Biological ensilage of fish. Optimization of stability, safety and functionality

Maria L. N. Enes Dapkevicius

Promotor:	Prof. Dr. Ir. F.M. Rombouts Hoogleraar in de levensmiddelenhygiëne en -microbiologie
Copromotoren:	Dr. Ir. M.J.R. Nout Universitair hoofddocent Leerstoelgroep Levensmiddelenmicrobiologie
	Dr. Ir. J.H. Houben Docent / onderzoeker Vleestechnologie Hoofdafdeling Voedingsmiddelen van Dierlijke Oorsprong Universiteit Utrecht
Promotiecommissie:	Prof. Dr. J.T.M. Wouters (Wageningen Universiteit) Prof. Dr. Ir. M.J.A. Verstegen (Wageningen Universiteit) Prof. Dr. Ir. M.A.J.S. van Boekel (Wageningen Universiteit) Dr. H.A.P. Urlings (ID-Lelystad)

Biological ensilage of fish. Optimization of stability, safety and functionality

Maria L. N. Enes Dapkevicius

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 29 januari 2002
des namiddags te vier uur in de Aula

M. L. N. Enes Dapkevicius – Biological ensilage of fish. Optimization of stability, safety and functionality – 2002
PhD Thesis Wageningen University, Wageningen, The Netherlands

Keywords: biological silage, fish offal, fermentation, histamine degradation, lactic acid bacteria

ISBN: 90-5808-570-8

Stellingen

1. Supernatant formation in fish silage as a means of assessing proteolysis is misleading, since supernatant formation also reflects loss of water binding capacity by fish myofibrillar protein.

   This thesis

   Backhoff, H. P. 1976. Some chemical changes in fish silage. *J. Food Technol.* **11**: 353-363.

2. New strains for the preparation of fermented products have traditionally been selected by technical criteria. The future trend will be to include strains that provide added health benefits for the consumers, such as probiotic effects on the gut, and inactivation of harmful dietary compounds.

   This thesis

   Peltonen, K., El-Nezami, H., Haskand, C., Ahokas, J., and Salminen, S. 2001. Aflatoxin B_1 binding by dairy strains of lactic acid bacteria. *J. Dairy Sci.* **84**: 2152-2156.

3. "Studies on histamine have shown that the frequency of undesirable strains is higher than previously thought, that their presence is not predictable and finally, that the activity is not restricted to the species level but indeed characterizes strains."

   These conclusions apply to fish products as well.

   Lonvaud-Funnel, A. 2001. Biogenic amines in wines. *FEMS Microbiol.Letters* **199**: 9-13.

4. As 60% of the commercially important fish species see their stocks dwindle irreversibly, so does marine biodiversity and the livelihoods of the fishermen that catch them.

   Agardy, T. 2000. Effects of fisheries on marine ecosystems: a conservationist's perspective. *ICES J. Marine Sci.* **57**: 761-765.

5. Science knows no country, because knowledge belongs to humanity, and is a torch which illuminates the world.

   Louis Pasteur

6. Politics is for the moment. An equation is for eternity.

   Albert Einstein

Stellingen belonging to the thesis "Biological ensilage of fish. Optimization of stability, safety and functionality", by Maria L. N. Enes Dapkevicius. Wageningen, 29 January 2002.

Contents

Abstract

This thesis deals with stability, safety, and functionality aspects of biological fish silage (BFS) obtained by lactic acid fermentation. BFS may provide an economically viable, environment friendly way of upgrading fish waste.

BFS has been found advantageous when compared to the so-called acid process, since it yielded lower levels of peroxides in the silage oil and led to lower non-protein nitrogen values. Fermentation by lactic acid bacteria (LAB) was shown to efficiently inhibit pathogens such as *L. monocytogenes* and *E. coli* O157:H7 as well as some spoilage microorganisms. Histamine degradation by some of the most promising available LAB strains was also tested, as an innovative means of ensuring low levels of this amine in the fermented product. Several potential starter strains were found to degrade histamine as single strain cultures and could find application in fish silage and other fish products in which histamine accumulation might pose a certain risk.

Temperatures of 35 – 37°C were regarded as most suitable from the point of view of starter growth and acidification. Strains with a short acidification lag time, such as *Lb. plantarum* 009, or with very fast growth and good antibacterial properties, such as *Lb. curvatus* 15.35, are most suitable.

Sodium chloride inhibited growth of biogenic amine producing bacteria and additionally decreased non-protein nitrogen and total volatile basic nitrogen values in BFS. Potassium sorbate was shown to inhibit growth and/or acidification by potential starter lactic acid bacteria.

Foreword

Research work is seldom the product of a single individual. Many persons have contributed in different ways to the work presented in this thesis. I wish to express my thanks to all of them.

First of all, I wish to thank my supervisors (Professor F. M. Rombouts, Dr. M. J. R. Nout, Wageningen University, and Dr. J. H. Houben, Utrecht University) for their support and guidance, both in scientific matters and in coping with the adaptation to life in The Netherlands. To Eng. Irineu Batista (IPIMAR, Portugal) and Professor Ponte (University of the Azores, Portugal) I owe the initial motivation for this thesis, besides many fruitful work discussions. I wish to thank the students that worked in this project (Nandanie, Ana Costa, Wieke, Willem and Gera) for their enthusiasm and dedication. Not all of your work has contributed directly to this thesis, but it was very inspiring to work with all of you.

Working in the Food Microbiology group of WU has been a very positive experience, one I shall remember and miss in the future. This is due to the wonderful working atmosphere created by all of the group members. I wish to thank all of you for making me feel at home there, in particular to Birgit Hasenack, my lab colleague, for the technical assistance, the useful scientific discussions, as well as the personal support. To the colleagues and co-workers at DCA (University of the Azores), my gratitude for the support throughout these past years.

A lot of people helped me (and later on, the rest of my family) to settle in The Netherlands. Their contribution to this work has been very valuable – without it, I would not have managed to keep the appropriate frame of mind. I wish to thank in particular Joke van den Berg (my "Dutch mother"), Jan and Nanda Cozijnssen, the late Jacora de Wit, the Van Beek family, and Mrs. Nout. The period during which this thesis was produced was a period of changes in my life, not the smallest of which was building a family and giving birth to a child in a foreign country. Without your precious support, life would have been much more difficult. A special word to my sister Fátima, who took care of all necessary legal matters and paper work back in Portugal and to Asta, who gave me a precious extra month of work at WU by baby-sitting Ieva.

Finally, the most special folks of all: my family. Airidas, it is difficult to find words to express my thankfulness. Your contribution was that of a fellow researcher, and can be found in the form of suggestions, critical reading of manuscripts and help with computer programs, but it went far beyond that. I doubt whether I would have undertaken this project at all if it weren't for your support and constant motivation. This thesis is dedicated to you. Myliu tave! To my

parents, I thank you for supporting me throughout a long education. Ievute dear, to you my thanks for having brought a new light and more meaning to my life.

Chapter 1

General introduction

FISH: A SCARCE BUT UNDERUTILISED RESOURCE

In 1950, the world fish catch amounted no more than 20 million tons a year. By 1989, the marine catch had reached its maximum, 86 million tons. Since then, it has decreased, partly due to severe over-fishing. Seventy per cent of the world's fish stocks are being strained beyond their ability to sustain commercial quantities of fish. Governments have responded world-wide to this problem by setting quota on fish species that are under pressure, or even by ordering temporary bans to allow for the recovery of stressed populations (Jackson, 1996).

In spite of its increasing scarcity, fish is an underutilised resource. By-catch fish which is dumped back dead into the sea amounts to an estimated 27 million tons a year, that is, more than 25% of the total caught world-wide (Jackson, 1996). Furthermore, up to 60% of the fish caught goes to offal during subsequent processing into human food commodities (Raa and Gildberg, 1982). These fish residues could be used as a source of commercially valuable products, such as feed ingredients for animals, peptones for microbiological purposes or fish oils (Almås, 1990, Børrensen, 1992).

