments in which cabbage salads were inoculated with *B. cereus* (approximately 250 cfu/g salad) and subsequently fermented with different starters, growth or survival of this pathogen could not be detected. This is in agreement with findings of Wong and Chen (1988), who found that spore germination, outgrowth, and vegetative cell multiplication of *B. cereus* in lactic acid-acidified skim milk, ceased at pH 5.0. Moreover, the storage temperature of 7°C is probably close to the minimum temperature of growth of many strains of *B. cereus*. Other factors, such as a decrease in the oxidation-reduction potential (Sims *et al.*, 1989) or the production of hydrogen peroxide by lactic acid bacteria may also play a role in the prevention of growth of *B. cereus*. In broth cultures of various aerobically grown lactic acid bacteria used as starters in these studies, however, production of hydrogen peroxide could not be detected (Bonestroo *et al.*, 1992b).

Control of staphylococcal enterotoxicosis must rely primarily on the inhibition of growth of the organism in foods. Multiplication and toxin formation are almost completely inhibited below 7°C (Mossel and Van Netten 1991). In supermarkets, however, salads are often stored in open display refrigerators at higher temperatures. Sims *et al.* (1989) showed that multiplication of *S. aureus* within a pH range of 4.7 to 5.2 is unlikely at a storage temperature of 7°C. *S. aureus* is also very sensitive to competition by common spoilage agents, such as lactobacilli. During storage of fermented salads adequate limitation of proliferation and metabolism can be assured by a combination of inhibitory factors, such as low temperature (7-10°C), low pH (<4.5) and competition by lactic acid bacteria. During the first stage of fermentation, however, critical conditions exist for the proliferation of *S. aureus*, i.e. a temperature of 30-45°C and an adapting competitive flora. According to Fig. 4.3.2. and tables 4.3.3. and 4.3.4., however, the number of generations of *S. aureus* can be limited by a rapid decrease of pH and a low final pH. The lowering of the oxidation-reduction potential (Stadhouders *et al.*, 1978) and the production of small amounts of acetic acid could have an additional effect. Although the salads in our experiments were inoculated with up to 420 cfu of *S. aureus*, populations of 10^6 cfu/g were never attained. This level is

required to produce enterotoxin concentrations of about 100 ng/100 g which might cause clinical symptoms (Mossel and Van Netten, 1990). As natural contamination levels are generally much lower (Hartog and Jansen, 1986; Hildebrandt *et al.*, 1989), the risk of staphylococcal enterotoxicosis caused by consumption of fermented salads is negligible, provided that proper fermentation characteristics are achieved.

According to the curves of *L. monocytogenes* Scott A, inoculated into cabbage salads fermented with different strains of *L. plantarum* (Fig. 4.3.3.), 2 to 3 generations of growth occurred during fermentation, followed by a rapid decline to below detectable levels. Buchanan and Klawitter (1990) showed that *Listeria monocytogenes* Scott A did not grow in microbiological media acidified with hydrochloric acid to pH 4.5 at 5 and 10°C, but survived for extended periods. However, organic acids employed as acidulants in foods, such as lactic acid, have been reported to be substantially more detrimental, compared to hydrochloric acid.

The behaviour of *Cl. botulinum* in fermented salads was not tested. Salads could be contaminated with this pathogen through the use of spices or ingredients such as potatoes, which are insufficiently decontaminated. In pasteurized slices of potatoes, heated for 2 min at 104°C (core temperature), *Cl. botulinum* could not be detected (Baumgart, 1987). Also, *Cl. botulinum* was not found in none of 45 samples of untreated spices. Moreover, *Cl. botulinum* type E was inhibited by the growth of lactobacilli, in an unpreserved potato salad on mayonnaise-basis (pH 4.8, acetic acid 0.3 %). Therefore there is no risk of botulism caused by consumption of fermented salads.

In this study we have shown that fermentation of sauce-based salads can be an adequate safeguard against growth and survival of a number of spoilage and pathogenic bacteria. In a preceding paper we described the fate of notorious spoilage yeasts in fermented salads (Bonestroo *et al.*, 1992b). It is obvious that with the fermentation and storage procedures applied, microbiologically safe products with a reasonable shelf life can be obtained. Measures should be taken to guarantee absence, or low initial contamination levels of spoilage and pathogenic microorganisms in raw materials and ingredients. Starter cultures should be used which are physiologically active and which produce lactic acid at a high rate and in an amount which lowers the pH below 4.2. Also, proper hygienic care during

processing should be assured.

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5

INHIBITION OF LIPID OXIDATION IN FERMENTED SALADS

ABSTRACT

The chemical shelf life of salads, i.e. vegetables and/or meat in an oil-in-water emulsion with a low pH due to the addition of acids, is often much shorter than the microbiological shelf life. Salads contain substantial amounts of vegetable oils, often with high levels of polyunsaturated fatty acids, which are highly susceptible to oxidation during storage. Packed in transparent packings and stored under light exposure in supermarkets, salads are subject to photo-oxidation as well as autoxidation. Salads have been fermented with *Lactobacillus plantarum* strains to study the protecting effect of fermentation towards lipid oxidation. Experiments showed that fermented potato salads, subjected to light exposure for different times, and analyzed for lipid oxidation products with gas-chromatographic methods, differed from uninoculated, acidified control salads. Fermented salads contained lower amounts of lipid oxidation products, a.o. hexanal, indicating a lower level or possibly the absence of lipid oxidation. Significant differences between the level of oxidation in potato salads fermented with different startercultures could be detected. Fermented potato salads, stored at 7°C for several days, had lower amounts of oxygen in the headspace than uninoculated, acidified control salads.

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This chapter is submitted for publication

5.1. INTRODUCTION

Prepared salads containing vegetables and/or meat in a low pH oil-in-water emulsion with added acids, are now important in the food market of western countries. Selection of high quality raw materials, better preparation methods and the use of preservatives, i.e. sorbic and benzoic acids, have led to a microbiological shelf life of these salads of 6 to 8 weeks, if stored below 7°C. However, the actual shelf life of salads may be much shorter, because salads contain substantial amounts of vegetable oils which are highly susceptible to oxidation and quality deterioration during storage. Market demands, such as the use of vegetable oils with high levels of polyunsaturated fatty acids and the use of transparent packing materials have a negative effect on the shelf life of salads because they are usually stored under high illumination in open display refrigerators.

Oxidation is accelerated by increased temperature, light exposure, the presence of metal ions, sensitizers and especially by the presence of oxygen, but is inhibited by natural and synthetic antioxidants (Pokorny, 1987; Mörsel, 1990; Mörsel and Meusel, 1990). Nevertheless, the addition of antioxidants is practically never applied, obviously due to the growing resistance of consumers to the use of food additives. A technological solution to the lipid oxidation problem could be the production of salads in closed systems with rigorous exclusion of oxygen, requiring, however, large capital investments.

In a previous paper (Bonestroo *et al.*, 1992) lactic acid fermentation was proposed as a novel way of preparation of salads which could be attractive, because of their possible health effects and especially because the use of preservatives can be abandoned.

Growth of lactic acid bacteria causes a lowering of the oxidation-reduction potential (Kandler and Weiss, 1986; Cooke *et al.*, 1987) as many lactic acid bacteria are able to consume oxygen (Götz *et al.*, 1980; Kanbe and Uchida, 1985; Condon, 1987). As oxygen is absent in fermented dairy products after culture (Langeveld and Bolle, 1985) one might assume that the fermentation processes could protect salads from lipid oxidation.

The objective of this study, as part of an investigation on lactic acid fermented salads, was to investigate these reducing effects and the influence of fermentation on lipid oxidation in (potato) salads stored under light exposure; oxidation was assessed by gas-chromatographic and sensory analyses.

5.2. MATERIALS AND METHODS

Organisms and culture conditions

Lactobacillus plantarum no. 23 and ATCC 8014, obtained from our laboratory collection (Bonestroo *et al.*, 1992), were cultivated for 24 hours at 30°C in a broth with the same composition as MRS broth (Merck, Darmstadt, F.R.G.), except that glucose was replaced by an equal weight of sucrose (Merck), followed by subculture in the same broth for 16 hours at 30°C.

Preparation and storage of salads

Potato salads, containing 65 % (w/w) peeled, cut (10x10x10 mm) and cooked (5-10 min 90-100°C) potato tubers (cultivar Bintje) and 35 % (w/w) sauce, composed of water (47.2 % [w/w]), fresh soybean oil (35 % [w/w]), sucrose (9 % [w/w]), salt (3 % [w/w]), egg yolk (3 % [w/w]) and thickening agents (2.8 % [w/w]), were inoculated with 10^6 to 10^7 cfu per g and filled in glass jars (100 g salad, headspace approximately 50 ml) sealed with air-tight metal Vapor-Vacuum™ Twist-Off™ caps (White Cap International, U.S.A.). These caps had previously been pierced and the 5 mm holes were sealed with silicone rubbers (diameter 10mm, thickness 3 mm), glued to the inner side, to allow headspace sampling with a gastight syringe. The jars were incubated in a water bath for 7 h at 42°C, chilled in ice water to 7°C and stored at 7°C in darkness or illuminated (TL FTD 33, Philips, Eindhoven, The Netherlands) with a light intensity of approximately 500 lux, corresponding to conditions prevailing in stores. Uninoculated, but acidified (0.4 % (w/w) lactic acid) control samples were handled according to the same procedure.

Determination of lipid oxidation

GC Static headspace volatile analysis. A gas chromatograph (Model HRGC, Carlo Erba Strumentazione, Rodano (Milan), Italy) equipped with a flame ionization detector at 300°C and a 26 m x 0.34 mm i.d., 0.12 μm Sil 5 CB liquid phase column (Chrompack, Middelburg, The Netherlands) was used, with helium carrier gas and make-up gas at 95 kPa and 100 kPa, respectively, with no split. The injector was at 250°C and the column at 35°C. Headspace samples of 25 μl were taken with gastight syringes.

GC Dynamic headspace volatile analysis. Glass tubes (16 cm x 6 mm o.d.) containing 0.1 g Tenax TA (Alltech Nederland, Zwijndrecht, The Netherlands), positioned with DMCS treated glass wool were conditioned (4 h, 250°C, nitrogen purging 2.5 ml/min).

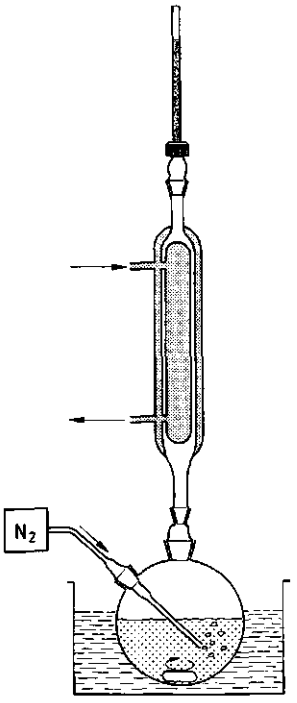

Fig. 5.2.1. Tenax-TA collection tube construction.

The all-glass gas-purging system (Fig. 5.2.1) consisted of a 500 ml round-bottom flask

with a nitrogen purge inlet and a Davies double surface condensor cooled at -15°C (Haake Mess-Technik GmbH, Karlsruhe, F.R.G.) with ethanol. Before use it was carefully cleaned and dried at 105°C for at least 20 hours. Samples of salads (approximately 100 g) were homogenized under nitrogen with approximately 200 ml of Nanopure water (Barnstead, Dubuque, Iowa, U.S.A.), filled into the flask (approximately 250 g) and purged at 4°C with molecular sieve purified nitrogen gas for approx. 60 min (20-60 ml/min) whilst the sample was stirred with a magnetic stirrer. The condensor reduced the absorption of water by the Tenax tubes.

Trapped volatiles were analyzed on a gas chromatograph (GC 6000 VEGA, Carlo Erba Strumentazione) with a flame ionization detector at 120°C, a 60 m x 0.25 mm i.d. Supelcowax 10 (0.25 μm Carbowax 20M) capillary column with helium carrier gas at 0.6 ml/min (160 kPa) and a thermodesorption and cold trap (TCT) unit (Chrompack). The cold trap was cooled to -120°C for 4 min with liquid nitrogen, the Tenax tube was heated to 180°C for 10 min, and the collected volatiles were injected by heating the cold trap to 250°C for 4 min. The column was used at 40°C for 4 min, increased to 100°C at a rate of 2.5°C/min and finally increased to 250°C at a rate of 4°C/min. Components were identified by GC-MS under identical conditions with an VG MM7070F mass spectrometer working in the 70 eV EI ionization mode (VG, Altrincham, U.K.).

Measurement of headspace oxygen

Headspace samples (100 μl) were taken by gastight syringe and analysed in a gas chromatograph (Model 433, Packard-Becker B.V., The Netherlands) with a thermal conductivity detector at 125°C and the injector at 110°C. Parallel coupled columns, split 1:1; 1.5 m x 3.2 mm i.d. teflon column packed with Chromosorb 108 (60-80 mesh) and a 1.2 m x 3.2 mm i.d. stainless steel column packed with Molecular Sieve 5 A (60-80 mesh) were used at 40°C with Helium as carrier gas (180 kPa).

Evaluation of the quality of fermented salads

The salads were analysed microbiologically directly after 7 h fermentation and after several days of storage at 7°C. Mesophilic aerobic spoilage bacteria were

enumerated on pour-plates of Gelysate agar (GEL, 5 g Bacto-peptone (Difco, Detroit, Michigan, U.S.A.), 5 g sodium chloride (Merck) and 14 g agar (L13, Oxoid Ltd., Basingstoke, U.K.) per l, pH 7.6 ± 0.1), incubated at 30°C for 3 days; *Enterobacteriaceae* on pour plates of Violet Red Bile Glucose Agar (VRBG; CM485, Oxoid) with a top layer of the same medium and incubated for 24 hours at 30°C. Lactic acid bacteria were selectively enumerated on pour-plates of MRS medium (Merck) with 12 g of agar (L13, Oxoid) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l, incubated under anaerobic conditions (Anaerocult system, Merck) at 30°C for 3 days. Yeasts and moulds were enumerated on Oxytetracycline Glucose Yeast Agar (OGGA; CM545, Oxoid), incubated at 25°C for 5 days and *Pseudomonas* spp. on Pseudomonas Agar Base (PSA; CM559, Oxoid), supplemented with *Pseudomonas* C-F-C supplement (SR103, Oxoid), incubated at 30°C for 2 days. pH was determined electronically and sugars and organic acids in fermented salads were determined by HPLC as described earlier (Bonestroo *et al.*, 1992).

5.3. RESULTS

Fresh potato salads, obtained from a Dutch salad producer, were filled in glass jars and examined after light exposure at 7°C with static headspace gas-chromatographic methods. A gradual increase (Fig. 5.3.1.) was seen in the amount of total volatiles with prolonged light exposure (≤ 15 days). It should be remarked that all these salads were analyzed after 20 days storage at 7°C, thus only the duration of light exposure was varied, to avoid possible disturbances caused by aging of the salads, i.e. microbial growth and chemical deterioration other than lipid oxidation. These findings were in good agreement with flavour ratings determined by a trained panel, after 20 days storage at 7°C, as shown in Table 5.3.1. Four flavour categories were used, i.e. good, light off-flavour, moderate off-flavour and rancid.