Several problems limit the full exploitation of underutilised fish resources (Venugopal, 1995): 1) the seasonal nature or lack of steady supply of individual items, 2) mixed type catch, with several species present in a single lot, 3) smaller size and inadequate composition of the fish residues, 4) highly perishable nature, 5) insufficient availability of icing and cold distribution chains, which are, at present, intended only for commercially important species, and 6) lack of consumer interest, which makes value addition unprofitable.

Processing methods that are, simultaneously, economically viable and environment friendly are required for the valorization of fish waste. An almost complete conversion of fish wastes can be achieved by reduction to fish meal or conversion into protein hydrolysates (Almås, 1990, Børrensen 1992). Fish meal is widely used, but it brings about environmental pollution problems, it is not economically viable when a steady supply of by-products is not available and it has been associated with the dissemination of pathogens such as *Salmonella* (Raa and Gildberg, 1982). Fish hydrolysates are less well known, but may offer promise as alternative valorization products. Conversion of fish residues into acid-preserved

hydrolysates is cheaper than producing fish meal and is applied to some extent in Northern Europe (Jangaard, 1987, Viken and Bjørge, 1987).

Fish hydrolysates are obtained by degradation of the fish protein into peptides, oligopeptides and amino acids. Hydrolysis can be achieved by tissue or digestive enzymes of the fish ("autolysates"), or by added exogenous enzymes of animal, plant or microbial origin ("heterolysates"). "Chemical hydrolysates" are defined as those in which hydrolysis arises exclusively from the action of added acid or alkaline compounds. "Mixed hydrolysates" comprise (i) natural enzymatic action accelerated by a modification of the pH (commonly designated "acid fish silages") and (ii) products in which a pH decrease results from the production of organic acids by lactic acid bacteria, designated "biological hydrolysates" (Sainclivier, 1985). This category of mixed hydrolysates is commonly known as "biological fish silages". The term "silage" applied to fish hydrolysates arises from the similarity with the process of preservation of wet fodder in silos adopted for green forage (Raa and Gildberg, 1982). Although this designation has been considered as less appropriate when applied to fish products (Sainclivier, 1985), it is universally used and will be employed in the present thesis.

Interest in using biological systems for preserving foods is increasing and has been mainly directed at lactic acid bacteria (Jeppesen and Huss, 1993). Transformation and preservation through a microbial fermentation by acid-producing microorganisms (lactic acid bacteria) has been considered as the most suitable procedure for recycling wastes and by-products from the food industry. The value-added products thus obtained were also regarded as more suitable in their composition and use than those obtained by high cost processes such as drying (Kherrati *et al.*, 1998). Biological fish silage, which results from incubation of minced fish waste to which a fermentable carbohydrate source and a suitable starter culture were added, offers nutritional and economical advantages over the acid fish silages (Szakács *et al.*, 1988, Montaner *et al.*, 1995). In biological fish silages, acid production results in a decrease in pH to values below 4.0. This pH decrease inhibits the growth of bacteria such as *Staphylococcus aureus* (Raa and Gildberg, 1982), *Escherichia coli*, *Serratia*, *Enterobacter*, *Citrobacter*, *Achromobacter*, *Pseudomonas* (Kompiang *et al.*, 1980), *Clostridium botulinum* and *Salmonella* (Tatterson, 1982), undesirable due to their deteriorating activity or their pathogenic nature.

Fish silages are mainly produced for animal feeds (Aryanta *et al.*, 1991), human consumption (Mackie *et al.*, 1971) and as plant fertilisers (Synnes and Opstadt, 1995), but can also be used as a source for the extraction of amino acids or biologically active compounds such as enzymes and polyunsaturated fatty acids.

STEPS IN THE PRODUCTION OF FISH SILAGE

Fish silages can be produced from fish wastes by two different methods (Raa and Gildberg, 1982):

- By adding organic and/or inorganic acid (acid fish silage). The pH is decreased to levels that prevent microbial growth and spoilage. The fish silage gradually liquefies due to the action of enzymes naturally present in the fish.

- By lactic acid fermentation (biological silage). Fermentation is initiated by mixing comminuted fish with a fermentable sugar, which enables the growth of lactic acid bacteria. Lactic acid bacteria are usually added as a starter culture. They produce acid and antimicrobial substances which, together, control competing spoilage/pathogenic microflora.

Fig. 1 shows a schematic representation of both methods for ensiling fish.

Fish silage can also be produced by adding ammonia, which results in a fast liquefaction (Raa and Gildberg, 1982, Man, 1999). However, alkali treatment of protein and amino acids may result in the formation of the potentially carcinogenic lysinoalanine and in racemization of free amino acids (Raa and Gildberg, 1982). Thus, this ensilage method is not recommended.

The traditional fermented fish products commonly prepared in Asia can be regarded as a type of silage in which high NaCl concentrations are used to ensure preservation.

REQUISITES FOR BIOLOGICAL FISH SILAGE PRODUCTION

Carbohydrate source

Fish contains only 1-3% carbohydrates, w/w (Suzuki, 1981). It is necessary to add a suitable carbohydrate source in order to obtain enough lactic acid to ensure the preservation of fish ensiled by the biological method. It is possible to use many different materials as carbohydrate sources for ensiling, from rice (Adams *et al.*, 1987), cassava (Lupín, 1983, Adams *et al.*, 1987) or maize flour (Lupín, 1983) to whey from cheese production (Lamprecht *et al.*, 1982, Van Wyk *et al.*, 1983a and b). Molasses have been widely employed (Johnson *et al.*, 1985, Twiddy *et al.*, 1987). Addition of carbohydrate-rich by-products of agro-industries is especially interesting, since it could provide a means of valorizing these residues as well. The choice of by-product influences the type of lactic acid bacteria (LAB) that will grow readily in the silage by excluding those species or strains that are not able to ferment the added carbohydrates. When using whey, for instance, the choice of starter cultures is limited to the LAB species or strains that metabolise lactose.

Different levels of carbohydrate addition are recommended in the literature (Table 1). For molasses, the addition levels vary from 5% (Ahmed and Mahendrakar, 1996a) up to 30% (Lupín, 1983, Faid *et al.*, 1994). Studies performed with purified sugars point out that at least 5% added lactose (Hassan and