Fermented potato salads, subjected to light exposure for different times, and analyzed for total volatiles with static headspace gas-chromatographic methods, differed from uninoculated, but acidified control salads. Fermented salads contained lower amounts of total volatiles (data not shown), indicating a lower level or possibly the

absence of lipid oxidation. No significant differences in the level of total volatiles were

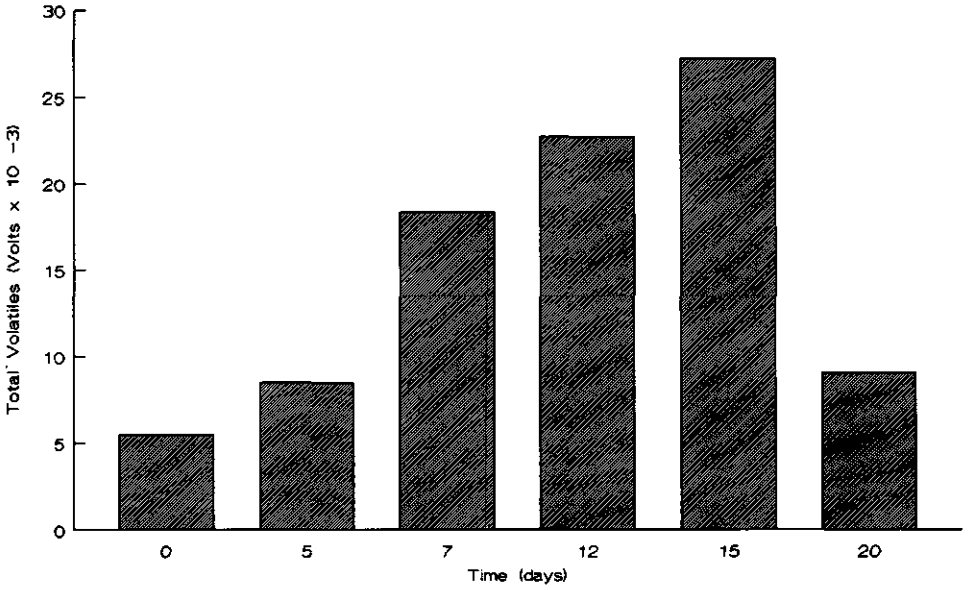

Fig. 5.3.1. Total volatiles of industrially produced potato salads stored at 7°C for 20 days and subjected to light exposure for various times, measured with static headspace gas-chromatographic methods.

found between potato salads fermented with various starter cultures. These findings correlated to a certain extent with flavour score determinations, which were difficult to assess, possibly because of the specific taste characteristics of the major acidulant, i.e. lactic acid. On the other hand, the level of total volatiles in uninoculated, acidified potato salads was relatively low in comparison with the level of total volatiles

Table 5.3.1. Flavour ratings of industrially produced potato salads stored for 20 days at 7°C and subjected to light exposure for 0, 5, 7, 12, 15 and 20 days, respectively.

	Flavour score			
	Good	Light off-flavour	Moderate off-flavour	Rancid
Potato salad	(0)[a]	(5) (7)	(20) (12)	(15)

Salads were analyzed in duplicate.
[a] days of storage at 7°C under light exposure.

found in industrially produced potato salads shown in Fig. 5.3.1., which could be due to differences in composition. Industrially produced potato salads not only contain potatoes, but also other vegetables, such as peas, carrots and onions, in contrast to fermented potato salads. Moreover, seasonings are not added to the latter. For the assessment of these low levels of volatiles, other methods had to be applied.

With more sensitive dynamic headspace gas-chromatographic methods it was also found that fermented potato salads, subjected to light exposure for 11 days, and analyzed for total volatiles, differed from uninoculated, acidified control salads (Fig. 5.3.2.). Also, significant differences between the level of volatiles in potato salads fermented with *Lactobacillus plantarum* no. 23 and ATCC 8014 could be detected (Fig. 5.3.2.). The 11 days period was chosen as a compromise between the time necessary for the development of significantly detectable amounts of lipid oxidation products and the time during which interferences due to microbial spoilage could be neglected. The type of column used, in combination with temperature gradient programming, made a better separation of individual components possible. The following major components could be identified by GC-MS: n-pentane, acetone, ethanol and hexanal (see Fig. 5.3.2.a); retention times 18.96, 21.23, 25.04 and 30.54 min, respectively. Acetone and ethanol were used to clean the Davies double surface condensor between analyses. Hexanal appears to be a unique component, and might be a good indicator of oxidative changes that occur in salads exposed to light.

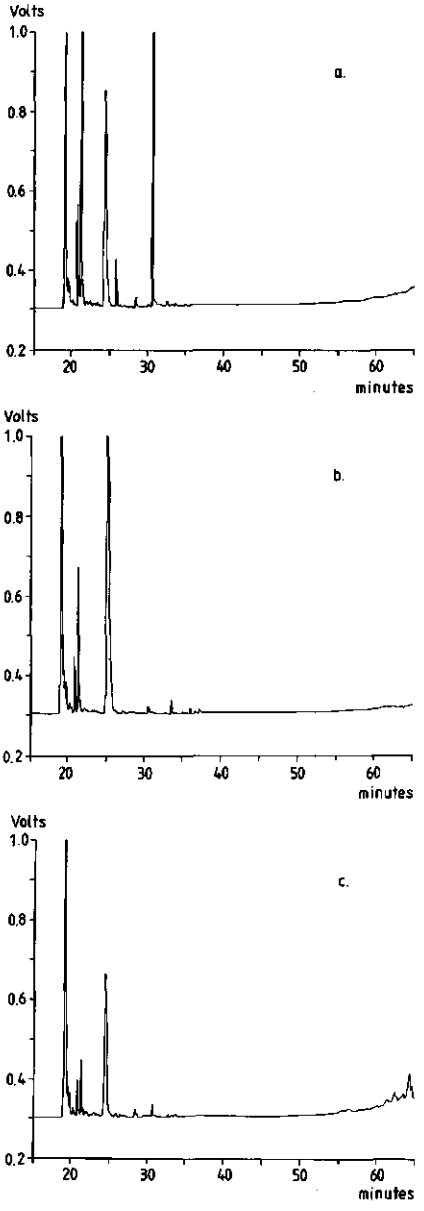

Fig. 5.3.2. Profiles of volatiles of potato salads, measured with dynamic headspace gas-chromatographic methods. **a**, uninoculated, acidified potato salad exposed to light during storage at 7°C for 11 days; **b**, fermented with *Lactobacillus plantarum* strain ATCC 8014, exposed to light during storage at 7°C for 11 days; **c**, fermented with *Lactobacillus plantarum* strain nr. 23, exposed to light during storage at 7°C for 11 days.

Microbiological and chemical analyses of fermented potato salads showed good growth and souring of starter cultures, since the salads had a pH-value $\leq$ 4.2, with an average lactic acid concentration of approximately 4 g per kg of salad. During the course of the experiments no growth of yeasts, *Enterobacteriaceae* and *Pseudomonas* spp. was observed.

Fermented potato salads, stored at 7°C for two weeks, and analyzed for the amount of oxygen in the headspace with gas-chromatographic methods, differed from uninoculated, acidified control salads (Table 5.3.2.). Fermented salads showed a higher consumption of oxygen from the headspace.

Repetition of these experiments, in which fermented and uninoculated, acidified control salads were stored at 7°C for several days, and analyzed for the amount of oxygen in the headspace, revealed comparable results (Table 5.3.3.). Noteworthy is the fact that the consumption of oxygen from the headspace depends on the ratio between the volume of the product and the volume of the headspace, i.e. a low ratio corresponds to a higher consumption of oxygen. These salads were microbiologically stable, i.e. no growth of yeasts, *Enterobacteriaceae* and *Pseudomonas* spp. was observed, pH-values were below 4.2, with an average lactic acid concentration of approximately 4 g per kg of salad.

5.4. DISCUSSION

Oxidation of salads is mainly associated with the unsaturated moieties of the lipid fraction, occurring by autoxidation (self-catalyzed, free radical chain reaction), photo-oxidation or, in case raw ingredients are used, enzyme-catalyzed oxidation (lipoxygenase). Packed in transparent packings and stored under light exposure in supermarkets, salads are subject to photo-oxidation as well as autoxidation. Although the unsaturated fatty acids do not absorb visible light and are not normally subject to direct ultraviolet irradiation, they can undergo accelerated photo-sensitized oxidation owing to light absorption by colouring matters in the salads, like chlorophyll. The oxidation of unsaturated fatty acids gives rise to hydroperoxides which decompose into volatile compounds. The mechanism of photo-oxidation differs from that of autoxidation. In the latter, the initiation stage

Table 5.3.2. Oxygen uptake during storage at 7°C from the headspace of control (uninoculated, acidified) and inoculated, fermented potato salads.

Sample	Storage time (days)	Volume product (ml)	Volume headspace (ml)	% Oxygen in headspace	Oxygen-uptake [a] (mg/kg)
Control	7	106.25	43.45	21.2	- [b]
		111.90	37.80	21.2	-
	14	84.91	64.79	21.0	-
		87.07	62.63	21.2	-
ATCC 8014	7	96.23	53.47	20.0	-
		79.46	70.24	20.2	3.0
	14	83.23	66.47	17.5	33.9
		85.63	64.07	17.4	31.7
Nr. 23[c]	7	100.16	49.54	18.7	8.5
		66.51	83.19	19.7	18.5
	14	89.27	60.43	15.4	47.8
		86.35	63.35	16.1	44.9

[a] Oxygen uptake calculated according to the following equations (derived from Langeveld and Bolle, 1985)

$$Oxygen\ offered\ (mg/l) = \frac{V_h \times M \times p_{t1} \times 1000}{V_m \times p_{at} \times V_p}$$

$$Oxygen\ remained\ (mg/l) = \left(\frac{V_h \times M \times p_{t2}}{V_m \times p_{at}} + \frac{V_p \times S \times (p_{t2} - p_b)}{1000 \times p_{ox}}\right) \frac{1000}{V_p}$$

V_h = volume headspace (ml);
V_m = molar volume (ml/mmol);
at 7°C, 101.3 kPa; V_m = 24.5 ml/mmol;
V_p = volume of product (ml);
p_{t1} = initial oxygen tension (kPa);
p_{t2} = basal oxygen tension of product (kPa);
S = oxygen solubility at an oxygen pressure of 21.3 kPa;
M = molar mass of oxygen (32 mg/mmol);
p_{at} = atmospheric pressure (101.3 kPa);
p_{ox} = atmospheric oxygen pressure (21.3 kPa).

[b] - = ≤ 1.0 mg/kg

[c] Number refers to *Lactobacillus plantarum* strain numbers described in Bonestroo *et al.* (1992).

Table 5.3.3. Oxygen uptake during storage at 7°C from the headspace of control (uninoculated, acidified) and inoculated, fermented potato salads.

Sample	Storage time (hours)	Volume product (ml)	Volume headspace (ml)	% Oxygen in headspace	Oxygen-uptake [a] (mg/kg)
Control	24	45.05	103.45	21.1	- [b]
		45.75	102.75	21.0	-
	48	45.96	102.55	21.3	-
		46.36	102.14	21.2	-
	72	44.95	103.56	21.1	-
		46.16	102.34	21.2	-
Control	24	87.87	60.63	21.0	-
		90.90	57.60	21.1	-
	48	90.40	58.11	21.2	-
		100.19	48.31	21.2	-
	72	100.39	48.11	20.9	-
		90.80	57.70	21.0	-
ATCC 8014	24	45.25	103.25	20.8	-
		44.84	103.66	20.7	-
	48	45.85	102.65	20.7	-
		46.26	102.24	20.7	-
	72	45.35	103.15	20.5	7.5
		45.25	103.25	20.4	8.5
ATCC 8014	24	87.67	60.83	20.6	-
		91.61	56.89	20.5	-
	48	88.98	59.52	21.3	-
		90.40	58.11	20.2	-
	72	92.21	56.29	19.8	1.1
		90.19	58.31	19.8	1.2
Nr. 23[c]	24	45.55	102.95	20.5	6.4
		45.35	103.15	20.5	6.5
	48	46.06	102.44	20.4	8.0
		44.44	104.06	20.3	12.6
	72	45.15	103.35	20.1	19.5
		44.95	103.56	20.1	18.4
Nr. 23	24	88.78	59.72	20.0	-
		90.90	57.60	20.0	-
	48	90.50	58.00	19.8	1.3
		90.09	58.41	19.8	1.9
	72	89.49	59.01	19.4	5.9
		90.29	58.21	19.3	6.8

[a] see Table 5.3.2.
[b]
[c]

results in the formation of the first hydroperoxides. The following propagation phase involves the breakdown of these hydroperoxides to form free radicals, which further generate new hydroperoxides in an autocatalytic chain reaction. In the photo-oxidation of lipids, the reaction is apparently not autocatalytic: the quantity of hydroperoxides formed is proportional to the total amount of light absorbed. The hydroperoxides undergo further oxidation by light to form free radicals. Since the reaction is not autocatalytic, antioxidants, which interrupt chain reactions in the autoxidation mechanism, are

ineffective in inhibiting photo-oxidation.This may be one of the reasons why antioxidants are apparently not used in salads. In photo-oxidation the oxygen uptake is considerably larger, than in autoxidation (Faria and Mukai, 1983; Chan, 1987; Mörsel, 1990; Mörsel and Meusel, 1990).

Potato salads are extremely sensitive to lipid oxidation, due to their composition and specific structure (spongy, with high oxygen inclusion) and some specific potato properties, which vary, however, between cultivars. The lipid content of potato tubers is low - 5.8 to 9.0 mg/g of dry weight - but about 75 % of the fatty acids are linoleic and linolenic acids (C18:2 and C18:3), which are polyunsaturated. The tubers also contain a large amount of lipolytic enzymes. In an intact potato tuber, the lipids are very stable but when the membranes are damaged, by cutting for example, the lipolytic enzymes are immediately activated. Lipolytic acylhydrolase catalyzes the hydrolysis of free fatty acids (FFA) from the glycerides and lipoxygenase catalyzes the oxidation of these free fatty acids. The enzymes are inactivated by heat treatments, i.e. cooking. However, the main problem seems to be that it takes a long time to heat potato tubers in a blanching bath, during which enzymatic hydrolysis is favoured (Hallberg, 1990; Hallberg and Lignert, 1991).

For the evaluation of lipid oxidation in salads objective methods were chosen which were known to show high correlation with flavour ratings as determined in taste panel procedures, but which required only a minimal amount of sample preparation. Therefore, standard methods for the analysis of lipid oxidation, i.e. determination of the peroxide and p-anisidine value (Paquot, 1979) and the 2-thiobarbituric acid (TBA) test (Marcuse and Johansson, 1973; Hoyland and Taylor, 1989), were not applied. In principle, all methods require extraction and isolation of the fat phase, which -in view of the mechanism of autoxidation - demands careful procedures in order to prevent a further progress of lipid oxidation. According to several studies, good correlations exist between the amount of lipid oxidation products, as determined with gas-chromatographic methods, and the sensoric appearance of rancid off-flavours (Dupuy *et al.*, 1976; Warner *et al.*, 1978; Angelo *et al.*, 1981; Waltking and Goetz, 1983; Marsili, 1984).

A gradual increase (Fig. 5.3.1.) was seen in the amount of lipid oxidation products in industrially produced potato salads with prolonged light exposure ($\leq$ 15 days) during storage at 7°C. All salads were examined after 20 days storage at 7°C; only the duration of light exposure was varied. Control salads, stored in the dark for 20 days, showed a very low level of lipid oxidation. Therefore, oxidation of potato salads appears to be almost entirely due to photo-oxidation rather than autoxidation.

As can be concluded from Fig. 5.3.1. and Table 5.3.1. the amount of total volatiles in the headspace of industrially produced potato salads, as measured with static headspace volatile analysis, and the flavour score determined by a trained sensory test panel, are in good agreement. However, one disadvantage of analyzing headspace vapours by direct gas chromatography, is that only relatively low molecular weight and highly volatile compounds can be readily detected (Angelo *et al.*, 1981; Snyder *et al.*, 1988). With further progress of lipid oxidation, i.e. after 20 days exposure of potato salads to light, high molecular weight products are formed, which are less volatile and therefore not detected with the instrumental method used. On the other hand, they were also not perceived by the sensory test panel. The temperature at which samples were taken, 7°C, certainly plays a role in this. Another disadvantage of analyzing headspace vapours by direct gas-chromatographic methods is the limitation placed on the size of the gas sample that can be injected, thus reducing the sensitivity of the method. Although hexanal, which appears to be the major oxidation product in potato salads, is highly volatile, the generally low levels in fermented potato salads thus limit the use of these static headspace gas-chromatographic methods.

A possible explanation for the lower level or absence of lipid oxidation in fermented salads could be the reduction of aldehydes to their corresponding alcohols by alcohol dehydrogenases present in many lactic acid bacteria. However, the lower levels or absence of hexanal in fermented potato salads did not coincide with higher levels of hexanol, as measured by dynamic headspace gas-chromatographic methods in combination with mass spectrometry (Fig. 5.3.2.).

Other reactions with lipids, for instance by lipases of lactic acid bacteria, are also

not likely to occur, since most lipases from lactic acid bacteria exhibit their activity against short-chain glycerides and fatty acids (El Soda *et al.*, 1986; Sztajer *et al.*, 1988), which are not present in the soybean oil used.

One might assume that the reducing effects of the fermentation processes could have a protecting effect on salads towards lipid oxidation, although difficult to prove. The reducing capacity of lactic acid bacteria can be seen by growing the bacteria in appropriate media containing redox dyes (Costilow, 1981). When lactic acid bacteria are grown in litmusmilk (Difco) fortified with 10 g/l glucose (Merck), the originally blue colour of the litmus indicator turns red by acidification, while the bottom layer turns colourless, due to reduction. Lactic acid bacteria are able to consume oxygen, as can be concluded from literature data concerning oxygen consuming reactions of specific strains, *Pediococcus halophilus* (Kanbe and Uchida, 1985) and *Lactobacillus plantarum* ATCC 8014 (Götz *et al.*, 1980).