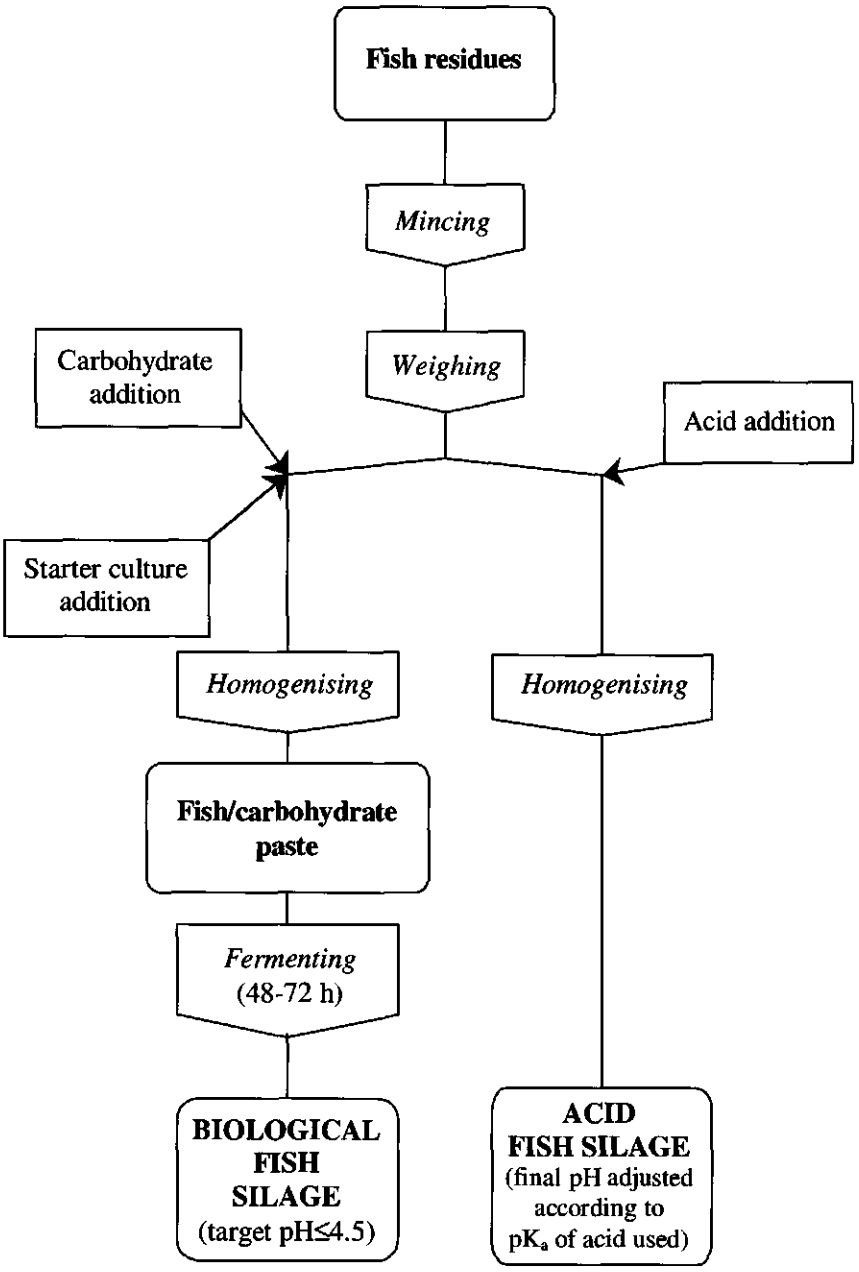

Fig. 1. Steps involved in the production of fish silage by acid and biological methods.

Table 1. Carbohydrate sources and levels used for the biological fish silage production.

Reference	Carbohydrate source	Addition level
Edin, 1940 (in Jangaard, 1987), Kompiang *et al.*, 1980	Molasses	2%
Dong *et al.*, 1993	Molasses	4%
Giurca and Levin, 1992	Molasses	7.5%
Ahmed and Mahendrakar, 1996a	Molasses	7.5-10%
Raa and Gildberg, 1982	Molasses	At least 10%
Roa, 1965, Ahmed *et al.*, 1996	Molasses	10%
Lupín, 1983	Molasses	10-30%
Johnson *et al.*, 1985	Molasses	12-15%
Brown and Sumner, 1985	Molasses	12-15%
Twiddy *et al.*, 1987	Molasses	9.5% or 19.5
Fagbenro and Bello-Olusoji, 1997, Fagbenro and Jauncey, 1998, Kherrati *et al.*, 1998	Molasses	15%
Carl, 1952 (in Petersen, 1953)	Molasses	18.75%
Faid *et al.*, 1997	Molasses	25%
Faid *et al.*, 1994	Molasses	30%
Van Wyk *et al.*, 1983a	Molasses+whey	2.5+12.5%
Van Wyk and Heydenrych, 1985	Molasses+whey	5%+5%
Van Wyk *et al.*, 1983a	Molasses+whey	5+12.5%
Lindgren and Pleje, 1983	Pre-fermented cereals+molasses	10%+10%
Green, 1989	Molasses or maize	25%
Lamprecht *et al.*, 1982	Whey+sucrose	11.5%+3.85%
Lindgren and Refai, 1984	Cereals	18%
Lupín, 1983	Cassava flour	15.5%-15%
Batista *et al.*, 1987	Cassava flour	20%
Twiddy *et al.*, 1987	Cassava+sugar	20%+2%
Fagbenro and Bello-Olusoji, 1997	Cassava flour	15%
Lupín, 1983	Maize flour	13.5%
Twiddy *et al.*, 1987	Rice+sugar	20%+2%
Hassan and Heath, 1986	Lactose	At least 5%
Adams *et al.*, 1987	Glucose or sucrose	4%
Dong *et al.*, 1993	Glucose	3%
Lássen, 1995a	Glucose	5%
Lássen, 1995b	Glucose	5%

Heath, 1986) or glucose (Lássen 1995 a and b) are necessary to ensure a proper fermentation. It must be noted that not all of these figures result from optimised processes. The differences among values recommended by different authors reflect not only the differences in chemical composition of the various carbohydrate sources, but also differences in the type of fish used and in the conditions under which the fermentation took place.

The ensilage temperature is an important aspect. Kreuzer (1954) showed that the pH decrease proceeds faster and less carbohydrate is consumed at temperatures between 25 and 30°C than at lower temperatures. Since it affects the buffering capacity of the system, the chemical composition of the fish residues also affects the amount of carbohydrate that has to be added in order to obtain a sufficient decrease in pH. Protein and ash content are of special importance in determining the buffering capacity of fish. Hassan and Heath (1986) obtained stable trout silages (2.35% ash) by adding only 3% lactose, but it was necessary to add 5% lactose in order to ensile white perch (6.25% ash).

When adding starch-containing materials as carbohydrate sources, addition of amylolytic enzymes (under the form of malt, for instance) must be envisaged, since LAB usually lack the ability to utilise starch (Raa and Gildberg, 1982).

Starter culture

Starter cultures are defined as preparations containing living microorganisms, which are applied with the intention of making use of their microbial metabolism (Hammes, 1990). LAB belonging to the species *Lactobacillus plantarum* are the most commonly used starter cultures for fish silage production (Johnson *et al.*, 1985, Hassan and Heath, 1986, Hassan and Heath, 1987). *Pediococcus* sp. have been used for ensiling fish by-products (Van Wyk and Heydenrych, 1985, Adams *et al.*, 1987). Some work has also been published on fermentation of fish waste with yeasts prior to ensiling with LAB. Yeasts belonging to the genera *Saccharomyces* and *Candida* were used, with the aim of deodorising the fish waste prior to ensiling (Faid *et al.*, 1994, Fagbenro and Jauncey, 1998).

LAB are present in fish (Schrøder *et al.*, 1980) in such low numbers that most of the authors use or recommend the addition of starter cultures to the fish pastes intended for ensilage (Pond and Manner, 1984, Gendron *et al.*, 1987, Lall, 1987, Nwokola and Sim, 1990, Kherrati *et al.*, 1998). Relatively high numbers of LAB are necessary to initiate a successful fermentation in fish waste. A level of 10^8 colony forming units (CFU) g^{-1} silage was, in most cases, considered as suitable for obtaining successful fermentations (Van Wyk and Heydenrych, 1985, Twiddy *et al.*, 1987, Lássen, 1995b). Larger quantities of starter culture become too expensive (Lássen, 1995b). In some cases however, successful silages were obtained with 10^3 (Hassan and Heath, 1986) or 10^4 CFU of starter LAB per g of silage (Dong *et al.*, 1993). No control silages lacking LAB inoculants were prepared in these works.

In addition to pure culture starters, mixed cultures obtained by back slopping or addition of products containing high numbers of LAB, such as sauerkraut, crushed snails pre-fermented cereals and pre-fermented molasses can be used to inoculate the fish waste (Lupín, 1983, Lindgren and Refai, 1984, Fagbenro and Jauncey, 1998). The addition of products containing high numbers of LAB is cheaper and easier than the purchase and maintenance of starter cultures, but provides less control over the fermentation process (Cooke *et al.*, 1987).