In our experiments, fermented salads, inoculated with different starter cultures, showed consumption of oxygen from the headspace (tables 5.3.2. and 5.3.3.). It should be remarked that this consumption of oxygen from the headspace not only depends on the strain used and the storage time, but also on the ratio between the volume of the product and the volume of the headspace, i.e. a low ratio corresponds to a higher consumption of oxygen. Higher oxygen consumption rates by lactic acid bacteria in the top layer of fermented salads could be responsible for this phenomenon. The concentration of oxygen in the salads is not only influenced by the oxygen consumption by the lactic acid bacteria in the salads, but also by the accessibility of oxygen. The latter depends on the amount of oxygen in the headspace and the diffusion through packing materials, other than glass. Flushing the headspace with nitrogen or mixtures of nitrogen and carbon dioxide probably would increase the chemical stability of the product. Further investigations are necessary, especially concerning the determination of oxygen profiles in fermented salads. A subsequent paper will deal with this aspect.

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6

OXYGEN CONSUMPTION BY STARTER CULTURES AND INHIBITION OF LIPID OXIDATION IN FERMENTED SALADS

ABSTRACT

Sauce-based salads are composed of vegetables and/or meat in an oil-in-water emulsion with a low pH due to the addition of acids. Their chemical shelf life is often much shorter than their microbiological shelf life. Salads contain substantial amounts of vegetable oils, often with high levels of polyunsaturated fatty acids, which are highly susceptible to oxidation during storage. Packed in transparent packings and stored under light exposure in supermarkets, salads are subject to photo-oxidation as well as autoxidation.

Potato salads have been fermented with *Lactobacillus plantarum* and *Lactobacillus acidophilus* to study the protecting effect of fermentation towards lipid oxidation. Experiments showed that fermented potato salads, subjected to light exposure for 10 days, and analyzed for lipid oxidation products with gas-chromatographic methods, differed from uninoculated, acidified control salads. Fermented salads contained lower amounts of lipid oxidation products, a.o. hexanal, indicating a lower level of lipid oxidation. During fermentation the oxygen tension in the salads decreased, with a corresponding decrease of the oxygen tension in the headspace. The decrease of the oxygen tension in fermented salads is caused by the oxygen consuming abilities of the lactic acid bacteria used as starters. Particularly some of the *L. acidophilus* strains showed remarkably high oxygen consumption levels. It is assumed that this oxygen consumption could be responsible for the protecting effect of fermentation on salads towards lipid oxidation.

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This chapter is submitted for publication.

6.1. INTRODUCTION

Sauce-based salads are composed of vegetables and/or meat in an oil-in-water emulsion with a low pH due to the addition of acids. Their chemical shelf life is often much shorter than their microbiological shelf life. Salads contain substantial amounts of vegetable oils, often with high levels of polyunsaturated fatty acids, which are highly susceptible to oxidation during storage. Packed in transparent packings and stored under light exposure in supermarkets, salads are subject to photo-oxidation as well as autoxidation.

The oxidation process is accelerated by reaction conditions such as light exposure, sensitizers and especially by the presence of oxygen, but is inhibited by natural and synthetic antioxidants (Pokorny, 1987; Mörsel, 1990; Mörsel and Meusel, 1990). However, antioxidants are practically never applied, obviously due to the growing resistance of consumers to the use of food additives.

In a previous paper (Bonestroo *et al.*, 1992a) lactic acid fermentation was proposed as a novel way of preparation of salads. If sufficiently attractive, a certain interest may be expected from the market for fermented salads, because of their possible health effects and especially because the use of preservatives can be abandoned. Moreover, the reducing effects of the fermentation processes could have a protecting effect on salads towards lipid oxidation. Experiments showed that fermented potato salads, subjected to light exposure for various time periods, and analyzed for lipid oxidation products with gas-chromatographic methods, differed from uninoculated, acidified control salads. Fermented salads contained lower amounts of lipid oxidation products, mainly hexanal, indicating a lower level or possibly the absence of lipid oxidation (Bonestroo *et al.*, 1992b).

Many lactic acid bacteria are able to consume oxygen (Götz *et al.*, 1980a; Kanbe and Uchida, 1985; Condon, 1987). Oxygen is absent in fermented dairy products after culturing (Langeveld and Bolle, 1985). The headspace of fermented potato salads, stored at 7°C for several days, contained lower amounts of oxygen, compared with uninoculated, acidified control salads (Bonestroo *et al.*, 1992b). One might assume that oxygen consumption by starter cultures could be responsible for the protecting effect of fermentation on salads

towards lipid oxidation.

The objective of this study, as part of an investigation on lactic acid fermented salads, was to investigate the oxygen consumption by starter cultures and the possible effect of fermentation on the delay of lipid oxidation in potato salads stored under light exposure. The appearance of lipid oxidation was evaluated after 10 days by dynamic gas-chromatographic methods. The 10 days period was chosen as a compromise between the time necessary for the development of detectable amounts of lipid oxidation products and the time during which interferences due to microbial spoilage in acidified control salads could be neglected.

6.2. MATERIALS AND METHODS

Organisms and culture conditions

Most lactic acid bacteria used in this study were obtained from our laboratory collection (Bonestroo *et al.*, 1992a). *L. plantarum* ATCC 8014, obtained from the State Institute for Quality Control of Agricultural Products (Wageningen, The Netherlands) was renumbered as *L. plantarum* no. 18. Prior to use the strains of the genus *Lactobacillus* were cultivated for 24 hours at 30°C or 42°C in a broth with the same composition as MRS broth (Merck, Darmstadt, F.R.G.), but containing sucrose (Merck) as carbohydrate, instead of glucose, followed by subsequent cultivation in the same broth for 16 hours at 30°C or 42°C.

Preparation and storage of salads

Potato salads, containing 65 % (w/w) peeled, cut (10x10x10 mm, series no. 1 and 2) or sliced (3 mm, series no. 3) and cooked (5-10 min 90-100°C) potato tubers (cultivar Bintje) and 35 % (w/w) sauce, composed of water (47.2 % [w/w]), fresh rapeseed oil (35 % [w/w]), sucrose (9 % [w/w]), salt (3 % [w/w]), egg yolk (3 % [w/w]) and thickening agents (2.8 % [w/w]), were inoculated with 10^6 to 10^7 cfu per g and filled in glass jars (100 $\pm$ 1 g salad, headspace 50 to 90 ml, depending on the size of the jars used) sealed with air-tight metal Vapor-Vacuum™ Twist-Off™ caps (White Cap International, U.S.A.). These caps had previously been pierced and the 5 mm holes were sealed with silicone rubbers (diameter

10 mm, thickness 3 mm), glued to the inner side, to allow headspace sampling with a gastight syringe. The jars were incubated in a water bath for 7 h at 42°C or 45°C, chilled in ice water to 7°C and stored at 7°C in darkness or illuminated (TL FTD 33, Philips, Eindhoven, The Netherlands) with a light intensity of approximately 500 lux, corresponding to conditions prevailing in stores. Uninoculated, but acidified (0.4 % (w/w) lactic acid) control samples were handled according to the same procedure.

GC Dynamic headspace volatile analysis

Glass tubes of 10 cm x 6 mm o.d. (Interscience B.V., Breda, The Netherlands) containing 0.1 g Tenax TA (Alltech Nederland, Zwijndrecht, The Netherlands), positioned with DMCS treated glass wool were conditioned (4 h, 250°C, nitrogen purging 2.5 ml/min).

For the collection of volatiles a gas purging system was used as presented in Bonestroo *et al.* (1992b). Samples of salads (approximately 100 g) were homogenized under nitrogen with approximately 200 ml of Nanopure water (Barnstead, Dubuque, Iowa, U.S.A.), filled into the flask (approximately 250 g) and purged at 7°C with molecular sieve purified nitrogen gas for approximately 60 min (100 ml/min) whilst the sample was stirred with a magnetic stirrer.

Trapped volatiles were analyzed on a gas chromatograph (HRGC 5300 MEGA, Carlo Erba Strumentazione, Rodano, Milan, Italy) with a flame ionization detector at 270°C, a 60 m x 0.25 mm i.d. Supelcowax 10 (0.25 μm Carbowax 20M) capillary column (Chrompack B.V., Middelburg, The Netherlands) with helium carrier gas at 150 kPa and a thermodesorption and cold trap (TCT) unit (TDAS 5000). The column was used at 40°C for 4 min, heated to 92°C at a rate of 2°C/min, finally to 272°C at a rate of 6°C/min and subsequently held at this temperature for 10 min. Components were identified by GC-MS under identical conditions with an VG MM7070F mass spectrometer working in the 70 eV EI ionization mode (VG, Altrincham, U.K.).

Measurement of headspace oxygen

Headspace samples (100 μl) were taken with a gastight syringe and analyzed in a gas

chromatograph (Model 433, Packard-Becker B.V., Delft, The Netherlands) with a thermal conductivity detector at 125°C and injector at 110°C. Parallel coupled columns, split 1:1; 1.5 m x 3.2 mm i.d. teflon column packed with Chromosorb 108 (60-80 mesh) and a 1.2 m x 3.2 mm i.d. stainless steel column packed with Molecular Sieve 5 A (60-80 mesh) at 40°C with helium as carrier gas (180 kPa).

Measurement of oxygen profiles in salads

Oxygen profiles in fermented salads were measured with laboratory-made Pt/Au microelectrodes sheathed in 10-cm long stainless steel syringe needles (Gemerden *et al.*, 1989). Profiles were recorded during the stepwise lowering of the electrodes into the fermented salads. Micro-electrode and reference electrode (Hg/HgCl, B161, Schott Geräte GmbH, Hofheim a. Ts., F.R.G.) were coupled to a sensitive ampèremeter (Keithley 485 picoammeter, Keithley Instruments B.V., Gorinchem, The Netherlands) adapted for oxygen estimation with a 750 mV polarization voltage. The oxygen electrodes (sensing tip approximately 40 μm) were calibrated at 7°C under non-stirred conditions in air-saturated 1.5 % (w/w) NaCl-solutions (=100%) and the same solutions after flushing with nitrogen gas for at least 20 min (=0 %). Typical electrode readings were 38 and 4 nAmp for 100 % and 0 % saturation, respectively. In practice, the lower limit of detection was 1.5 % air saturation, which is equivalent to a dissolved oxygen concentration of 5.3 μM.

Evaluation of the quality of fermented salads

- *measurement of pH.* The pH of the potato salads was determined with a pH-electrode (N61, Schott) coupled to a combined pH/mV-meter (pH522, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, F.R.G.).

- *Microbiological quality.* The salads were analyzed microbiologically during fermentation, directly after 7 h fermentation and at intervals for up to six weeks of storage at 7°C. Mesophilic aerobic spoilage bacteria were counted on pour-plates of Gelysate Agar (GEL): 5 g Bacto-peptone (Difco, Detroit, Michigan, U.S.A.), 5 g sodium chloride (Merck) and 14 g agar (L13, Oxoid) per l, pH 7.6 $\pm$ 0.1, incubated at 30°C for 3 days; *Enterobacteriaceae*

on pour-plates of Violet Red Bile Glucose Agar (VRBG; CM485, Oxoid) with a top layer of the same medium and incubation for 24 hours at 30°C; lactic acid bacteria on pour-plates of MRS medium (Merck) with 12 g of agar (L13, Oxoid) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l, incubated at 30°C for 3 days; yeasts and filamentous fungi on pour-plates of Oxytetracycline Glucose Yeast Agar (OGGA; CM545, Oxoid), incubated at 25°C for 5 days and *Pseudomonas* species on pseudomonas agar base (PSA, CM559, Oxoid), supplemented with *Pseudomonas* C-F-C supplement (SR103, Oxoid), incubated at 30°C for 2 days.

- *Determination of sugars, ethanol and organic acids.* Sugars, ethanol and organic acids in the potato salads were determined by HPLC as described earlier (Bonestroo *et al.*, 1992a).

6.3. RESULTS

Table 6.3.1. shows the oxygen profiles of potato salads, along with some other analytical data. During incubation at 42°C for 7 hours, the oxygen concentration in the fermented salads, as well as in the uninoculated, acidified control salad, decreased. The decrease in oxygen concentration in fermented salads, however, was more pronounced. At the surface of all salads, except the control salad, a minor decrease of the oxygen concentration occurred. During storage at 7°C for 10 days the oxygen concentrations in all salads increased. At the surface of the salads an increase towards the original oxygen saturation level was observed. Starter cultures, inoculated at 2.1×10^7, 2.2×10^7, 4.5×10^7, 2.3×10^7, 3.6×10^7 and 3.2×10^6 cfu/g salad, for starter no. 1, 9, 13, 18, 23 and 41, respectively, showed good growth and souring. During fermentation the number of generations were 5.9, 4.7, 3.8, 3.3, 4.6, and 8.6, respectively, while pH values $\leq$ 4.78 were attained. The amount of lactic acid produced in the fermented salads was 1.3 to 2.4 g/kg salad, after fermentation and subsequent storage at 7°C for 1 day, increasing to 3.1 g/kg for salads fermented with strains no. 1 and 41, or 4.0 to 4.6 g/kg for strains no. 9, 13, 18 and 23, respectively, after storage at 7°C for 10 days. This increase was accompanied by a further decrease of pH to below 4.22 or 3.91 for salads fermented with *Lactobacillus acidophilus* and *Lactobacillus plantarum*, respectively. In salads fermented with *L. acidophilus* strain no. 41 relatively large amounts of acetic acid (1.2 g/kg salad) were found

after fermentation and subsequent storage at 7°C for 1 day, increasing to 1.4 g/kg salad after storage at 7°C for 10 days.

Table 6.3.1. Oxygen profiles in potato salads exposed to light during storage at 7°C for 10 days (series no. 1).

Sample	Time	pH	Lactic acid (g/kg salad)	Acetic acid (g/kg salad)	Oxygen concentration (mg/kg salad) [a] at different measuring points			
					MP 1	MP 2	MP 3	MP 4
Control	A [b]	5.97	0.2	0.2	9.37	8.16	8.06	8.35
	B	4.00	3.2	0.2	10.49	5.74	4.89	5.88
	C	3.94	3.8	0.2	10.51	7.86	6.02	5.19
No. 1 [c]	A	5.97	n.d. [d]	n.d.	9.19	9.37	10.18	8.23
	B	4.56	1.9	0.4	5.36	1.50	1.19	1.24
	C	4.12	3.1	0.8	13.97	5.03	4.56	4.24
No. 9	A	5.97	n.d.	n.d.	8.39	8.28	8.59	9.87
	B	4.38	2.2	0.3	6.04	1.5	1.48	1.24
	C	3.89	4.2	0.4	10.66	7.23	6.29	5.97
No. 13	A	5.97	n.d.	n.d.	10.46	10.54	10.46	9.54
	B	4.37	2.2	0.3	6.04	1.91	1.48	1.40
	C	3.87	4.5	0.3	11.52	7.86	6.44	5.81
No. 18	A	5.97	n.d.	n.d.	9.81	8.63	8.16	8.07
	B	4.78	1.3	0.3	6.38	2.73	1.93	2.17
	C	3.91	4.0	0.4	8.64	7.23	5.50	4.71
No. 23	A	5.97	n.d.	n.d.	10.46	8.87	10.97	8.07
	B	4.23	2.4	0.3	8.09	1.77	1.34	1.24
	C	3.76	4.6	0.3	11.81	7.54	6.60	6.13
No. 41	A	5.97	n.d.	n.d.	12.42	9.11	11.22	9.78
	B	4.60	2.3	1.2	2.51	1.36	1.19	1.24
	C	4.22	3.1	1.4	10.37	5.19	4.87	4.56

[a] Oxygen concentration in potato salads; MP 1 = at surface of salad; MP 2 = approx. 2 cm below surface of salad; MP 3 = approx. 1 cm above bottom of jar; MP 4 = at bottom of jar.

[b] A = before fermentation; B = after fermentation for 7 hours at 42°C and subsequent storage at 7°C for 1 day; C = after fermentation and subsequent storage at 7°C for 10 days.

[c] Strain numbers 1 and 41: *Lactobacillus acidophilus*; all other strains: *Lactobacillus plantarum*. No. 18 corresponds with *L. plantarum* ATCC 8014.

[d] n.d. = not determined

At this time the acetic acid content of the salad fermented with *L. acidophilus* no. 1 was 0.8 g/kg. All other salads contained acetic acid in minor quantities (0.2 to 0.4 g/kg salad). During the course of the experiments no growth of *Enterobacteriaceae* and *Pseudomonas*

spp. was observed in any salad. Growth of yeasts was inhibited in the fermented salads, but not in the uninoculated, acidified control salad in which large numbers of yeasts ($\geq 10^4$ cfu/g) occurred after 10 days of storage at 7°C.

To study the oxygen consuming properties in more detail, potato salads were inoculated with various *L. acidophilus* strains and incubated at 45°C. As can be seen from Table 6.3.2., the oxygen concentration in the fermented salads, as well as in the uninoculated, acidified control salad decreased. The decrease in oxygen concentration in fermented salads, however, was again more pronounced. At the surface of all salads, except the control salad, a minor decrease of the oxygen concentration occurred. During storage at 7°C for 2 days the oxygen concentrations in the upper layers of all salads increased, while the concentration in the bottom layers of most fermented salads further decreased. At the surface of all salads an increase towards the original oxygen saturation level was observed.

When salads, made with sliced potatoes, were fermented at 45°C for 7 hours with *L. acidophilus* strains, a comparable pattern of oxygen consumption was attained (Table 6.3.3.). During storage at 7°C for 10 days the oxygen concentrations in the upper layers of most salads increased. At the bottom layer of all salads, however, a further decrease in oxygen concentrations was observed, with minor variations amongst strains. Starter cultures, inoculated at 3.9×10^6, 6.2×10^6, 4.2×10^6, 1.7×10^6, 3.6×10^6 and 3.9×10^6 cfu/g salad, for starters no. 4, 25, 39, 45 and 48, respectively, showed good growth and souring. During fermentation the number of generations were 4.6, 4.6, 4.5, 3.2 and 4.3, respectively, while pH values ≤ 4.15 were attained. The amount of lactic acid produced in the fermented salads was 3.2 to 4.0 g/kg salad, after fermentation and subsequent storage at 7°C for 1 day. Minor amounts of acetic acid were found (≤ 0.5 g/kg salad). No major changes in pH and concentration of lactic and acetic acids occurred during storage at 7°C for 10 days. During the course of the experiments no growth of *Enterobacteriaceae*, yeasts and *Pseudomonas* spp. was observed in any salad.

Oxygen in the headspace was analyzed by gas chromatography. Fermented potato salads (series no. 1) showed a higher consumption of oxygen from the headspace than uninoculated, acidified control salads (Table 6.3.4.). Significant differences between the

oxygen consumption level in potato salads fermented with different starter cultures could be

Table 6.3.2. Oxygen profiles in potato salads stored at 7°C in darkness (series no. 2).

Sample	Time	pH	Oxygen concentration (mg/kg salad) [a] at different measuring points			
			MP 1	MP 2	MP 3	MP 4
Control	A [b]	3.67	9.10	9.13	6.31	8.65
	B	3.72	8.63	8.28	5.99	5.32
	C	3.92	8.87	6.56	4.41	4.33
	D	3.91	9.13	7.16	4.98	5.88
No. 4 [c]	A	5.69	8.15	5.13	6.94	7.32
	B	5.56	6.23	4.28	3.78	4.66
	C	4.65	5.51	3.14	2.21	3.00
	D	4.29	8.68	5.57	2.05	1.24
No. 25	A	5.79	6.23	6.28	5.04	7.32
	B	5.79	6.23	3.99	4.10	5.00
	C	4.81	4.07	2.57	2.52	1.66
	D	4.34	8.68	4.24	2.05	1.24
No. 39	A	5.74	6.23	4.56	6.94	6.66
	B	5.81	6.71	5.13	4.41	4.99
	C	4.83	4.55	3.14	2.21	2.33
	D	4.39	9.13	5.04	2.05	1.24
No. 45	A	5.80	6.23	6.85	5.04	4.66
	B	5.80	4.55	4.56	3.15	5.32
	C	4.95	6.23	1.71	1.26	1.66
	D	4.33	7.79	4.77	1.76	1.55
No. 48	A	5.74	6.23	6.85	6.94	7.99
	B	5.67	5.51	3.99	5.05	4.66
	C	4.87	5.27	2.28	1.26	1.00
	D	4.25	9.35	3.98	2.05	1.55

[a] Oxygen concentration in potato salads; MP 1 = at surface of salad; MP 2 = approx. 2 cm below surface of salad; MP 3 = approx. 1 cm above bottom jar; MP 4 = at bottom of jar.

[b] A = before fermentation; B = after fermentation for 2 hours at 45°C; C = after fermentation for 5 hours at 45°C; D = after fermentation for 7 hours and subsequent storage at 7°C for 2 days.

[c] All strains were *Lactobacillus acidophilus*.

detected. Highest consumption was seen in salads fermented with *L. acidophilus* no. 1 and 41. In series no. 3, however, differences between oxygen consumption level in control salad

and fermented salads were less pronounced. Also, smaller differences amongst different starter cultures occurred.

Table 6.3.3. Oxygen profiles in potato salads exposed to light during storage at 7°C for 10 days (series no. 3).

Sample	Time	pH	Lactic acid (g/kg salad)	Acetic acid (g/kg salad)	Oxygen concentration (mg/kg salad) [a] at different measuring points			
					MP 1	MP 2	MP 3	MP 4
Control	A [b]	6.48	0.2	0.2	8.49	6.74	7.86	9.43
	B	3.78	5.0	0.3	10.40	6.49	5.57	7.11
	C	3.64	5.5	0.3	13.55	7.51	3.68	2.81
No. 4 [c]	A	6.48	n.d. [d]	n.d.	8.49	6.74	7.86	9.43
	B	4.15	3.5	0.3	6.29	3.09	2.34	2.47
	C	4.06	4.0	0.3	5.17	3.64	2.01	1.68
No. 25	A	6.48	n.d.	n.d.	8.49	6.74	7.86	9.43
	B	4.06	4.0	0.5	4.84	3.09	2.34	2.47
	C	4.01	3.6	0.5	9.83	3.98	1.79	1.80
No. 39	A	6.48	n.d.	n.d.	8.49	6.74	7.86	9.43
	B	4.13	3.4	0.3	8.47	3.40	2.05	2.16
	C	4.07	3.7	0.3	8.61	3.76	1.76	1.30
No. 45	A	6.48	n.d.	n.d.	8.49	6.74	7.86	9.43
	B	4.12	3.4	0.3	8.47	4.02	2.64	1.86
	C	4.10	3.3	0.3	6.04	3.85	1.79	1.42
No. 48	A	6.48	n.d.	n.d.	8.49	6.74	7.86	9.43
	B	4.15	3.2	0.3	10.65	4.12	2.54	2.47
	C	4.06	3.6	0.3	7.01	3.80	1.89	1.47

[a] Oxygen concentration in potato salads; MP 1 = at surface of salad; MP 2 = approx. 2 cm below surface of salad; MP 3 = approx. 1 cm above bottom jar; MP 4 = at bottom of jar.

[b] A = before fermentation; B = after fermentation at 45°C for 7 hours and subsequent storage at 7°C for 1 day; C = after fermentation and subsequent storage at 7°C for 10 days.

[c] All strains were *Lactobacillus acidophilus*.

[d] n.d. = not determined

With dynamic headspace gas-chromatographic methods it was found that fermented potato salads, subjected to light exposure for 10 days, differed from uninoculated, acidified control salads. Fermented salads generally contained lower amounts of lipid oxidation products, indicating a lower level or possibly the absence of lipid oxidation. These findings correlated with flavour score determinations, which were difficult to assess, possibly because

of the specific taste characteristics of the major acidulant, i.e. lactic acid. Significant differences between the level of oxidation in potato salads fermented with various starter cultures could be detected with gas-chromatographic methods. In Fig. 6.3.1. some typical chromatograms with headspace volatiles from differently treated potato salads are shown. The main compounds identified, are listed in the order of elution on Carbowax 20M (Table 6.3.5.). Identification of the different peaks was accomplished on the basis of mass spectral and retention time data. The major component of the headspace concentrates from oxidized potato salads was hexanal. This component might be a good indicator of oxidative changes that occur in salads exposed to light. In fermented potato salads of series no. 1, illuminated during storage, the relative peak areas of hexanal, compared to the corresponding area of the illuminated, uninoculated, acidified control salad (100%), were 90 %, 134 %, 144 %, 93 %, 52 % and 61 %, for strain numbers 1, 9, 13, 18, 23 and 41, respectively. In series no. 3 the relative peak areas of hexanal, compared to the corresponding area of the illuminated, uninoculated, acidified control salad (100%), were 31 %, 39 %, 8 %, 32 %, 26 % and 31 %, for an uninoculated, acidified control salad stored in darkness and salads fermented with *L. acidophilus* strain numbers 4, 25, 39, 45 and 48, respectively. In all salads the relative peak areas of other less-volatile oxidation products, such as 2-heptenal, nonanal and decanal, were at most 8.2 %, 12.2 % and 3.8 %, respectively, compared to the peak area of hexanal in the illuminated, uninoculated, acidified control salad.

6.4. DISCUSSION

Tables 6.3.1. to 6.3.3. show that lactic acid bacteria used as starters in salad fermentation are able to decrease the oxygen tension in the salads. These observations are in agreement with literature reports concerning oxygen consuming reactions of lactic acid bacteria in general (Condon, 1987; Borch and Molin, 1989; Piard and Desmazeaud, 1991) or specific strains, amongst which *L. plantarum* ATCC 8014 (Götz *et al.*, 1980a,b; Murphy and Condon, 1984a,b). The starter cultures used showed different oxygen consuming capacities. However, differences in comparable products from the same starter that have been cultured on different occasions were observed (tables 6.3.2. and 6.3.3.). As Langeveld and Bolle (1985) remarked earlier, it is expected that in practice this variation between successive productions will be smaller if the technique of culturing is maintained as uniform as possible.

Moreover, this also holds for the salad composition. The decrease of the dissolved oxygen tension in the food corresponds with a decrease of the oxygen tension in the headspace (Table 6.3.4.), as oxygen diffuses from the headspace into the salad.

The decrease of the oxygen tension in foods or in the headspaces thereof could be indicative for the microbial quality of these foods. Several food poisoning organisms, such as *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus cereus* are able to consume oxygen from the headspace of foods (Mattila and Ahvenainen, 1989). Psychrotrophic populations (amongst others *Pseudomonas* spp.) exceeding 5 x 10^6 cfu/ml caused a large fall (from 89 % to 10 % saturation) in the oxygen tension of raw milk incubated in a fermenter with constant aeration and agitation at 7°C after 1 day (Rowe and Gilmour , 1986).

Yeasts are also able to consume oxygen. However, Langeveld and Bolle (1989) concluded that if the concentration of cells of *Geotrichum candidum* and *Pichia fermentans* in pasteurized cultured milk was less than 10^5 cfu/ml, the oxygen tension did not decrease to less than 85 % of the initial level. During the course of our experiments, no growth of *Enterobacteriaceae*, yeasts and *Pseudomonas* spp. was observed in any fermented salad. Moreover, earlier studies showed that growth of spoilage and pathogenic microorganisms, such as *B. cereus* and *S. aureus* was inhibited in fermented salads (Bonestroo *et al.*, 1992d). Therefore, the decrease of the oxygen tension in fermented salads must be due to the oxygen consuming abilities of the lactic acid bacteria used as starters.

The fate of oxygen

Enzymes catalysing reactions with O_2, such as NADH oxidases and pyruvate oxidase, all produce H_2O_2 or H_2O as end products. H_2O_2, an inhibitory compound, can be further reduced to H_2O by NADH peroxidases (Condon, 1987; Piard and Desmazeaud, 1991). However, in broth cultures of various aerobically grown lactic acid bacteria, production of hydrogen peroxide could not be detected (Bonestroo *et al.*, 1992c). A reason for this could be that the NADH oxidase and the NADH peroxidase might exist as a complex in the cells of the lactic acid bacteria tested and that H_2O_2 formed in the oxidase reaction is reduced to H_2O by the peroxidase, with very little H_2O_2 released from the complex, as was suggested by Götz *et al.* (1980a) for *L. plantarum* ATCC 8014.

Table 6.3.4. Oxygen uptake during storage at 7°C from the headspace of uninoculated, acidified control salads and fermented potato salads exposed to light for up to 10 days.

Series no. 1				Series no. 3			
Sample	Storage time (days)	% Oxygen in headspace	Oxygen uptake [a] from the headspace (mg/kg salad)	Sample	Storage time (days)	% Oxygen in headspace	Oxygen uptake [a] from the headspace (mg/kg salad)
Control	1	20.9	- [b]	Control	1	21.0	- [b]
	10	20.7	-		10	18.6	22.4
No. 1 [c]	1	17.4	19.4	No. 4 [c]	1	20.1	2.8
	10	7.0	101.0		10	18.1	28.8
No. 9	1	n.d. [d]	n.d. [d]	No. 25	1	17.0	43.4
	10	13.1	54.7		10	16.0	55.9
No. 13	1	20.5	-	No. 39	1	19.9	5.6
	10	14.2	45.8		10	19.1	16.5
No. 18	1	20.4	-	No. 45	1	17.5	36.1
	10	15.0	40.1		10	17.6	34.6
No. 23	1	19.9	0.5	No. 48	1	20.1	3.7
	10	14.6	42.8		10	18.1	29.3
No. 41	1	15.6	33.7				
	10	4.7	119.7				

[a] Oxygen uptake calculated according to equations derived from Langeveld and Bolle, 1985.

[b] - = ≤ 1.0 mg/kg salad.

[c] Strain numbers 13, 18 and 23 were *Lactobacillus plantarum*; all other strains were *Lactobacillus acidophilus*. No. 18 corresponds with *L. plantarum* ATCC 8014.

[d] n.d. = not determined

Table 6.3.5. Volatile compounds identified from the headspace of potato salads exposed to light during storage at 7°C.

Peak [a]	Retention time (min)	Compound Name [b]
1	7.84	nC8
2	8.17	aceton
3	12.10	ethylacetaat
4	12.29	2-methyl-2-propanal
5	12.42	ethanol
6	14.08	2-pentanon
7	14.80	3-methyl-3-buteen-2-on
8	16.42	1-penten-2-on
9	19.20	2-methyl-1-penten-3-on
10	20.28	hexanal
11	24.00	1-butanol
12	27.38	2-heptanon
13	36.59	octanal
14	39.76	octa-1,5-dien-3-ol
15	44.83	2-heptenal
16	48.69	nonanal
17	56.49	decanal

[a] Refers to peak number in Fig. 6.3.1.
[b] Identification based on MS data only

As can be seen from tables 6.3.1. and 6.3.3. lactic acid was the major acid produced in the fermented salads. In salads fermented with *L. plantarum* generally low amounts of acetic acid were found. ($\leq$ 0.5 g/kg salad). No major changes in this pattern occurred during storage at 7°C for 10 days. According to Condon (1987) *L. plantarum* produces almost exclusively D- and L- lactates when growing aerobically in glucose broth. The cells then have low levels of lactate oxidizing enzymes. When the glucose level decreases the lactate oxidation pathway is derepressed and acetate rather than lactate accumulates. When glucose is exhausted, lactate is converted to acetate and *L. plantarum* continues to grow slowly when O_2 is available, but not in its absence (Murphy and Condon, 1984a,b). This could be an

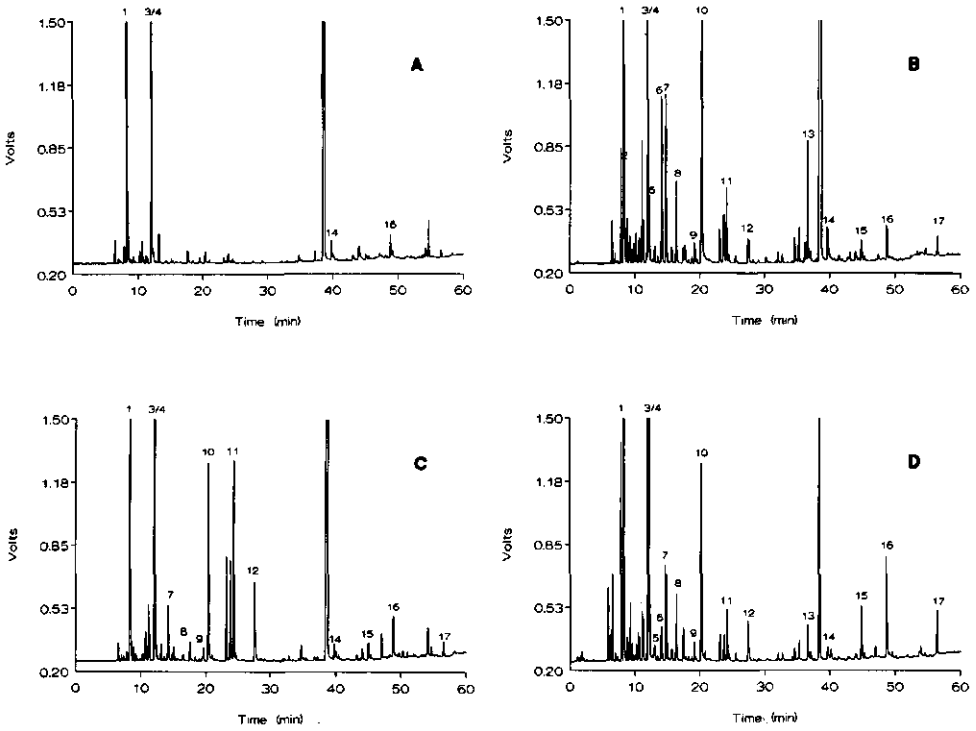

Fig. 6.3.1. Profiles of volatiles of potato salads. A, uninoculated, acidified potato salad before the start of the experiment; B, uninoculated, acidified potato salad exposed to light (approx. 500 lux) during storage at 7°C for 10 days; C, uninoculated, acidified potato salad stored at 7°C in darkness for 10 days; D, fermented with *Lactobacillus acidophilus* no. 39, exposed to light (approx. 500 lux) during storage at 7°C for 10 days. Peak numbers refer to components listed in Table 6.3.5.

explanation for the low amounts of acetic acid found in salads fermented with *L. plantarum*, as the glucose concentrations found in fermented salads after 10 days of storage at 7°C are rather high, i.e. > 6 g/kg salad , and growth of starter cultures generally ceased during cold storage. In salads fermented with *L. acidophilus* no. 1, 25 and 41 significant higher amounts of acetic acid are produced (tables 6.3.1. and 6.3.3.). This accumulation of acetate corresponds with a significant higher O_2-uptake from the headspace (Table 6.3.4.). Apparently, in these homofermentative *L. acidophilus* strains, the lactate oxidation pathway is not or not as completely repressed in the presence of relatively high amounts of glucose.