Other additives

The ensilage of fish takes place in an anaerobic environment (E_h around -550 mV, Lassén, 1995a) that favours the development of LAB and provides, by itself, a means of excluding the multiplication of obligatory aerobic contaminant microorganisms, such as *Pseudomonas* sp., for instance. In this type of environment, anaerobic bacteria such as *Clostridium* sp. are inhibited by the high acidity and can only survive if the pH did not decrease enough. Some clostridia compete with the LAB for the carbohydrate source. Some of them can use lactate as an energy source, thus raising the pH and creating the adequate conditions for the growth of the bacteria responsible for putrefaction (Mackie *et al.*, 1971). Some of these bacteria can use amino acids as sources of energy, yielding fatty acids, ammonia and CO_2 as final products. These undesirable alterations have been observed in a well-controlled ensilage, although to a minimum extent. In fish silage, which aims at preserving the protein, the extent of such phenomena should be minimised. Thus, the time during which the pH of the product is above the recommended values should be reduced to a minimum. Efficient starters, the addition of adequate carbohydrate sources at the proper levels and the use of adequate additives can contribute to fulfil this objective (Mackie *et al.*, 1971).

The excessive gas formation resulting from deterioration not only brings about a loss in quality, but also raises technical problems, since the volume of the ensiled material can reach the double (Stanton and Yeoh, 1977). Sodium chloride addition in levels up to 4 or 5% has been used to control gas formation (Twiddy *et al.*, 1987). Another possibility is the use of small amounts of acids at the beginning of the fermentation. Jangaard (1987) reports that gas production in biological fish silages with 20% molasses added was efficiently inhibited by using 2% (v/w) of a 50% H_2SO_4 solution. Roa (1965) used an adjustment of the initial pH value with formic acid instead. However, experiments made by Adams *et al.* (1987) led these authors to the conclusion that decreasing the initial pH of the fermented fish in 0.5-1.0 units by adding lactic, acetic or citric acid did not bring about considerable beneficial effects.

Sodium nitrite can be used with the purpose of rendering the environment more selective towards the LAB. Sodium nitrite inhibits the growth of sporeforming bacteria (*Bacillus* and *Clostridium*), *Micrococcus*, and some Gram-negative bacteria such as *Escherichia coli*, *Achromobacter*, *Enterobacter*,

Flavobacterium, and *Pseudomonas* (Davidson, 1997). The effective levels depend on the bacterial species considered and on the testing conditions. *Clostridium perfringens* growth at 20°C in a laboratory medium containing 3% NaCl was inhibited by the addition of 200 mg l^{-1} $NaNO_2$ (Gibson and Roberts, 1986). In spite of the objections against nitritre because of its potential for forming carcinogenic nitrosamines by reaction with secondary or tertiary amines, some authors recommend its addition to fish silage at the level of 100 mg l^{-1}. The concentration of this compound decreases during storage (Freeman and Hoogland, 1956, Cassens *et al.*, 1979). The maximum level of sodium nitrite allowed by the US Food and Drug Administration in food products is 120 mg kg^{-1} (Cooke *et al.*, 1987).

A successful silage is a stable product that can be kept for very long periods, up to several years. Instability problems occur mainly due to the admission of air into the system. Then, aerobes such as moulds and oxidative yeasts can thrive. These yeasts can grow at low pH values, and they oxidise carbohydrates and proteins. Some yeasts can also oxidise lactic acid. As a result of their growth, a rise in the pH value of the silage follows course. Fish silage instability has often been associated with yeast growth (Faid *et al.*, 1997). Lindgren and Pleje (1983) reported that the predominant yeasts in fish silage belong to the genera *Saccharomyces* and *Pichia*. Spoilage by moulds can be a problem, especially on the surfaces of the silage that are exposed to air (Raa *et al.*, 1983). Several additives have been proposed to control mould and yeast growth. Propionic acid has been used for this purpose (Johnsen and Skrede, 1981, Strøm and Eggum, 1981, Van Wyk and Heydenrych, 1985). This acid has a specific microbiostatic action against moulds (Jay, 2000). Gildberg and Raa (1977) reported that it has an inhibitory effect upon sporeforming bacteria. It is regarded as effective against yeasts of the genus *Saccharomyces*, but not against *Pichia* (Mc Donald, 1981). The non-dissociated molecule of this acid is necessary for its activity (Mc Donald, 1981). The fungistatic activity of propionic acid is conditioned by the pH of the substrate. The microbiostatic activity of propionic acid is regarded as relevant when the pH of the substrate is below five since the pK_a of this acid is 4.87. Propionic acid has been added to fish silage at the level of 0.75% or 0.3% (Johnsen and Skrede, 1981, Van Wyk and Heydenrych, 1985).

Sorbic acid (or its calcium, potassium and sodium salts) displays antifungal properties. The pK_a of this acid is 4.76 (Jay, 2000). Sorbic acid and sorbates are known for their efficiency against moulds and yeasts, but they are active against a wide range of bacteria too. In general, catalase-positive cocci are more sensitive than the catalase-negative ones and aerobes are more sensitive than anaerobes. LAB have been reported to be resistant against sorbate especially at pH 4.5 or higher (Davidson, 1997, Jay, 2000). This allows their use as fungistatic agents in products submitted to lactic acid fermentation. Sorbate has been added to fish silage as a fungistatic agent at the levels of 0.1% or 3% (Austreng and Åsgård, 1986, Espe *et al.*, 1989, Haaland *et al.*, 1990).

Metabisulfite is currently used as a fungistatic agent in food fermentations. Although no references could be found of its use in fish silage, Wessels *et al.* (1978) used 2% metabisulfite for the preservation of fish residues. At this level of addition it is active against *Clostridium botulinum* and Enterobacteriaceae. Metabisulfite is reported to have a fungistatic action against *Saccharomyces*, *Pichia* and *Candida*. However, LAB are also sensitive to this compound (Davidson, 1997).

Antibiotics such as natamycin proved to be useful for controlling aerobic deterioration in grass silages (Woolford, 1972). However, the use of antibiotics has contributed to the appearance of antibiotic-resistant microorganisms and their high cost would negatively influence the economy of fish silage production.

Chemicals such as ethoxyquin and butylated hydroxytoluene, BHT (phenolic antioxidants) are added to fish silage mainly with the purpose of minimising lipid oxidation (Reece, 1981, Jackson *et al.*, 1984a, Machin *et al.*, 1990, Espe *et al.*, 1992a). Nevertheless, they display some antimicrobial properties. BHT has been reported as having inhibitory activity against bacteria, moulds, yeasts as well as having virustatic properties (Jay, 2000, Davidson, 1997). Spices and extracts from certain plants are known to have antimicrobial activity and they can advantageously replace synthetic compounds in fish silage preparation (Davidson, 1997, Fagbenro and Jauncey, 1994a).

FISH DETERIORATION AND ITS PREVENTION BY LACTIC ACID FERMENTATION

Fish deterioration

Fish spoil quickly by the action of microorganisms, compared with the carcasses of warm-blooded animals. There are at least two reasons for this difference in susceptibility to bacterial spoilage. Firstly, fish tissues become less acid post-mortem than the corresponding tissues of warm-blooded animals. Secondly, fish contains trimethyl amine oxide (TMAO) which stimulates the anaerobic growth of spoilage bacteria (Strøm *et al.*, 1979).

The biochemical processes during microbial spoilage of fish preserved under low available oxygen can be summarised as follows (Strøm *et al.*, 1979). Spoilage bacteria initially use the small amount of free sugars in fish (mainly glucose and ribose) to obtain energy for their growth. While residual dissolved oxygen is still available, they do so by aerobic metabolism, yielding CO_2 and water as the final products. When the small amount of dissolved oxygen is depleted, bacteria resort to anaerobic energy metabolism to obtain energy from the sugars. TMAO can be used by spoilage bacteria as an electron acceptor or oxidising agent when oxygen is depleted. The bacteria gain more energy by this anaerobic energy metabolism with TMAO than by strict anaerobic fermentations. TMAO therefore

stimulates the rate and increases the yield of the growth of bacteria under anaerobic conditions, while giving rise to trimethylamine (TMA), a compound characteristic of the odour of spoiled fish.

Lactic acid is the second choice of energy for bacteria living with access to TMAO. In the absence of TMAO or oxygen, most bacteria cannot degrade lactic acid. Propionic acid bacteria may be able to do it, but they do not play a significant role in fish spoilage. During conversion of lactic acid in the absence of oxygen, TMAO is reduced to TMA while acetate and CO_2 are produced. This redox reaction yields two moles of ATP per mole of lactic acid (Strøm *et al.*, 1979). The quantity of available TMAO is the limiting factor for bacterial growth in lactic acid under anaerobic conditions. When all TMAO has been reduced, lactic acid cannot be further degraded and growth ceases (Raa, 1980).

After the depletion of TMAO there is a succession from facultative anaerobic to strict anaerobic bacteria that degrade amino acids. Ammonia is rapidly produced in this phase. Bacteria derive energy from amino acids under anaerobic conditions by the Stickland reaction, in which one amino acid is oxidised while another is reduced, and ammonia is released from both (Meister, 1965). Several products (H_2S, ptomaines) which have an offensive odour are formed during anaerobic amino acid decomposition, and the objective quality of the fish declines accordingly (Raa, 1980).

Fish preservation by fermentation

During biological ensilage, fish is preserved by the complex action of lactic acid bacteria, as a result of the decrease in pH they cause, of the antimicrobial activity of the non-dissociated lactic acid molecules or the production of other antimicrobial substances such as bacteriocins or H_2O_2.

The low pH favours the action of endogenous proteolytic enzymes leading to autolysis of the fish tissues and liquefaction of the fish silage. The resulting product is semi-liquid, and most of the nitrogen it contains is in the form of amino acids and peptides. A high concentration of free amino acids together with the acid anaerobic environment may favour the formation of biogenic amines if care is not taken to avoid the proliferation of decarboxylase-positive microorganisms. The oxidation-prone poly-unsaturated fish lipids are partly protected in fish silage by the anaerobic environment and by the protective effect of the fermentation by lactic acid bacteria (Raa and Gildberg, 1982).

Proteolysis

Fish silage gradually liquefies as the fish tissue proteins become solubilised due to enzymatic degradation under the favourable pH conditions present within the fish pastes (Raa and Gildberg, 1982). Although related, the liquefaction and proteolysis phenomena are not coincident. In practical terms, it is possible to stop protein degradation before attaining the maximum degree of liquefaction in fish

silages (Jangaard, 1987). At 37°C the liquefaction process in fish silage is completed within two or three days, but proteolysis still continues for about one month (Haard *et al.*, 1985). Liquefaction is regarded as completed when the viscosity drops below five kg $(ms)^{-1}$ (Haard *et al.*, 1985).

Liquefaction and protein autolysis within fish silage are due to the action of enzymes naturally present in the fish (Mc Bride *et al.*, 1961). Enzymes of the digestive tract, mainly pepsin, account for most of the proteolytic activity in fish silage (Tatterson and Windsor, 1974). This group of enzymes gives rise to soluble polypeptides that can be degraded by exopeptidases (Hall *et al.*, 1985a). Besides pepsin activity, another type of enzymatic activity has been reported at pH 4.4 and 5.0, both in eviscerated and non-eviscerated fish. This activity has been attributed to cathepsins. This group of muscle proteases occurs in high levels in the ensiled fish (Siebert and Schmidt, 1965) and they may play an important role in the autolysis of ensiled fish. Trypsin does not seem to play a considerable role in the autolysis of fish silage, since the addition of trypsin inhibitors had only a slight effect upon the proteolytic activity in this system (Van Wyk and Heydenrych, 1985).

The autolytic activity that takes place during fish silage fermentation and storage leads to an increase in the levels of ammonia, amines, amino acids and peptides. According to Haard *et al.* (1985), the first stage of autolysis is characterised by an increase in the oligopeptide amount. By the end of this stage, oligopeptides account for 30-40% of the organic nitrogen in the fish silage. However, one or two weeks later, these compounds account only for a negligible portion of the organic nitrogen present in the silage. This decrease in the level of oligopeptides probably corresponds to their degradation into smaller peptides and amino acids, and a concomitant increase in the buffering capacity of the material occurs. As a result, an increase in the pH of the silages may follow (Sinnel, 1980).

In a successful silage, storage for six months has very little effect upon the amino acid composition although several authors mention some formation of volatile compounds as a result of limited amino acid degradation (Nilsson and Rydin, 1963, Gildberg and Raa, 1977, Jackson *et al.*, 1984b). In successful silages, volatile nitrogen compounds are formed in minimal quantities and consist mainly of ammonia that originates from amino acid deamination. It was initially thought that this deamination would affect all amino acids present and that it did not significantly affect the amino acid composition of the silages (Raa and Gildberg, 1976). It has been reported that the main source of ammonia is the amide group of glutamine and possibly of asparagine. Nutritional tests performed with rats showed that this ammonia formation did not lead to a decrease in nutritional value of the silages (Espe *et al.*, 1989). In poorly preserved silages (insufficient acidification), ammonia is only partly responsible for the volatile nitrogen evolved. In this case, amino acid composition is severely affected. Arginine and tyrosine are the most affected and trimethylamine is formed as well (Haaland and Njaa, 1989).

Biogenic amines as a potential risk in fish silage

Although biogenic amines are needed for many critical functions in man and animals, consumption of food containing high amounts of these amines can have toxic effects. The most notorious food borne intoxications caused by biogenic amines are related to histamine. Histamine poisoning is often referred to as scombroid fish poisoning because of the frequent association of this illness with the consumption of scombroid fish such as tuna, mackerel and sardines (ten Brink *et al.*, 1990).

Symptoms of histamine poisoning that may occur after excessive oral intake are nausea, respiratory distress, hot flush and/or headache. More serious complications such as cardiac palpitations are rare (Lahsen, 1991). The toxic effect depends on histamine intake dose, presence of other amines, amino oxidase activity and the intestinal physiology of the individual. A fairly efficient detoxification mechanism exists in the intestinal tract of mammals, which is capable of metabolising normal dietary intakes of biogenic amines. Under normal conditions, exogenous amines from food are rapidly detoxified by the action of amine oxidases or by conjugation, but in the case of allergic individuals or if amine oxidase inhibitors are used or when too high levels are consumed, the detoxification process is disturbed and biogenic amines accumulate in the body (Silla-Santos, 2001). Other biogenic amines, such as putrescine, cadaverine and tyramine, inhibit the histamine-metabolising enzymes diamine oxidase and histamine N-methyl-transferase so their presence in food will enhance the toxic effects of histamine (Taylor and Sumner, 1986).

The formation of histamine in scombroid fish and other fish, containing abundant free histidine, has been attributed to microbial action rather than to endogenous histidine decarboxylase activity (Baranowski, 1985, Halász *et al.*, 1994).

Only a few bacterial species are capable of prolific histamine formation during fish silage. These are mainly *Morganella morganii*, some strains of *Klebsiella pneumoniae* and *Lactobacillus büchneri*. The ability to produce putrescine and cadaverine appears to be more widespread, especially among the Enterobacteriaceae, since many bacterial species possess ornithine decarboxylase and/or lysine decarboxylase. *Morganella morganii* has been implied as causative organism in the formation of toxicologically significant levels of histamine in fish (Ieniştea, 1971, Wei *et al.*, 1990).

The use of a starter culture in dry sausage production significantly decreased the levels of histamine, tyramine and cadaverine formed (Maijala *et al.*, 1995). Some lactic acid bacteria were found to degrade histamine or tyramine (Voigt and Eitenmiller, 1978, Leuschner *et al.*, 1998). The use of starter cultures with the ability to degrade biogenic amines could be useful in controlling the risk posed by these compounds during the ensilage of fish. The main factors affecting biogenic amine production by a strain of *Enterococcus faecalis* were pH, NaCl

concentration and fermentation temperature (Halász *et al.*, 1994, Silva *et al.*, 1998, Gardini *et al.*, 2001). These factors could also provide useful means of controlling the formation of biogenic amines in fish silage.