Lipid oxidation

As stated earlier, upon light exposure for 10 days fermented salads generally contained lower amounts of lipid oxidation products than acidified control salads, indicating a lower level of lipid oxidation. In fermented potato salads of series no. 1, illuminated during storage, the relative peak areas of hexanal, compared to the corresponding area of the illuminated, uninoculated, acidified control salad (100%), were higher than 100 % for strain numbers 9 and 13. This might have been caused by yeast growth in the uninoculated, acidified control salad, since yeasts are able to reduce or oxidize hexanal (Nomura *et al.*, 1991). In uninoculated, acidified potato salads, which were stored in darkness, low levels of lipid oxidation products were found, comparable to the levels found in fermented potato salads. These lipid oxidation products were absent in control salads before fermentation and probably originated from autoxidation rather than from photo-oxidation (Bonestroo *et al.*, 1992b). Therefore, it seems reasonable to conclude that fermentation of potato salads inhibits the onset of photo-oxidation. Autoxidation in potato salads can be minimized by selection of good quality ingredients and especially by proper potato handling (Hallberg, 1990; Hallberg and Lingnert, 1991).

There are two pathways in the photo-oxidation of lipids: type I (photosensitized oxidation) and type II (singlet oxygen oxidation) (Chan, 1977). The rate of the type II reaction depends mainly on the solubility and concentration of oxygen present in the food system (Bradley and Min, 1992). Therefore, we assume that oxygen consumption by starter cultures could be responsible for the protecting effect of fermentation on salads towards lipid

photo-oxidation. Singlet oxygen oxidation of unsaturated oils is also minimized by compounds that quench singlet oxygen, such as ascorbic acid and carotenoids (Bradley and Min, 1992). However, this quenching effect probably did not occur in the experiments with potato salads, as uninoculated, acidified control salads were not protected from photo-oxidation. Another possible explanation for the lower level or absence of lipid oxidation in fermented salads could be the reduction of aldehydes to their corresponding alcohols by alcohol dehydrogenases present in many lactic acid bacteria. However, the lower levels of hexanal in fermented potato salads did not correspond with higher levels of hexanol (Fig. 6.3.1. and Table 6.3.5.).

As stated earlier, we assume that oxygen consumption by starter cultures could be responsible for the protecting effect of fermentation on salads towards lipid oxidation. A direct relationship between the amount of oxygen in potato salads and the level of lipid oxidation is, however, not easily demonstrable, since it is difficult to attain different concentrations of oxygen in uninoculated, acidified control salads. The oxygen solubility in water is very low compared to the solubility in vegetable oils (Rols and Goma, 1991). The oxygen concentration in the sauce can be drastically reduced by purging the oil with nitrogen before homogenization of the sauce. However, for the maintenance of these conditions in whole salads, preparation should be performed in closed systems with rigorous exclusion of oxygen, which was not possible under laboratory conditions. Further investigations are thus necessary.

The concentration of oxygen in the salads is not only influenced by the oxygen consumption by the lactic starters in the salads, but also by the accessibility of oxygen. The latter depends on the amount of oxygen in the headspace and the diffusion through packing materials, other than glass. Flushing the headspace with nitrogen or mixtures of nitrogen and carbon dioxide probably would increase the chemical stability of the product.

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INHIBITION OF THE FORMATION OF BIOGENIC AMINES IN FERMENTED SALADS

ABSTRACT

The formation of biogenic amines in fermented salads was studied by assessing the decarboxylative properties of starter bacteria and by performing experiments in which salads were deliberately contaminated with amino acid decarboxylase positive *Enterobacteriaceae* and then fermented. To create conditions suitable for the production of biogenic amines, these salads were spiked with free amino acids and the cofactor pyridoxal phosphate. Of the 191 lactic acid bacteria tested, only 11 strains possessed decarboxylative properties. Tyramine production was most frequently encountered. Some of these strains also produced phenylethylamine, however, in relatively small amounts, compared to tyramine. Only a few strains produced putrescine or cadaverine. In leek-cabbage-ham salads, inoculated with *Hafnia alvei* and *Escherichia coli* (inoculation level approximately 100 cfu/g), free amino acids (approximately 1 g/kg) and cofactor pyridoxal phosphate (5 mg/kg), fermented with an amino acid decarboxylase negative starter culture (*Lactobacillus plantarum*) and stored for 4 weeks at 7°C, no biogenic amines were produced. In fermented salads, the occurrence of biogenic amines can be prevented by assuring a low initial contamination, proper hygienic care during processing, and the use of amino acid decarboxylase negative starter cultures which actively suppress non-starter microorganisms.

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7.1. INTRODUCTION

Biogenic amines are organic bases of low molecular weight, possessing biological activity, which can be formed and degraded during the normal metabolism of animals, plants and microorganisms. In foods, biogenic amines usually are generated by microbial decarboxylation of amino acids (Askar and Treptow, 1986). According to Joosten and Stadhouders (1987) the factors that govern the formation of amines include: (a) the availability of free amino acids, (b) the presence of microorganisms that can decarboxylate these amino acids, and (c) favourable conditions for the growth of these microorganisms and for the production and activity of decarboxylase enzymes. Although biogenic amines such as histamine, are needed for critical functions in man and animals (Brink *et al.*, 1990), consumption of food containing high amounts of these amines can have toxicological effects.

In a previous paper (Bonestroo *et al.*, 1992a) lactic acid fermentation was proposed as a novel way to prepare salads. A process was developed in which salads, composed of vegetables and/or meat in an oil-in-water emulsion, were inoculated with starter cultures and fermented. A fermentation temperature above 40°C and an inoculation level of approximately 10^6 to 10^7 cfu of lactic acid bacteria/g, were chosen to give the starter organisms an advantage over possible spoilage organisms, i.e. lactobacilli, yeasts and *Enterobacteriaceae*. Especially during the early stages of fermentation these microorganisms could flourish. Several authors reported about decarboxylative properties of these non-starter bacteria. With reference to the prerequisites mentioned earlier, the formation of biogenic amines during fermentation and storage of salads should therefore be considered.

The primary aim of the experiments was to find out whether starter bacteria can generate biogenic amines in fermented salads. The formation of biogenic amines by non-starter bacteria was studied by performing experiments in which salads were deliberately contaminated with amino acid decarboxylase positive *Enterobacteriaceae* and then fermented. To create conditions suitable for the production of biogenic amines, these salads were spiked with free amino acids and pyridoxal phosphate, the latter compound being a cofactor in enzymic amino acid decarboxylation.

7.2. MATERIALS AND METHODS

Chemicals

Tyrosine (TYR), phenylalanine (PHE), lysine (LYS), tryptophan (TRP) and ornithine (ORN), cadaverine (CAD), histamine (HA), phenylethylamine (PHEA), putrescine (PUT) and tyramine (TA) were obtained from Janssen Chimica (Tilburg, The Netherlands), histidine (HIS), arginine (ARG), tryptamine (TPA), ninhydrin, hydrindantin and pyridoxal phosphate from Merck (Darmstadt, F.R.G.), benzylamine (BA) from Fluka (Buchs, Switzerland) and sodium dodecyl sulphate (SDS) from Bio-Rad Labs. (Richmond, CA, U.S.A.). All other reagents were obtained from Merck.

Organisms and culture conditions

The lactic acid bacteria which were used in this study were obtained from our laboratory collection (Bonestroo *et al.*, 1992a). Prior to use the strains were cultivated for 24 hours at 30°C or 42°C in a broth with the same composition as MRS broth (Merck, Darmstadt, F.R.G.), but containing sucrose (Merck) as carbohydrate, instead of glucose, followed by subsequent cultivation in the same broth for 16 hours at 30°C or 42°C. Strains of *Escherichia coli* ATCC 11229 and 11289 and *Hafnia alvei* strains 1 and 2 were obtained from our laboratory collection. *E. coli* strains no. 4,8,12 and 15 were obtained from Mr. H.A.P. Urlings (State University, Utrecht, The Netherlands). Prior to use these strains were cultivated twice at 30°C or 37°C, first for 24 hours and then for 16 hours, in Brain Heart Infusion Broth (Gibco Ltd., Paisley, Scotland, UK).

Determination of decarboxylative properties

The formation of amines was determined in modified Møller medium (Joosten and Northolt, 1987). Growth of the tested strain in the basal medium (without amino acid) was indicated by a yellow colour and slight gas accumulation in Durham tubes. These phenomena were also seen in the medium with the amino acid in question, when no decarboxylase was formed. When a certain strain did possess decarboxylase activity, the colour after incubation

was purple, while generally larger amounts of gas were accumulated. However, a strain was assumed to be decarboxylase-positive only if the HPLC analysis of the growth medium also gave evidence of amine formation. The sample clean-up consisted of adding 5 ml of 0.6 M trichloroacetic acid to an equal volume of growth medium. The mixture was centrifuged for 10 min at 10000 *g* and 4°C in a Sorvall SB-50 centrifuge and the supernatant was filtered through a 0.45 μm Nylaflow filter (Gelman Sciences, Ann Arbor, Michigan, U.S.A.).

Preparation and storage of salads

Two types of salads were prepared, one containing 23 % (w/w) shredded (10x6x3 mm) leek, 23 % (w/w) shredded (15x10x3 mm) cabbage, 10 % (w/w) cooked ham and 44 % sauce; the other containing 25 % (w/w) cut (12x12x12 mm) and cooked chicken meat and 75 % (w/w) sauce. The sauce was composed of water (35.9-53.2 % [w/w]), soybean oil (35-50 % [w/w]), sucrose (5.3-6 % [w/w]), salt (0.7-1.8 % [w/w]), egg yolk (3-3.5 % [w/w]) and thickening agents (2.8 % [w/w]). Occasionally small amounts of seasonings were added to the salads. The salads were inoculated with 10^6 to 10^7 cfu of lactic acid bacteria per g and filled in glass jars (100 g salad, headspace approximately 50 ml) air-tightly sealed with metal Vapor-Vacuum™ Twist-Off™ caps (White Cap International, U.S.A.). The jars were incubated in a waterbath for 7 hours at 42°C or 45°C, and then chilled in ice water to a temperature of 7°C and stored at this temperature. Uninoculated, acidified (0.4 % (w/w) lactic acid) salads were used as control.

Determination of biogenic amines

Biogenic amines in Mφller media and chicken salads were determined with an HPLC method based on the method of Joosten and Olieman (1986). The HPLC system used consisted of a Spectra Physics model 3500 pump (Spectra Physics Inc., San Jose, C.A., U.S.A.), a Spectroflow 773 UV-detector (Kratos Analytical Instruments, Westwood, New Yersey, U.S.A.) operated at 546 nm in the 0.25 AUFS range and a chart recorder (Kipp en Zonen, Delft, The Netherlands). A Pharmacia 125 mm x 4.0 mm I.D. column (Pharmacia Nederland B.V., Woerden, The Netherlands) packed with Sherisorb ODS 2 (3μm) was used. The guard column was filled with the same material. The column temperature was

maintained at 29°C. The flow rate of the mobile phase was 0.3 ml/min. The reaction coil consisted of Teflon tubing (0.3 mm I.D., volume approx. 0.2 ml, Inacom Instruments B.V., Veenendaal, The Netherlands) knitted in the form of a twisted figure eight and heated in an oil-bath to 145°C. To prevent bubble formation the mobile phase was carefully deaerated and a back-pressure was applied to the heating coil by positioning the outlet at approximately 1 m above the detector. For the analysis of salads, decimal dilutions in 0.07 M trisodium citrate solution (45°C) were prepared, followed by homogenisation with a Seward 400 stomacher (Laméris, Breukelen, The Netherlands). A portion (3 ml) of this suspension was mixed with 3 ml of 0.6 M trichloroacetic acid (TCA) and centrifuged for 10 min at 10.000 g and 4°C in a Sorvall centrifuge. The resulting pellet was resuspended in 3 ml of 0.3 M TCA and centrifuged. The combined supernatants were filtered through a 0.45 μm Nylaflow filter. Samples were stored below -18°C prior to analysis. The method permitted a detection limit for each of the amines of 5 mg/kg salad.

Biogenic amines in leek-cabbage-ham-salads were determined by ion exchange chromatography on an automated amino acid analyzer according to Villanueva and Adlakha (1978). The cationic resin was of the Dionex DC-6A type (Dionex B.V., Breda, The Netherlands). Benzylamine was used as internal standard.

Amino acid analysis

The concentration of individual free amino acids was determined according to the method of Spackman *et al.* (1958) with an amino acid analyzer equipped with a single column as described by Benson (1973). Sample clean up was performed in the same way as for the determination of biogenic amines. Prior to injection the pH of the extract was adjusted to 2.0 with 4N NaOH and norleucine was added as internal standard.

Evaluation of the quality of fermented salads

- *Microbiological quality.* The vegetable salads were judged on the basis of microbiological analyses up to six weeks of storage at 7°C. Mesophilic aerobic spoilage bacteria were enumerated using pour-plates of Gelysate Agar (GEL): 5 g Bacto-peptone (Difco, Detroit,

Michigan, U.S.A.), 5 g sodium chloride (Merck) and 14 g agar (L13, Oxoid) per l, pH 7.6 ± 0.1, incubated at 30°C for 3 days. *Enterobacteriaceae* were counted on pour-plates of Violet Red Bile Glucose Agar (VRBG; CM485, Oxoid) with a top layer of the same medium and incubation for 24 hours at 30°C. Lactic acid bacteria were selectively enumerated on pour-plates of MRS medium (Merck) with 12 g of agar (L13, Oxoid) and 2 g of Delvocid (Gist Brocades, Delft, The Netherlands) per l, incubated at 30°C for 3 days. Enumeration of yeasts and filamentous fungi was in pour-plates of Oxytetracycline Glucose Yeast Agar (CM545, Oxoid), incubated at 25°C for 5 days.

- *Determination of organic acids.* Organic acids in fermented salads were determined by means of HPLC as described earlier (Bonestroo *et al.*, 1992a).

7.3. RESULTS

From the experiments with modified Mφller medium it was concluded that most starter cultures do not possess decarboxylative properties. Of the 191 lactic acid bacteria tested only 34 were able to create a purple colour after incubation in media with certain amino acids, mostly tyrosine. This was generally accompanied by accumulation of relatively small amounts of gas. A strain was assumed to be decarboxylase positive only if the HPLC analysis of the growth medium also gave evidence of amine formation. This was true for only 11 strains. Tyramine production was most frequently encountered. Some of these strains also produced phenylethylamine, however, in relatively small amounts, compared to tyramine. Only a few strains produced putrescine or cadaverine. Although not all strains which produced biogenic amines were identified, it is assumed that many of them belong to the genus *Enterococcus*, as many of them were able to grow on Kanamycin Aesculin Azide Agar.