Alterations in the fish lipids during ensilage

Fish lipids are highly unsaturated and thus extremely prone to oxidation. Carbonyl compounds formed as a result of the degradation of hydroperoxides resulting from the oxidation of fish lipids can react with the protein (Gardner, 1978), causing a reduction in the nutritive value of the silages (Wirahadikusumah, 1968, Kompiang *et al.*, 1980). Lipid oxidation products may accumulate in the tissues of the animals fed with oxidised lipids, causing carcass taints (Raa and Gildberg, 1982).

Since lipid oxidation leads to decreased nutritional value of fish silages, it is of major importance to find efficient ways of minimising its extent. Synthetic antioxidants such as BHT or ethoxyquin have been widely applied (Kritzinger *et al.*, 1979, Torrissen *et al.*, 1981, Ahmed and Mahendrakar, 1996b). Formaldehyde addition displayed a protective effect on fish lipids in acid fish silages (Haard *et al.*, 1985). The high price of synthetic antioxidants together with the growing demand for "natural products", even in animal feeds, makes the use of these additives less advantageous. Spices and vegetable extracts have demonstrated antioxidative properties in fish products (Larson, 1988) and can advantageously replace synthetic antioxidants in fish silage preparation (Fagbenro and Jauncey, 1994a). Lactic acid fermentation by itself is regarded as having a beneficial effect upon the lipid fraction of fish silages, stabilising the oil and even improving its acceptability in animal feeds (Raa and Gildberg, 1982). Lactic acid bacteria have been found to act as scavengers of reactive oxygen species (Bonestroo, 1992, Sanders *et al.*, 1995, Lin and Yen, 1999, Stecchini *et al.*, 2001). It is possible to remove the oil from the silage, to flush the silage headspace with inert gases, such as nitrogen or to design the silage containers in a way that excludes air admission during storage (Potter *et al.*, 1978, Windsor and Barlow, 1981, Jangaard, 1987).

PRACTICAL APPLICATIONS OF FISH SILAGE

Fish silage finds its major use in the area of animal nutrition, where it is mainly used as a nitrogen source. In studies published as early as 1942, Olsson described feeding experiments with hens using this type of product (Jangaard, 1987). Fish silages can be incorporated into feeds intended for several types of animals: swine, fur animals, fish, fowl and even cattle and sheep. Fish silage has been described as having good nutritional quality and its biological nitrogen value is comparable to that of skim milk powder or fish meal (Raa and Gildberg, 1982).

According to Van Lunen (1987), fish silage has a great potential as nitrogen source for swine and fur animals. Reports on fish silage feeding trials with

acid silages fed to swine have led to good results (Lisac, 1961, Trow-Smith, 1974, Whittemore and Taylor, 1976, Rangkuti *et al.*, 1980, Batterham *et al.*, 1983, Tibbetts *et al.*, 1988, Myer *et al.*, 1988, 1990, Espe and Haaland, 1992, Kjos *et al.*, 1999). However, problems with carcass tainting have been reported in some cases (Windsor and Barlow, 1981, Green, 1989). The level of fish lipids in the diet should not exceed 1% of the dry matter content of the feed, in order to avoid such problems (Raa and Gildberg, 1982). Another possibility is to stop feeding silage some time before slaughtering (Green, 1989). Biological silages have found successful application in swine feeding (Faid *et al.*, 1997). Feeding trials with mink (Rouvinen *et al.*, 1996, Clausen *et al.*, 1999, White *et al.*, 1999) and foxes have yielded good results (Ulvestad, 1987). Fish silage was well accepted by the animals and gave comparable or even better results than the commercial feed (Jangaard, 1987). The absence of histamine and low levels of lipid oxidation products are essential when fish silage is to be fed to fur animals. The presence of these toxic products can decrease the quality of the furs (Jangaard, 1987). Some authors have found limitations in the use of fish silage for fur animals. Winter and Feltham (1983) reported that these animals can be sensitive to low pH. Mink can tolerate a pH of 5.5 and foxes a pH of 5.8. On a total feed basis, 10-30% fish silage can be included without negative effects in the diets of growing foxes and mink, but it should be withdrawn during the reproduction period (Van Lunen, 1987).

Although swine and fur animals are the biggest potential consumers of fish silage, its use for feeding other animals can also be envisaged. In one of the first published studies on fish silage, Petersen (1953) recommended fish silage incorporation levels for fowl and cattle diets. For laying hens, 20g fish silage a day was recommended, whereas breeding birds could have up to 30 g fish silage a day. In duck diets, fish silage can be added up to 10% of the total weight of feed. One kg fish silage a day was indicated as appropriate for lactating cows (Petersen, 1953). Biological fish silages have led to good results in fowl compared with the conventional nitrogen sources (Rattagool *et al.*, 1980a, b, and c, Brown and Sumner, 1985, Sainclivier, 1985, Hassan and Heath, 1987, Ologhobo *et al.*, 1988, Rodriguez *et al.*, 1990, Guevara *et al.*, 1991, Bigueras-Benitez and Nacorda, 1992, Ahmed and Mahendrakar, 1996b, Vizcarra-Magana *et al.*, 1999) and their nutritional value was found to be significantly better than the value of acid silages (Kompiang *et al.*, 1980). Good results were also obtained when fish silages were incorporated in ruminant feeds. Shqueir *et al.* (1984) reported that adding an ensiled fish supplement to the normal basal ruminant diet improves the digestibility of organic matter, protein and crude fibre. Wet *et al.* (1982) showed that ensiled fish can be used as an ingredient for the preparation of replacement milk for sheep. Chirase *et al.* (1985) suggest the use of fish silage as a supplement to wheat straw silage. Higher nitrogen retention and utilisation values were obtained when this silage mixture was fed to sheep. Fontenot *et al.* (1992) and Van Wyk and Heydenrych (1985) reported good results when biological silages were fed to

cattle. However, Winter and Feltham (1983) considered that ruminants should not be regarded as the preferential consumers for fish silage. Due to the microbial activities that take place in the rumen, leading to the breakdown of amino acids, those animals can use less efficiently the nitrogen nutrients from fish silage than a monogastric animal, which can absorb directly the organic nitrogen. As a way of overcoming this problem, also present in some traditional protein sources, Johnsen and Skrede (1981) and Haard *et al.* (1985) suggest the addition of formaldehyde to the silages in order to inhibit amino acid breakdown in the rumen.

The use of fish silages in aquaculture (salmon, trout, and carp) is advantageous. Being a wet feed, fish silage is more similar to the natural diet of the fish than the alternative commercial dry feeds (Ulvestad, 1987). Good results were obtained when feeding sweet water fish with fish silage (Djadjasewaka and Djadjadiredja, 1980, Hardy *et al.*, 1983, 1984, Åsgård and Austreng, 1981, 1985, Wood *et al.*, 1985, L'Aventure, 1987, Lapie and Bigueras-Benitez, 1992, Ali *et al.*, 1994). Studies with sea-bream and catfish gave promising results (El-Hili, 1989, Fagbenro and Jauncey, 1994b). Biological fish silages are regarded as a safe feed for fish. They do not provide a suitable environment for fish pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* (Smail *et al.*, 1990, Lo *et al.*, 1993, Whipple and Rohovec, 1994).

Fish silage can also be used as a fertiliser for ornamental plants (Jangaard, 1987), in horticulture (Brown and Sumner, 1985, Drebot, 1987) or pasture fertilisation (Synnes and Opstadt, 1995). Other liquefied fish products are already in use, for ornamental plants (Jangaard, 1987). The results obtained with fish silage-fertilised cabbage, cauliflower and broccoli were very similar to the results obtained with the usual commercial products (Drebot, 1987). It has also been applied as a fertiliser to forests (Prescott *et al.*, 1996, Prescott and Brown, 1998).

QUALITY CRITERIA FOR FISH SILAGE

The concept of fish silage quality is difficult to define. Each group of potential users of this product has its own set of requirements. For fowl, cattle and swine, the silages must have low oil content in order to avoid tainting (Green *et al.*, 1988). For mink and foxes, it is important to ensure the absence of lipid oxidation products, histamine and thiaminase, as well as an appropriate pH value, to guarantee the quality of the furs (Anglesea and Jackson, 1985, Skrede and Nes, 1988). In general terms a fish silage can be considered of good quality, keeping stable for more than six months, without changes in odour, free from pathogenic or deterioration microorganisms, and exempt from toxic products when the following requirements were fulfilled during production (Yeoh, 1980):

- A quick decrease in pH, from 6.0-6.5 until values below 5.0 within 2 –3 days. The fastest the decrease, the most stable the silage will be.

- Obtention of high amounts of lactic acid. Generally, the concentration of lactic acid in the silage raises abruptly during the first two days and remains

approximately constant thereafter. Final lactic acid concentrations ranging from 3.7 – 4.0% (w/w) have been reported as necessary for ensuring the preservation of the silage (Lassén, 1995c).

- A minimal formation of volatile nitrogen. In well-preserved acid silage, volatile nitrogen formation reaches up to 30 – 40 mg N (g total N)$^{-1}$ (Haaland and Njaa, 1989). At this level of volatile nitrogen formation, only the amide N from certain non-essential amino acids is volatilised.

- Low numbers of coliforms and sporeforming bacteria. In well-preserved silages these microbial contaminants are not detected beyond the first 2 – 3 days of ensilage (Ahmed and Mahendrakar, 1996a).

- Emission of minimal amounts of gas. An increase in the volume of silage of 105 – 112% has been reported to occur during the first day of fermentation (Faid *et al.*, 1994). The composition of the gas was not determined, but it was assumed to be CO_2 arising from microbial utilisation of the added carbohydrate.

Table 2. Critical pH values for biological fish silage preservation and maximum delays for its obtention.

pH value	Maximum delay (days)	Reference
4.0	2	Ahmed and Mahendrakar, 1996a Faid *et al.*, 1997
	3 – 4	Petersen, 1953
4.5	2	Sainclivier, 1985 Hassan and Heath, 1986 Adams *et al.*, 1987 Lassén, 1995a
	2 – 3	Van Wyk and Heydenrych, 1985
	3	Kompiang *et al.*, 1980
5.0	–	Yeoh, 1980

The pH decrease is the key factor for the success of the process, since it affects both the inhibition of undesirable microorganisms and the extent to which protein is degraded. The pH decrease and its permanence at low values are regarded as being most responsible for the control of deterioration phenomena in fish silage (Owens and Mendoza, 1985, Faid *et al.*, 1997). Table 2 summarises the pH values regarded as critical by different authors and indicates the maximum delay to their obtention.

SCOPE AND MOTIVATION FOR THIS THESIS

This thesis deals with safety and functionality aspects of the biological ensilage of fish. The main objective was to study the potential of biological ensilage with lactic acid bacteria for the preservation of fish and to devise strategies leading to an increase of the safety and quality of the silages obtained.

The extent of proteolysis and lipid oxidation strongly influence the nutritive value of fish silage. Proteolysis and lipid oxidation in fish silage are studied in chapter 2. The establishment of an adequate LAB microflora plays a major role in the biological ensilage of fish. For this reason, aspects related to the LAB in fish silage assumed a central role in the development of this thesis. The need for a starter culture in order to obtain stable fish silages is established in chapter 4. The potential of different LAB species as starter cultures for the biological ensilage of fish is studied in chapter 3. Isolation of LAB from naturally-fermented fish substrates, studying their growth, acidification, antimicrobial properties against pathogens and deterioration microflora with the aim of selecting potential starter cultures was carried out in chapter 4. Detailed studies on the growth, acidification and competitive properties of potential starter cultures were the object of chapter 6.

Fermentation conditions (temperature, Eh, and additives) can strongly influence the outcome of the ensilage process, both by influencing the rate of various chemical reactions, the growth of the various microbial contaminants, LAB growth and metabolic activities. In chapter 3, the influence of fermentation conditions on silage stability is analysed. The influence of fermentation conditions on starter growth, acidification and competitive properties is assessed in chapter 7.

Biogenic amines may constitute a potential risk in biological fish silage. In this thesis, several strategies for the control of biogenic amine formation have been considered. In chapter 4, the antimicrobial properties of LAB isolates against biogenic amine producing microorganisms is studied. In chapter 5, an innovative approach to the control of histamine is proposed, based on the ability of selected LAB strains to degrade this compound. The effect of fermentation conditions on the growth of biogenic amine producing microorganisms is studied in chapter 8.

Chapter 9 presents a general discussion and the conclusions from the present thesis.

Chapter 2

Lipid and protein changes during the ensilage of blue whiting (*Micromesistius poutassou* Risso) by acid and biological methods

ABSTRACT

Careful control of the degree of proteolysis and lipid oxidation is required to produce fish silages of high nutritional value. This chapter studies the changes in lipids and protein during storage (15 days) of acid silages (with 0, 0.25 and 0.43%, w/w, of formaldehyde) and biological silages (with 10 and 20% molasses or dehydrated whey) prepared from blue whiting. A remarkable reduction in protein solubilisation values was achieved by adding formaldehyde. However, formaldehyde addition led to an increase in the peroxide value of the oil extracted from the silages. Ensiling by biological methods seems promising. It yielded both a considerable reduction in protein solubilisation and in basic volatile nitrogen when compared with acid ensilage. Furthermore, the oil from biological silages had lower peroxide values than the oil from acid silages with added formaldehyde.

INTRODUCTION

Fish silage is a promising product that has already found some industrial application (Espe *et al.*, 1992a) and its use can become widespread. The presence of oil with high levels of polyunsaturated fatty acids (PUFA), thus very prone to oxidation, is one of the constraints for its broad use in animal feeding. Oil retained in fish silage can become oxidised, rendering the feed unpalatable or unsafe to livestock (Haard *et al.*, 1985). Several antioxidants have been used to prevent deterioration of the lipid fraction of the silages (Machin *et al.*, 1990, Espe *et al.*, 1992a and b). De-oiling is also possible and may be desirable (Potter *et al.*, 1976). Formaldehyde can contribute to the inhibition of lipid oxidation in acid-preserved fish silages (Haard *et al.*, 1985) and its content was found to decrease during the storage of fish (Lovern, 1965). Lactic acid fermentation has a beneficial effect on

Enes Dapkevicius, M. L., Batista, I., Nout, M. J. R., Rombouts, F. M. and Houben, J. H. *Food Chemistry* 63 (1) 97 – 102, 1998.

the lipids in fish silage, stabilising the oil and improving its acceptability in animal diets (Raa and Gildberg, 1982).

During ensilage, autolysis takes place and the fish gradually liquefies as the protein matrix partially solubilises due to its breakdown by endogenous enzymes (Raa and Gildberg, 1982). The autolytic activity occurring during the ensilage of fish leads to an increase in the concentrations of ammonia, amines, amino acids and peptides. Up to 90% of the organic nitrogen becomes solubilised in acid-preserved fish silages (Haard *et al.*, 1985), whereas ensiling by the biological method yields solubilisation values of around 60-70% (Hassan and Heath, 1986 and 1987).

Feeding trials performed on several species of monogastric animals showed that it might be advantageous to have some pre-digested protein in the diet, but there is a limit above which the animals would have difficulties in using the absorbed protein for assimilative purposes. However, no decreases in weight gains were observed when chickens were fed diets in which autolysed fish silages contributed up to 400 g kg^{-1} of the total dietary protein (Espe *et al.*, 1992a).