As can be seen in Table 7.3.1. the *E. coli* strain numbers 4, 8, 12 and 15 were able to decarboxylate tyrosine, histidine and lysine. Chemical analysis, however, revealed large amounts (> 200 mg/l) of histamine and cadaverine only. When *E. coli* no. 4 was deliberately added to chicken salads (approximately 700 cfu/g), which were then fermented with different amounts of *L. plantarum* no. 24, which does not possess decarboxylative

Table 7.3.1. Formation of biogenic amines by *Enterobacteriaceae* in modified Mφller medium.[a]

Strain	Amine					
	TA[b]	PHEA	CAD	HA	TPA	PUT
E. coli no. 4	P/+[c]	Y/+	PC/+	PC/+	Y/+	Y/+
E. coli no. 8	P/+	Y/+	PC/+	PC/+	Y/+	Y/+
E. coli no. 12	P/+	Y/+	PC/+	PC/+	Y/+	Y/+
E. coli no. 15	P/+	Y/+	PC/+	PC/+	Y/+	Y/+
Hafnia alvei no. 1	P/+	P/+	PC/+	PC/+	P/+	PC/+
Hafnia alvei no. 2	P/+	P/+	PC/+	P/+	P/+	PC/+
E. coli ATCC 11229	P/+	Y/-	Y/-	P/+	Y/-	PC/+
E. coli ATCC 11289	P/+	P/+	PC/+	PC/+	P/+	PC/+

[a] Mφller medium, incubated for up to 7 days at 37°C.
[b] TA, tyramine; PHEA, phenylethylamine; CAD, cadaverine; HA, histamine; TPA, tryptamine; PUT, putrescine.
[c] P, purple colour (positive reaction); Y, yellow colour (negative reaction); +, gas production; -, no gas production; PC/+, production of biogenic amines confirmed by chemical analysis.

properties, massive outgrowth of *E. coli* occurred (Table 7.3.2.). Due to the high buffering capacity of chicken meat the decrease of pH during fermentation was moderate: pH-values after 3.5 hours of fermentation at 42°C were 5.65 and 4.76 for salads inoculated with 4.3 x 10^7 and 2.1 x 10^8 cfu of the starter culture per g salad, respectively. Final pH-values of these salads, reached after 7 hours, were 4.83 and 4.22, respectively. Lactic acid concentrations in these salads after 7 hours of fermentation at 42°C were 0.29 % (w/w) and 0.41 % (w/w), respectively, increasing to 0.56 % (w/w) and 0.69 % (w/w), after 28 days storage at 7°C, respectively. Little or no acetic acid could be found in the fermented salads ($\leq$ 0.03 % [w/w]). *E. coli* survived during the storage period, although numbers declined, especially in the salads fermented with 4 % (w/w) starter. Despite the apparent suitable conditions for the production of biogenic amines by *E. coli*, no detectable amounts of these amines were formed.

When leek-cabbage-ham salads, inoculated with two strains of *H. alvei* and *E. coli* ATCC nrs. 11229 and 11289 (inoculation level approximately 100 cfu/g) were fermented with *L. plantarum* no. 39, which does not possess decarboxylative properties, growth of *Enterobacteriaceae* during fermentation at 45°C and storage at 7°C was completely inhibited, although survival occurred during the fermentation (Table 7.3.3.). As can be seen in Table 7.3.3. a low pH ($\leq$ 4.4) was reached in the salads after 7 hours of fermentation at 45°C. Although the fermented salads contained raw ingredients, i.e. leek and cabbage, no excessive growth of spoilage bacteria occurred. During storage at 7°C the pH remained low and the fermented salads were microbiologically stable for at least 4 weeks. The fermented salads of type A and B, in that order, contained approximately 0.44 % (w/w) and 0.34 % (w/w) lactic acid after 7 hours of fermentation at 45°C, increasing to 0.49 % (w/w) and 0.41 % (w/w) after subsequent storage at 7°C for 4 weeks. Little or no acetic acid ($\leq$ 0.03 % [w/w]) or ethanol ($\leq$ 0.04 % [w/w]) could be found in the fermented salads. According to Table 7.3.1. both strains of *H. alvei* and *E. coli* ATCC 11289 are able to decarboxylate all the amino acids tested. *E. coli* ATCC 11229 on the other hand was able to decarboxylate tyrosine, histidine and ornithine only. Chemical analysis, however, only indicated the production of large amounts ($>$ 500 mg/l) of putrescine for all these *Enterobacteriaceae* and cadaverine for *H. alvei* no. 1 (690 mg/l) and *E. coli* ATCC 11289 (230 mg/l). Despite these characteristics and although pyridoxal phosphate was added to the salads (5 mg/kg) no

Table 7.3.2. Microbiological condition and pH after fermentation and subsequent storage of chicken salad, inoculated with *E. coli* no. 4 (approx. 700 cfu/g) before fermentation.

CFU[a] of	pH				CFU after 7 h[c]			CFU after 6 days storage[d]			CFU after 25 days storage[e]		
starter no. 24	Initial[b]	After 7 h[c]	After 6 days storage[d]	After 25 days storage[e]	ENT[f]	LAB	MAB	ENT	LAB	MAB	ENT	LAB	MAB
4.3×10^7	6.47	4.83	4.62	4.23	6.48	8.54	6.70	5.54	8.62	5.79	3.96	8.45	4.57
2.1×10^8	6.48	4.22	4.41	4.00	4.60	8.70	4.90	3.95	8.72	4.26	<2.00	8.85	2.70

Results are the means of determinations of duplicate samples.

[a] CFU = Colony forming units.

[b] Before fermentation.

[c] after 7 h of fermentation at 42°C.

[d] after 7 h of fermentation at 42°C and subsequent storage at 7°C for 6 days.

[e] after 7 h of fermentation at 42°C and subsequent storage at 7°C for 25 days.

[f] ENT = *Enterobacteriaceae*, presumably *E. coli*
LAB = Lactic acid bacteria, presumably *L. plantarum*
MAB = Mesophilic aerobic bacteria

Table 7.3.3. Effect of decarboxylase-positive *Enterobacteriaceae* on the microbiological condition and pH after fermentation and subsequent storage of leek-cabbage-ham salads spiked with pyridoxal phosphate (5 mg/ kg salad).

Salad type	pH				CFU[e] after 7 h[b]			CFU after 14 days storage[c]			CFU after 28 days storage[d]		
	Initial[a]	After 7 h[b]	After 14 days storage[c]	After 28 days storage[d]	ENT[f]	LAB	MAB	ENT	LAB	MAB	ENT	LAB	MAB
Control[g]	6.59	3.98	4.31	4.19	<2.70	<3.70	4.83	<1.70	5.05	<2.70	<1.70	2.75	3.34
A		4.40	4.48	4.50	<2.70	8.06	4.94	<1.70	7.22	<2.70	<1.70	7.17	2.05
B		4.39	4.34	4.47	3.14	8.21	4.23	<1.70	6.31	<2.70	<1.70	7.10	<1.70

Results are the means of determinations of duplicate samples.

[a] Before fermentation or acidification.

[b] after 7 h of fermentation at 45°C.

[c] after 7 h of fermentation at 45°C and subsequent storage at 7°C for 14 days.

[d] after 7 h of fermentation at 45°C and subsequent storage at 7°C for 28 days.

[e] CFU = Colony forming units (log N/g).

[f] ENT = *Enterobacteriaceae*
LAB = Lactic acid bacteria
MAB = Mesophilic aerobic bacteria

[g] Control: not inoculated, acidified with 0.4 % (w/w) lactic acid.
A: fermented with *Lactobacillus plantarum* no. 39.
B: fermented with *Lactobacillus plantarum* no. 39, inoculated with *Enterobacteriaceae* (approx. 100 cells/g salad; *Hafnia alvei* [2 strains] and *Escherichia coli* ATCC 11229 and 11289).

detectable amounts of biogenic amines were found. The recovery of the amines was studied by homogenizing a fresh leek-cabbage-ham salad, which contained no detectable amounts of biogenic amines. Portions of the suspension were spiked with amines at several concentrations. With the method used the recoveries of histamine, tyramine, phenylethylamine and putrescine were 60 ± 3 %, 104 ± 8 %, 109 ± 13 % and 91 ± 10 %, respectively.

For the production of biogenic amines not only decarboxylating organisms, but also free amino acids are necessary. From data of Haytowitz and Mattheus (1984), Posati (1979) and Richardson *et al.* (1980) amino acid concentrations of two types of salads were calculated. For chicken salad values for TYR, PHE, LYS, HIS, TRP, ARG and pyridoxal phosphate were 2810, 3317, 7039, 2581, 974, 5012 and 1.27 mg/kg, respectively. For leek-cabbage-ham salads these values were 796, 1034, 2052, 890, 306, 1746 and 0.61, respectively. It should be noted that these values include both free and bound amino acids. Arginine is the precursor amino acid for ornithine. As leek-cabbage-ham salads contain a low level of amino acids, compared to chicken salads and because proteolytic activity during fermentation and storage is generally very restricted (Table 7.3.4.), the absence of formation of biogenic amines might be due to low levels of the appropriate precursors.

To study this in further detail, the above-mentioned experiments were repeated with leek-cabbage-ham salads to which pyridoxal phosphate (5 mg/kg) and several amino acids (histidine, lysine, ornithine, phenylalanine, tryptophan and tyrosine) were added (approximately 1 g/kg each). The level of inoculation with strains of *H. alvei* and *E.coli* was about 100 cfu/g. As can be seen in Table 7.3.5. a low pH (≤ 4.3) was reached in the salads after 7 hours of fermentation at 45°C. Growth of *Enterobacteriaceae* during fermentation at 45°C and storage at 7°C was completely inhibited. During storage at 7°C the pH remained low and the fermented salads were microbiologically stable for at least 4 weeks. The fermented salads of type A and B, in that order, contained approximately 0.44 % (w/w) and 0.34 % (w/w) lactic acid after 7 hours of fermentation at 45°C, increasing to 0.47 % (w/w) and 0.40 % (w/w) after subsequent storage at 7°C for 4 weeks. Little or no acetic acid (≤ 0.03 % [w/w]) or ethanol (≤ 0.05 % [w/w]) could be found in the fermented salads. Although several conditions for the production of biogenic amines were favourable no detectable amounts of these amines were found.

7.4. DISCUSSION

Determination of decarboxylative properties of microorganisms

Although the determination of decarboxylative properties of lactic acid bacteria can be much simplified by the use of modified Mφller media, a few remarks have to be made with regard to its performance. In most cases the regeneration of a purple colour after incubation indicates the presence and activity of amino acid decarboxylases. However, other reactions, such as the production of ammonia, could also initiate this change in colour. Chemical analysis of the test medium is required to confirm the real nature of this phenomenon. The addition of Durham tubes to the modified Mφller media did not improve the overall performance, since the change in colour and not just the production of gas indicates the presence of decarboxylases. The modified Mφller medium is useful for assessing the capacity to produce biogenic amines under optimal conditions, which could differ from conditions during salad fermentation. Concentrations of biogenic amines formed in modified Mφller medium are therefore less relevant.

When strains producing relatively large amounts of tyramine in modified Mφller medium with tyrosine, were inoculated into the same medium with phenylalanine relatively small amounts of phenylethylamine were formed. It is known that the tyrosine-decarboxylating enzyme possesses activity towards phenylalanine (Joosten and Northolt, 1987). However, the enzyme has a much lower affinity for phenylalanine than for tyrosine, thus for the production of detectable amounts of phenylethylamine high enzyme levels are required. Most tyramine-producing strains are assumed to belong to the genus *Enterococcus*. As growth rate and acid production of these strains generally are moderate, these strains were not used in salad fermentation.

Most *Enterobacteriaceae* tested, except *H. alvei* no. 2 and *E.coli* ATCC 11229, were able to produce histamine from histidine in modified Mφller medium. This corresponds with the findings of Joosten and Northolt (1989) that histidine-decarboxylating strains could be found amongst *Enterobacteriaceae*. Most strains tested, except *E. coli* ATCC 11229, were able to decarboxylate lysine.

Table 7.3.4. Free amino acid content of leek-cabbage-ham salads (mg/kg).

Time [a]	Control						Fermented						Fermented, inoculated with *Enterobacteriaceae*					
	TYR	PHE	LYS	HIS	TRP	ORN	TYR	PHE	LYS	HIS	TRP	ORN	TYR	PHE	LYS	HIS	TRP	ORN
A	13	63	28	15	0	0	13	63	28	15	0	0	13	63	28	15	0	0
B	8	33	30	20	0	0	35	77	63	32	0	3	30	75	56	27	0	0
C	25	67	54	19	0	0	43	62	76	27	0	1	44	58	80	30	0	1
D	25	28	65	21	0	1	36	39	78	29	0	1	37	38	79	27	0	1

[a] A = before fermentation or acidification; B = after 7 hours fermentation at 45°C; C = after fermentation and subsequent storage at 7°C for 14 days; D = after fermentation and subsequent storage at 7°C for 28 days.

Table 7.3.5. Effect of decarboxylase-positive *Enterobacteriaceae* on the microbiological condition and pH after fermentation and subsequent storage of leek-cabbage-ham salads spiked with pyridoxal phosphate (5 mg/ kg salad) and amino acids (approx. 1 g/kg salad).

Salad type	pH				CFU[c] after 7 h[b]			CFU after 14 days storage[c]			After 28 days storage[d]		
	Initial[a]	After 7 h[b]	After 14 days storage[c]	After 28 days storage[d]	ENT[f]	LAB	MAB	ENT	LAB	MAB	ENT	LAB	MAB
Control[g]	6.13	3.83	4.07	4.93	1.75	2.86	5.32	<1.70	4.76	<3.70	<1.70	5.41	5.05
A		4.27	4.36	4.27	<1.70	5.40	4.79	<1.70	7.75	<3.70	<1.70	6.07	1.80
B		4.19	4.30	4.23	<1.70	5.40	4.68	<1.70	7.59	<3.70	<1.70	7.18	<1.70

Results are the means of determinations of duplicate samples.

[a] Before fermentation or acidification.

[b] after 7 h of fermentation at 45°C.

[c] after 7 h of fermentation at 45°C and subsequent storage at 7°C for 14 days.

[d] after 7 h of fermentation at 45°C and subsequent storage at 7°C for 28 days.

[e] CFU = Colony forming units (log N/g).

[f] ENT = *Enterobacteriaceae*
LAB = Lactic acid bacteria
MAB = Mesophilic aerobic bacteria

[g] Control: not inoculated, acidified with 0.4 % (w/w) lactic acid.
A: fermented with *Lactobacillus plantarum* no. 39.
B: fermented with *Lactobacillus plantarum* no. 39, inoculated with *Enterobacteriaceae* (approx. 100 cells/g salad; *Hafnia alvei* [2 strains] and *Escherichia coli* ATCC 11229 and 11289).

Biogenic amines in salads and related foods

According to Brink *et al.* (1990) the presence of biogenic amines could be expected in certain fermented foods, including those in which a lactic fermentation is achieved by allowing a selection of naturally occurring microorganisms. Especially during the early stages of these fermentations all kinds of microorganisms can flourish. The presence of biogenic amines has indeed been observed in, amongst others, sauerkraut. The main biogenic amines in sauerkraut are putrescine, tyramine, cadaverine and histamine. Phenylethylamine is found only in minor quantities (Brink *et al.*, 1990; Kuensch *et al.*, 1990). In the fermentation of chicken salads comparable circumstances exist. When *E. coli* no. 4 was deliberately added to chicken salads, and then fermented with different amounts of an amino acid decarboxylase negative starter culture, massive outgrowth of *E. coli* occurred (Table 7.3.2.). Moreover, despite the high numbers of lactic acid bacteria and the low pH (< 4.5) attained, *E. coli* survived during storage at 7°C. For that matter, in later experiments this fermentation was strongly improved by the use of other starter cultures and different process conditions. As calculated from literature data, chicken salads are rich in amino acids, mainly tight up in proteins. Also high amounts of vitamin B6, the cofactor needed for decarboxylase enzymes, are found (1.27 mg/kg). In Gouda, Edam and Maasdam cheese vitamin B6 is present at concentrations between 0.66 and 1.57 mg/kg, which is sufficient to allow amine formation (Joosten, 1988). Although suitable conditions exist for the production of biogenic amines, no detectable amounts were found. This could be due to an apparent lower availability of amino acids and/or cofactor, low amounts of decarboxylases or sub-optimal conditions for activity of these enzymes. Also, the low storage temperature (7°C) and the relatively short storage period should be considered.

A better fermentation was attained in leek-cabbage-ham salads, inoculated with strains of *H. alvei* and *E.coli*, fermented with *L. plantarum* no. 39 (Table 7.3.3.). Lactic acid bacteria reached high numbers and acid production was such that no growth of *Enterobacteriaceae* occurred during fermentation. Moreover, survival of these non-starter bacteria was restricted to the first period of storage. Although pyridoxal phosphate was present in excess, no biogenic amines were produced. As stated earlier, the free amino acid content of leek-cabbage-ham salads is rather limited. This is an additional reason for the

absence of amine production. It is not likely that amines are degraded by amine-oxidases, since oxygen is required for this reaction. Oxidation will probably not occur under the anaerobic conditions prevailing in fermented salads (Bonestroo *et al.*, 1992b). Moreover, production and degradation of amines should be confirmed by changes in the amino acid balance. However, no significant differences exist in the amino acid contents of the microbiologically stable control salads, fermented salads and salads, inoculated with *Enterobacteriaceae* and fermented with *L. plantarum* no. 39 (Table 7.3.4.).

To create conditions suitable for the production of biogenic amines, leek-cabbage-ham salads were inoculated with *Enterobacteriaceae*, free amino acids and pyridoxal phosphate and fermented with *L. plantarum* no. 39 (Table 7.3.5.). An excellent fermentation was attained, i.e. a rapid decrease of pH and a low final pH (< 4.3), through which the numbers of *Enterobacteriaceae* declined below the detection limits and a microbiologically stable salad was created. Biogenic amines could not be detected. Apparently, a properly controlled fermentation can prevent the formation of biogenic amines.

This is in agreement with the findings of Andersson (1988), who concluded that vegetables and root crops fermented with the aid of lactic acid starter cultures should be considered as low-risk products with regard to the presence of biogenic amines. Also, Kuensch *et al.* (1990) concluded that the formation of biogenic amines in sauerkraut fermentation could be limited by the use of *L. plantarum* as starter culture, together with restriction of the lenght of the fermentation process. The decarboxylative properties of the strain used by Kuensch *et al.* (Vege-Start 10, Chr. Hansen's Lab., Copenhagen, Denmark) were tested in our laboratory with modified Mϕller medium spiked with several amino acids. No colour changes occurred and no decarboxylase activity could be revealed by chemical analysis.

As Brink *et al.* (1990) concluded, the occurrence of biogenic amines can be prevented by assuring a low initial contamination, proper hygienic care during processing, and control of contamination and microbial activity. This study shows that in fermented salads, production of biogenic amines can be controlled by these measures and by using starter cultures which are amino acid decarboxylase negative and which actively repress non-starter

microorganisms.

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8

GENERAL DISCUSSION

As stated in Chapter 1, the major objective of this study was to develop a new method of salad preparation by which the major microbiological and chemical problems of the present-day delicatessen salads could be controlled. Next to this the taste of the new salads should be natural, i.e. mildly sour and the solid ingredients should retain a crispy texture. In this study the use of starter cultures and a short time ($\leq$ 8 hours) fermentation process at higher temperatures (42-45°C) were chosen. After the fermentation the product was cooled. The high fermentation temperature was chosen to give the starter organisms an advantage over possible spoilage organisms, i.e. lactobacilli, including *L. brevis* and *L. fructivorans*, yeasts and *Enterobacteriaceae*.

The project plan included the selection of suitable starter cultures, screening of promising strains on major performance criteria, determination of the feasibility of the designed process for preparation of salads with increasing ratios of solids to sauce and assessment of optimal fermentation conditions. Furthermore, a number of tests were introduced to determine the microbiological and chemical stability, and the safety of the newly developed products.

Many lactic acid bacteria used as starters in salad fermentation produce sufficient lactic acid to attain a low final pH. Within 7 hours at temperatures of 42-45°C a pH $<$ 4.2 is reached, which is necessary to achieve a microbiologically stable product. However, large differences exist between strains in the rate of pH decrease attained in salads, caused amongst others by differences in lag phase, in sucrose fermentation inducibility and intrinsic acidifying capacity. Further improvements in the fermentation characteristics can be attained by the use of cultures with high fermenting capacities. In this respect the use of mixed-strain starter cultures could also be considered.

Process conditions

A limitation in producing salads through short-time high-temperature fermentation is the warming-up time at the start of the fermentation. Reduction of the warming-up time is

desirable. This reduction can be achieved, for instance by heating the sauce with a plate heat exchanger, prior to addition of starter culture and solid ingredients. Results in this study, however, were attained under less optimal conditions by heating in a waterbath at 42°C or 45°C. Although the warming up in a waterbath is considerably faster than in an air circulated incubator, it still takes approximately 65 min to increase the temperature of 150 ml glass jars filled with approximately 100 g salad from 7°C to 42°C in a 42°C waterbath. In a 45°C waterbath this increase in temperature takes approximately 40 min, while heating to 45°C takes approximately 85 min (Fig. 8.1).

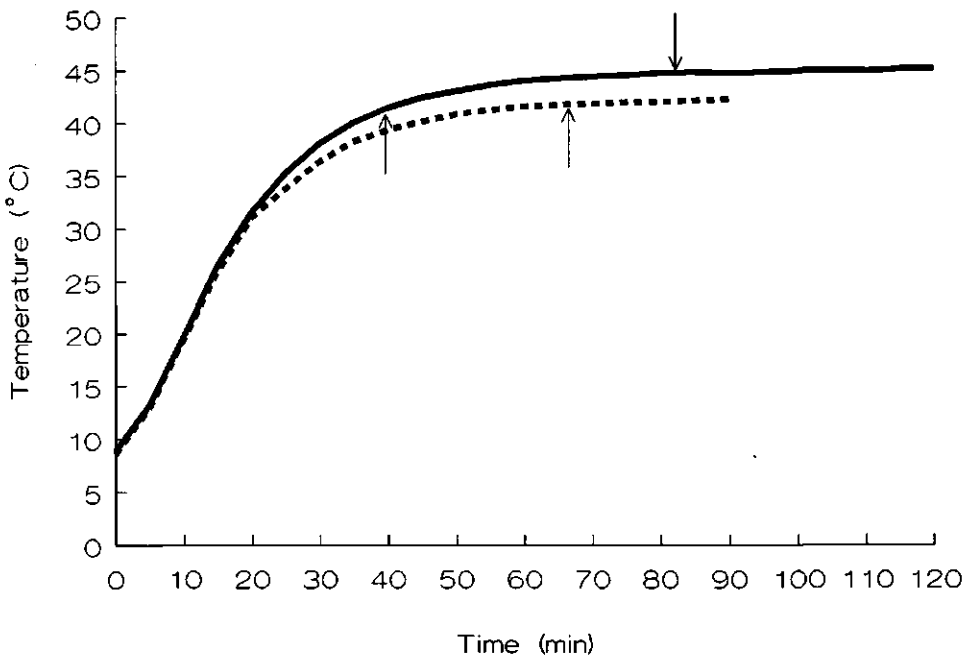

Fig. 8.1. Temperature profile as determined in the centre of a jar filled with leek-cabbage-ham salad when warmed up in a waterbath of 42°C (---) or 45°C (—).

In Chapter 4, salads were fermented with *Lactobacillus acidophilus* at 42°C and 45°C and fermentation characteristics were compared. The final pH, reached after 7 hours of fermentation, showed minor variations for salads fermented at 45°C and 42°C (Table 4.3.2.). The decrease of pH, however, was quicker at 45°C, than at 42°C. This difference

can be attributed to strain characteristics, such as optimal growth temperature, although the reduced time to reach 42°C in a waterbath of 45°C, compared to 42°C, could also play a role. In Fig. 8.2. growth curves of some strains of *Lactobacillus* spp. at various temperatures above 40°C are shown. The strains used in this figure are representatives of different groups.

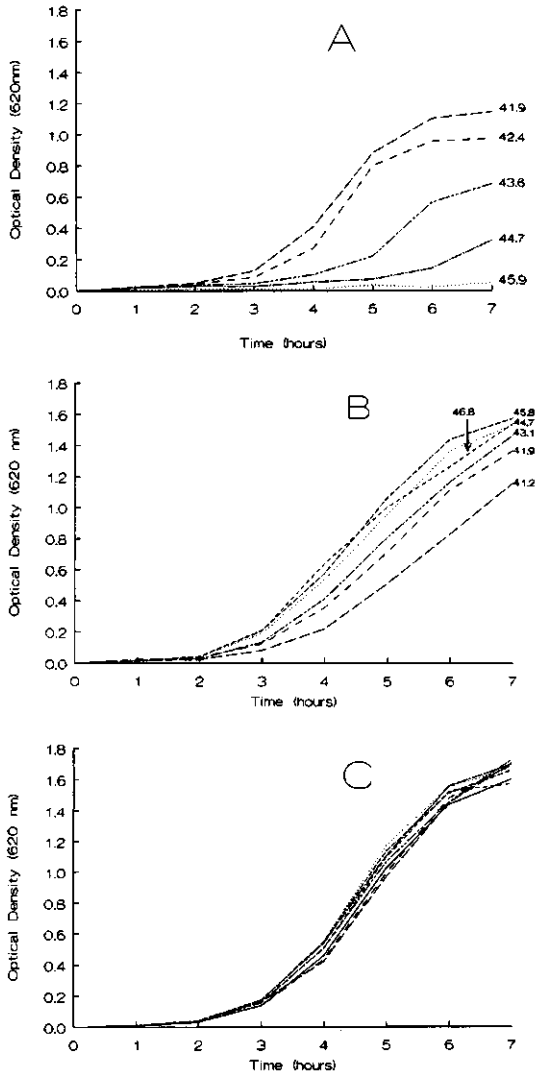

Fig. 8.2. Growth curves of *Lactobacillus* spp. in MRS broth at temperatures ranging from 41.9 to 47.6°C. A, no. 32, optimum growth temperature near 42°C; B, no. 63, optimum growth temperature near 46°C; C, no. 51, no optimum growth temperature in the range measured.

Ingredients

Different types of salads were fermented in this study, varying from vegetable salads and meat salads to mixed salads with both compounds. The composition of the recipes was based on several criteria, amongst which the buffering capacity of the ingredients used. The total buffering capacity, i.e. the amount of lactic acid necessary to decrease the pH of the mixture below a value of 4.2, should not exceed the maximum amount of lactic acid which can be produced by the starter cultures used. The diffusion rate between sauce and solids also plays a role. For vegetables, the buffering capacities are relatively low and diffusion rates relatively high, compared to meat (Bückenhuskes and Gierschner, 1985; Pfeil and Liepe, 1987). The designed process is feasible for the preparation of potato and vegetable salads with ratios of solids to sauce up to 70:30, whereas the limit of this ratio for sauce-based salads with meat or fish as sole ingredient is considerably lower. In the latter, pH in the sauce decreases rather quickly, which reduces or even terminates the acid production of lactic acid bacteria with limited acid tolerance. Lactic acid bacteria maintain a cytoplasmic pH that is higher than the pH of the medium, but the internal pH decreases as the growth medium is acidified during growth and fermentation. When the cytoplasmic pH decreases below a threshold pH, which depends on the organism, cellular functions are inhibited (Kashket,1987; McDonald *et al.*, 1990). By using both vegetables and meat in the mixture and by using mixed starter cultures, composed of strains with high fermenting capacities and acid-tolerant strains, the ratio of meat, i.e. chicken meat, in salads can be raised to 25 %. Further increase of the proportion of chicken or fish is not possible with the present-day laboratory scale salad fermentation process. Fermentation of these types of salads is feasible at lower temperatures ($\leq$ 25°C), which will reduce the efficiency of the process. Due to the low buffering capacities of the sauces and the relatively limited acid tolerance of the lactic starters, the use of acidified canned ingredients is restricted. Preferably fresh or deep-frozen vegetables are to be used.

Taste and stability

Most of the lactic acid bacteria used give the fermented salads a mildly sour taste and do not affect the crispy texture of the ingredients negatively. A characteristic difference of

the fermented salads with the commercially available salads, acidified with acetic acid, is that the ingredients keep more of their own characteristic taste. Uninoculated control salads frequently have off-flavours and sometimes show curdling of the sauce. The mild taste of the fermented salads can be improved by de addition of seasonings before fermentation. The composition of mixtures of spices and herbs is quite complicated, since the salads should have a pleasant taste throughout the whole storage period, i.e. up to 6 weeks at 7°C, and decline in taste of salads fermented with added seasonings is faster than in corresponding salads without added seasonings. Spices and herbs used should have low enzymatic activities. Furthermore, they should not affect the growth characteristics and acid forming capacities of lactic starter cultures negatively. In laboratory tests several spices and herbs exhibited growth stimulating or inhibitory activities, depending on the concentrations used. These findings are in agreement with literature data (Zaika and Kissinger, 1984; Deans and Ritchie, 1987; Kivanç *et al.*, 1991). It should be noted that some vegetables can also possess inhibitory activities on lactic starter bacteria. In our studies this was particularly demonstrated in fermentation trials with salads containing celery. Constituents, such as (pro-)anthocyanins, could be responsible for this phenomenon (Pratt *et al.*, 1960).

Secondary properties of starter culture bacteria

As stated in Chapter 1, starter cultures were selected on several criteria, of which only the primary criteria are discussed in the following chapters. One of the secondary criteria is that starter cultures preferably should be able to produce dextran or other extracelluar polysaccharides, which is based on the desire to reduce the amount of additives in the sauce, i.e. stabilisers and thickening agents. Several lactic acid bacteria appear to form ropy colonies on MRS plates supplemented with sucrose, but only under laboratory conditions, i.e incubation at temperatures < 40°C for at least 24 hours. When applied in the fermentation of sauce the effects were not detectable, possibly due to the relatively large amounts (approximately 2.8 %) of thickening compounds added. Moreover, fermentation time and temperature could be responsible for these effects, since formation of ropy slime by most ropy slime-producing lactic acid bacteria occurs after prolonged incubation at temperatures below 40°C (Korkeala *et al.*, 1990; Toba *et al.*, 1990).

Lactic acid bacteria with high fermenting capacities were screened for several other

criteria in laboratory tests. Most cultures were not able to degrade malic and citric acids, which could give rise to gas bubbles, a quality defect. Most cultures did not degrade starch, the main component of the thickening agent. The proteolytic activity of the starter cultures was not tested. In fermentation trials, however, deviating taste characteristics, such as bitterness, due to degradation of proteins, were never detected. Moreover, results of amino acid analysis of several salads (Chapter 7) showed that liberation of amino acids was limited.

Conclusion

The results of the numerous fermentation trials, as presented in the preceding chapters, clearly show that preparation of salads by the new process, i.e. fermentation of salads in their package with lactic acid bacteria at high temperatures ($> 40°C$) for a short time ($\leq$ 8 hours), followed by cooling to below 7°C, is feasible.

Several lactic acid bacteria tested, especially strains of *Lactobacillus plantarum* and *Lactobacillus acidophilus*, isolated from plant sources at 45°C, possess the necessary traits, mentioned in Chapter 1. Salads produced according to this procedure are microbiologically stable for 5-6 weeks, i.e. growth of yeasts and other spoilage organisms does not exceed the tolerances specified by health authorities, provided that measures are taken to assure absence or low initial contamination with these microorganisms (a.o. Chapter 3). The salads can be regarded as safe, as growth of pathogenic microorganisms (Chapter 4) and production of biogenic amines (Chapter 7) are inhibited. The occurrence of lipid oxidation in sensitive salads, e.g. potato salads, packed in transparent packings and stored under light, can be inhibited by the use of specific starter cultures. Most of the lactic acid bacteria used give the fermented salads a mildly sour taste, whilst the crispy texture of vegetable ingredients is not affected. A characteristic difference of the fermented salads with the commercially available salads, acidified with acetic acid, is that the ingredients keep more of their own characteristic taste. Before introduction to the consumer market the process has to be tested on pilot-plant scale. Some improvements are necessary, such as a reduction of the warming-up time. Also, combinations with other technologies, such as flushing the headspace of salads with nitrogen or mixtures of nitrogen and carbon dioxide probably would further increase the chemical stability of the product.

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SUMMARY

The microbial shelf life of sauce-based delicatessen salads, composed of solid ingredients, such as potatoes, vegetables, fish, meat and an oil-in-water emulsion containing acidulants, such as acetic and lactic acids, and chemical preservatives, i.e. sorbic and benzoic acids, varies from 6 to 8 weeks, if stored below 7°C. Micro-organisms causing spoilage are lactic acid bacteria, yeasts, moulds and bacilli. However, the actual shelf life of salads, especially potato salads, may be much shorter due to lipid oxidation of polyunsaturated vegetable oils. Market demands, such as the use of transparent packing materials have a negative effect on the shelf life of salads because they are usually stored under high illumination in open display refrigerators. Oxidation could be inhibited by natural and synthetic antioxidants, but these are never applied. Next to these microbial and chemical changes, salads can also suffer from physical deterioration, such as changes in viscosity of the dressings, coagulation and separation.

Nowadays, there is a strong demand for salads without chemical preservatives and with a less sour taste. This trend apparently emerges from the rising health consciousness of the consumer and his desire for quality products, in which ingredients retain some of their original taste.

In this thesis a new method of salad preparation is presented which meets these consumer demands and by which the major microbiological and chemical shortcomings of the present-day delicatessen salads can be controlled. The taste of the new salads is natural, i.e. mildly sour and the vegetables used retain a crispy texture. Basic principle of the process is that salads are prepared from separate ingredients and fermented in their package with the aid of starter cultures at relatively high temperatures ($\geq$ 42°C) in a short time ($\leq$ 8 hours), after which the product is cooled. It is the aim of this thesis to study the conditions for operation and the microbiological and chemical aspects that are relevant for the process.

In Chapter 2 the selection of suitable starter cultures and the screening of promising strains on major performance criteria have been described. Lactic acid bacteria from the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* were screened for their capacity to ferment glucose and sucrose in a model system and in a standard salad. Several of these lactic acid bacteria tested, especially strains of *Lactobacillus plantarum* and *Lactobacillus acidophilus*, isolated at 45°C amongst others from plant sources, e.g. sugarbeet pulp and soak water from tempeh production, are suitable for use in the fermentation process. The

temperature optimum of these strains was in the range of 34 - 48°C. Most of these strains produced about equal amounts of L(+)- and D(-)-lactic acid. Inhibition of spoilage bacteria in the standard salad could be achieved by using starter cultures with high fermenting capacity.

In Chapter 3 experiments are described which demonstrate the microbiological stability of fermented salads. Salads were inoculated with acid-resistant spoilage yeasts, such as *Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Torulaspora delbrueckii*, and fermented for 7 hours at 42°C or 45°C with strains of *Lactobacillus* spp.. High numbers of spoilage yeasts (and production of large volumes of CO_2) were not attained in salads fermented with good lactic starters, provided the initial concentration of spoilage yeasts was sufficiently low ($\leq$ 100/g). Inhibition of spoilage yeasts in lactic fermented salads is apparently due to lactic acid, the low storage temperature and the low residual oxygen concentration.

Chapter 4 deals with the microbiological safety of fermented salads. In order to investigate the possible growth and persistence of spoilage bacteria and pathogens, salads were inoculated with *Klebsiella pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* and fermented with lactic acid bacteria. *Klebsiella pneumoniae*, an acid-resistant psychrotrophic organism was also used as a model organism for the behaviour of pathogenic *Enterobacteriaceae* such as *Salmonella*, *Shigella* and pathogenic *E. coli*. Lactic fermentation has been shown to cause prevention of growth and usually death of these spoilage and pathogenic bacteria due largely to the production of organic acids and a decrease in pH. Primarily, however, measures should be taken to attain absence or low initial contamination with these pathogens. Also, proper hygienic care during processing should be assured.

It is assumed that the reducing effect of the fermentation processes can have a protecting effect on salads towards lipid oxidation. In Chapter 5 experiments are mentioned in which salads have been fermented with *Lactobacillus plantarum* strains to study this protecting effect. It was shown that fermented potato salads, subjected to light exposure for different times, differed from uninoculated, acidified control salads. Fermented salads contained lower amounts of lipid oxidation products, amongst others hexanal, indicating a lower level or possibly the absence of lipid oxidation. Significant differences between the level of oxidation in potato salads fermented with different starter cultures could be detected. During fermentation the oxygen tension in the salads decreased and at the same time there was a decrease of the oxygen tension in the headspace. These phenomena were studied in further detail in Chapter 6. It was found that the decrease of the oxygen tension in fermented salads is caused by the oxygen consuming abilities of the lactic acid bacteria used as starters. It is assumed that this oxygen consumption was responsible for the protecting effect of

fermentation on salads towards lipid oxidation.

Fermented salads contain proteins and are subject to conditions which could invoke the formation of biogenic amines. As consumption of food containing high amounts of these amines can have toxic effects, fermentation conditions should be such that biogenic amines are absent. This was studied by assessing the decarboxylative properties of starter bacteria and by performing experiments in which salads were deliberately contaminated with amino acid decarboxylase positive *Enterobacteriaceae* and then fermented (Chapter 7). To create conditions suitable for the production of biogenic amines, these salads were spiked with free amino acids and the cofactor pyridoxal phosphate. Of the 191 lactic acid bacteria tested, only 11 strains possessed decarboxylative properties. In leek-cabbage-ham salads, inoculated with *Hafnia alvei* and *Escherichia coli*, free amino acids and cofactor pyridoxal phosphate, fermented with an amino acid decarboxylase negative starter culture (*Lactobacillus plantarum*) and stored for 4 weeks at 7°C, no biogenic amines were produced. It was concluded that in fermented salads, the occurrence of biogenic amines can be prevented by assuring a low initial contamination, proper hygienic care during processing, and the use of amino acid decarboxylase negative starter cultures which actively suppress non-starter microorganisms.

Finally, in Chapter 8 some implications of the work described in this thesis have been discussed. The results of the numerous fermentation trials, clearly show that preparation of salads by the new process, i.e. fermentation of salads in their package with lactic acid bacteria at higher temperatures ($\geq$ 42°C) in a short time ($\leq$ 8 hours) subsequently followed by cooling to below 7°C, is feasible. Salads produced according to this procedure are microbially stable for 5-6 weeks at 7°C, i.e. yeast and other spoilage bacteria are inhibited, provided that measures are taken to assure absence or low initial contamination with these microorganisms. The salads could be regarded as safe, as growth of pathogenic microorganisms and production of biogenic amines is inhibited. Some restrictions that require further investigations are discussed. The first one is the relatively low proportion of ingredients with high buffering capacities, such as meat and fish, which can be used in fermented salads. Regarding the future acceptance by the consumer, solutions have to be found to raise this ratio. Secondly, further improvements in process conditions, i.e. a reduction of the warming-up time, need further investigation.

SAMENVATTING

De microbiologische houdbaarheid van salades, bestaande uit vaste componenten zoals aardappels, groenten, vis, vlees en een olie-in-water emulsie, waaraan voedingszuren (azijnzuur en melkzuur) en konserveermiddelen (sorbine- en benzoëzuur) zijn toegevoegd, bedraagt bij gekoelde bewaring ($\leq$ 7°C) ca. 6 tot 8 weken. Melkzuurbacteriën, gisten, schimmels en bacilli zijn de voornaamste bederfverwekkende micro-organismen. De uiteindelijke houdbaarheid van salades, met name aardappelsalades, kan aanzienlijk korter zijn door het optreden van oxydatie van de gebruikte meervoudig onverzadigde oliën van plantaardige oorsprong. Verder heeft het gebruik van transparante verpakkingen een negatieve invloed op de houdbaarheid, omdat salades worden opgeslagen in koelvitrines onder sterke belichting. Toevoeging van antioxydantia kan oxydatie verhinderen, maar wordt in de praktijk niet of nauwelijks gebruikt. Naast genoemde microbiologische en chemische veranderingen zijn salades onderhevig aan fysisch bederf, zoals veranderingen in viscositeit van de gebruikte dressing, coagulatie and fase-scheiding.

Bij frequent consumentenonderzoek wordt vastgesteld dat de consument in toenemende mate voorkeur geeft aan salades zonder konserveermiddelen en met een mild zure smaak. Deze wens is onder andere gebaseerd op het toenemende kwaliteits- en gezondheidsbewustzijn van de consument.

In dit proefschrift wordt een nieuwe methode van saladebereiding beschreven, welke beantwoordt aan de genoemde wensen van consumenten en waardoor de belangrijkste microbiologische en chemische bederf-factoren onder controle kunnen worden gehouden. De smaak van de nieuwe salades is natuurlijk: mild zuur, terwijl de gebruikte groenten een knapperige structuur behouden. Belangrijk principe van het proces is dat salades, samengesteld uit de ingrediënten, worden gefermenteerd in de verpakking met behulp van startercultures bij relatief hoge temperaturen ($\geq$ 42°C) in korte tijd ($\leq$ 8 uur), waarna het produkt wordt gekoeld. Het doel van het werk zoals beschreven in dit proefschrift is het bestuderen van de vereisten en de microbiologische en chemische parameters welke relevant zijn voor het proces.

In Hoofdstuk 2 wordt de selectie van geschikte startercultures en het screenen van veelbelovende stammen op belangrijke gebruikscriteria beschreven. Melkzuurbacteriën van de geslachten *Lactobacillus*, *Pediococcus* en *Streptococcus* werden gescreend op het vermogen tot fermentatie van glucose en sacharose in zowel een modelsysteem als in salades.

Veel van de geteste bacteriën, met name *Lactobacillus plantarum* en *Lactobacillus acidophilus*, geïsoleerd bij 45°C uit plantaardige bronnen, waaronder suikerbietenpulp en weekwater uit het tempe-proces, zijn geschikt voor gebruik in het fermentatieproces. Het temperatuursoptimum van deze stammen bedraagt 34-48°C. L(+)- en D(-)-melkzuur worden in ongeveer gelijke mate geproduceerd. Door toepassing van startercultures met een hoog zuurvormend vermogen kunnen bederforganismen worden onderdrukt.

In Hoofdstuk 3 worden experimenten beschreven waarmee de microbiologische stabiliteit van salades wordt aangetoond. Salades werden opzettelijk besmet met *Saccharomyces cerevisiae*, *Saccharomyces exiguus* en *Torulaspora delbrueckii* en vervolgens gefermenteerd met stammen van *Lactobacillus* spp. gedurende 7 uur bij 42 of 45°C. Een hoog aantal gisten (en produktie van grote hoeveelheden CO_2) werd niet bereikt in met goede startercultures gefermenteerde salades, mits de initiële besmetting laag was ($\leq$ 100/g). Remming van bederfgisten wordt naar alle waarschijnlijkheid veroorzaakt door melkzuur, de lage opslagtemperatuur en het lage restzuurstofgehalte.

In Hoofdstuk 4 wordt de microbiologische veiligheid van gefermenteerde salades beschreven. Om de mogelijke groei en overleving van bederforganismen en pathogenen te bepalen, werden salades beënt met *Klebsiella pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes* en *Staphylococcus aureus* en gefermenteerd met melkzuurbacteriën. *Klebsiella pneumoniae*, een zuurtolerant psychrotroof micro-organisme werd gebruikt als model voor het gedrag van pathogene *Enterobacteriaceae*, zoals *Salmonella*, *Shigella* en pathogene *E. coli*. Aangetoond is dat melkzuurfermentatie leidt tot groeiremming en meestal afdoding van genoemde bederforganismen en pathogenen, voornamelijk door de produktie van organische zuren en verlaging van de pH. Toch moeten maatregelen worden genomen om hetzij aanwezigheid van pathogenen te voorkomen of een laag kiemgetal te bereiken. Daarnaast moet gedurende bereiding hygiënisch worden gewerkt.

Verondersteld wordt dat het optreden van vetoxydatie wordt tegengegaan door het reducerende effect van het fermentatieproces. In Hoofdstuk 5 worden experimenten beschreven waarin salades zijn gefermenteerd met *Lactobacillus plantarum* om dit beschermende effect te bestuderen. Er is verschil aangetoond tussen gefermenteerde aardappelsalades en ongefermenteerde, aangezuurde salades, beide blootgesteld aan belichting. Het gehalte aan vetoxydatie produkten, waaronder hexanal, is lager in gefermenteerde salades, hetgeen wijst op een lager niveau van of het niet optreden van vetoxydatie. Significante verschillen in de mate van vetoxydatie werden waargenomen bij salades welke met verschillende startercultures waren gefermenteerd. Gedurende fermentatie daalt het zuurstofgehalte in salades, tegelijkertijd daalt het zuurstofgehalte in de kopruimte. Deze verschijnselen zijn nader beschreven in Hoofdstuk 6. Er werd gevonden dat de daling van het zuurstofgehalte in salades veroorzaakt wordt door zuurstofconsumptie van de als

starters gebruikte melkzuurbacteriën. Er wordt verondersteld dat deze zuurstofconsumptie verantwoordelijk is voor het beschermende effect van fermentatie tegen vetoxydatie.

Gefermenteerde salades bevatten eiwitten en zijn onderhevig aan condities die het ontstaan van biogene aminen kunnen bevorderen. Omdat consumptie van voedsel met hoge gehaltes aan biogene aminen toxicologische effecten kan bewerkstelligen, moeten fermentatiecondities dusdanig zijn dat vorming van biogene aminen wordt voorkomen. Daartoe werden de decarboxylerende eigenschappen van startercultures bestudeerd en werden salades opzettelijk besmet met decarboxylerende *Enterobacteriaceae* en vervolgens gefermenteerd (Hoofdstuk 7). Ter optimalisatie van de voor vorming van biogene aminen benodigde condities werden tevens aminozuren en de cofactor pyridoxaalfosfaat aan de salades toegevoegd. Van de 191 geteste melkzuurbacteriën vertoonden slechts 11 decarboxylerende eigenschappen. In ham-prei salades, waaraan toegevoegd decarboxylerende *Enterobacteriaceae* (*Hafnia alvei* en *E. coli*), aminozuren en cofactor pyridoxaalfosfaat, welke gefermenteerd werden met een niet-decarboxylerende starter (*Lactobacillus plantarum*) en opgeslagen bij 7°C gedurende 4 weken, werden geen biogene aminen gevonden. Geconcludeerd werd dat in gefermenteerde salades de vorming van biogene aminen kan worden verhinderd door borging van een lage besmetting, goede hygiëne tijdens bereiding, en het gebruik van niet-decarboxylerende starter cultures welke niet-starter micro-organismen actief onderdrukken.

In Hoofdstuk 8 tenslotte, worden enige implicaties van het in dit proefschrift beschreven werk besproken. De resultaten van de vele fermentatietesten tonen aan dat bereiding van salades volgens het nieuwe proces, bestaande uit fermentatie van salades in de verpakking met behulp van melkzuurbacteriën bij relatief hoge temperatuur ($\geq$ 42°C) in korte tijd ($\leq$ 8 uur), gevolgd door koeling tot koelkasttemperatuur, haalbaar is. Salades, geproduceerd volgens deze methode zijn microbiologisch 5-6 weken lang bij 7°C stabiel: groei van gisten en bederforganismen wordt tegengegaan, mits maatregelen zijn genomen om aanwezigheid van deze micro-organismen te voorkomen of te beperken. De salades zijn veilig: groei van pathogenen en vorming van biogene aminen wordt verhinderd. Er zijn evenwel enige beperkingen. Ten eerste het relatief lage aandeel aan ingrediënten met hoge buffercapaciteit, zoals vlees en vis, welke kan worden toegepast in gefermenteerde salades. Om de acceptatie door consumenten te vergroten moeten oplossingen worden gevonden om dit aandeel te verhogen. Daarnaast moeten verbeteringen in procescondities worden onderzocht, waarbij in eerste instantie wordt gedacht aan een verdere verkorting van de opwarmtijd voor fermentatie.

CURRICULUM VITAE

Martin Hendrik Bonestroo werd geboren op 11 mei 1963 te Apeldoorn. In 1981 behaalde hij het VWO-diploma aan het Myrtus College te Apeldoorn. In datzelfde jaar begon hij met zijn studie aan de toenmalige Landbouwhogeschool in Wageningen.

In 1985 legde hij het kandidaatsexamen in de richting Levensmiddelentechnologie af. Het vakkenpakket in de doctoraalfase omvatte de hoofdvakken Levensmiddelenmicrobiologie en Levensmiddelenchemie en het bijvak Toxicologie. Zijn stageperiode bracht hij door bij de Rijkskeuringsdienst van Waren te Enschede en het Nederlands Instituut voor Zuivelonderzoek te Ede. In januari 1988 studeerde hij met lof af.

Van februari 1988 tot januari 1992 was hij werkzaam bij de sectie Levensmiddelenchemie en -microbiologie van de Landbouwuniversiteit waar het onderzoek zoals beschreven in dit proefschrift werd uitgevoerd. Momenteel is hij werkzaam bij Coberco Research te Deventer.