The degree of hydrolysis of the protein in fish silage is likely to result in a lower nutritive value for ruminant livestock. The small peptides and amino acids formed during ensilage are more readily available to the rumen microflora. When metabolised by the microflora in the rumen, the efficiency of nitrogen utilisation by the animals can decrease and toxicity problems can occur, particularly when the animal is on a low energy diet (Ørskov, 1977). The lower degree of hydrolysis in biological fish silages can be regarded as advantageous for these animals from the nutritional point of view.

When applied to soybean oil-meal, formaldehyde increased the flow of dietary amino acids in the intestine of adult ruminants by 50-90% (Verité *et al.*, 1977). A commercial mixture containing formaldehyde is available as an additive for ensiling grass (Delort-Laval, 1985). Relatively low concentrations of formaldehyde were found to be sufficient to arrest protein hydrolysis in acid-preserved and biological fish silages (Haard *et al.*, 1985, Fagbenro and Jauncey, 1994a). This finding is important, since undesirable side effects of excessive formaldehyde in ruminant rations, such as decrease in voluntary intake and loss in the efficiency of utilisation of dry matter, fibre and nitrogen can occur (Haard *et al.*, 1985).

The present work aims at comparing the effects on the nitrogen fraction and the lipids of blue whiting (*Micromesistius potassou* Risso) during ensiling by acid or biological methods. Formaldehyde addition is tested in the acid-preserved silages as a means of controlling lipid and protein degradation and two carbohydrate sources (molasses and dehydrated whey) are used for the preparation of biological fish silages.

MATERIALS AND METHODS

Silage preparation

Blue whiting (*Micromesistius poutassou* Risso) was caught near the Portuguese coast by the research ship of the Portuguese Institute for Fisheries and Marine Research, Lisbon, and kept frozen in plastic containers (-28°C) for several months. Prior to ensiling, whole fish were thawed for 16h at ambient temperature and minced in a Hobart meat grinder. The additives were incorporated using a kneading machine. Three-kilogram batches of several types of silages were prepared, according to the formulas described in Table 1.

Table 1. Formulas for the preparation of acid and biological silages from blue whiting (*Micromesisitius poutassou* Risso).

Designation	Composition	Proportions (on a weight basis)
AS 0	ground raw fish+formic acid	97:3
AS F0.25	ground raw fish+formic acid +formaldehyde	96.75:3:0.25
AS F0.43	ground raw fish+formic acid +formaldehyde	96.57:3:0.43
BS M10	ground raw fish+molasses+water+starter	55:10:15:20
BS M20	ground raw fish+molasses+ water+starter	45:20:15:20
BS W10	ground raw fish+dehydrated whey+ water+starter	55:10:15:20
BS W20	ground raw fish+dehydrated whey+ water+starter	45:20:15:20

The silages were stored in plastic bags at ambient temperature (± 20° C) during the 15 days of the experiment.

Formaldehyde was added as formalin (SIGMA, 37% formaldehyde).

Dehydrated whey was obtained from a dairy plant (Lacto-Açoreana, Azores, Portugal) and sugar beet molasses from a sugar plant (SINAGA, Azores, Portugal). The dehydrated whey contained 3% moisture, 1.5% fat, 11.5% protein, 75% lactose and 9% ash. A 10% solution of dehydrated whey had a pH of 6.0-7.0.

The starter used was prepared by inoculating a ground, cooked fish/molasses/water mixture (55:20:15, w/w/w) with 10% (w/w) of a *Lactobacillus plantarum* MRS broth culture and incubating the mixture for 72h at 35°C. After incubation, the mixture had a pH of 4.19. The *Lb. plantarum* culture available at the Portuguese Institute for Fisheries and Marine Research had been in use in olive fermentation. It was subcultured three times in MRS broth prior to use.

Analytical determinations

Moisture, crude protein, crude fat and ash were determined by standard procedures (AOAC, 1984). For the determination of pH values, 5 g of sample were homogenised with 50 ml distilled water and measurements were made with a

combined glass calomel electrode. Viscosity was determined with a synchro-lectric viscometer (model LVF, Brookfield Engineering Laboratories, Inc., UK). Non-protein nitrogen (NPN) was determined by the micro Kjeldahl method in trichloroacetic acid (7%) extracts of the silages and in extracts of fresh, ground fish. Total volatile basic nitrogen (TVBN) was determined by the microdiffusion method of Conway (Anon., 1988). For the determination of amino acids, well homogenised samples of silages and fresh, ground fish were hydrolysed in 6N HCl at 110°C for 24h. After removing the acid by means of evaporation in a rotovapor, a known volume of sodium citrate buffer (pH 2.2) was added and the samples were filtered through Millipore membranes (USA) with pores of 0.22 µm. The samples were then injected into an amino acid analyser (Alpha 4150-LKB, Biochrom, Sweden), equipped with an integrator. The peroxide value of oil extracted from freshly ground fish and from silage samples was determined iodometrically, according to a standard procedure (AOCS, 1993). For the extraction of the oil, the method of Bligh and Dyer (1959) was used. All determinations were made in duplicate.

RESULTS AND DISCUSSION

Proximate composition

Table 2 shows the proximate composition of the different silages at the beginning and the end of the experimental period.

A slight increase in moisture during fermentation was observed for the biological silages. This could be a result of the microbial activity within the silages. The increases in fat and protein proportions in the dry matter of biological silages during storage can be explained by the utilisation of carbohydrates during the fermentation process.

The differences in composition observed for the acid fish silage are not easy to interpret. Formaldehyde addition to fish brings about considerably complex changes. Proteins tend to aggregate and lose solubility, mainly due to the formation of noncovalent bonds among molecules (Owusu-Ansah and Hultin, 1987, Ang and Hultin, 1989). The amount of total lipids decreases, hydrolysis occurs in the neutral lipid, esters of stearic acid and phospholipid fractions, whereas the proportion of lipid-protein complexes increases in fish preserved by formaldehyde (Ostyakova and Kosvina, 1975). The low values of crude fat in the silage with 0.25% formaldehyde could result from the degradation of lipids. The concentration of crude fat in silages with 0.43% added formaldehyde is higher than in the acid silage without formaldehyde. A possible explanation for this might be that the fat extraction method used would not be able to disrupt completely the increased level of lipid-protein complexes.

Table 2. Proximate composition of blue whiting before and after ensilage by different methods.

Sample*	Storage time (days)	Moisture (g $100g^{-1}$)	Protein (g $100g^{-1}$ dry matter)	Fat (g $100g^{-1}$ dry matter)	Ash (g $100g^{-1}$ dry matter)
AS 0	0	76.1	70.7	11.3	13.4
	15	77.2	71.9	14.9	11.8
AS	0	77.7	64.6	7.2	17.0
F0.25	15	76.4	64.5	7.2	19.9
AS	0	76.3	65.3	13.9	14.8
F0.43	15	75.7	69.2	13.6	16.9
BS M10	0	74.6	55.9	9.1	15.4
	15	78.7	62.4	14.6	10.3
BS M20	0	71.2	39.9	4.9	11.8
	15	74.7	44.3	10.3	11.9
BS W10	0	74.4	53.5	10.5	12.1
	15	76.2	56.7	14.7	8.8
BS W20	0	68.4	40.2	2.5	11.7
	15	70.4	42.9	7.1	10.8

* For abbreviations, see Table 1.

Viscosity

The formaldehyde-treated silages did not liquefy during the observation period. For this reason, their viscosity was not measured. The viscosity values represented in Fig. 1 concern only the acid silage with no formaldehyde added and the biological silages. The liquefaction of AS 0 was already completed at t=0, whereas the other silages were completely liquefied within the first three days. Biological silages prepared with the addition of dehydrated whey showed a somewhat higher final viscosity level than the other silages.

Changes in pH

The evolution in pH values after 15 days for the acid and biological fish silages is shown in Fig. 2. The final values obtained for the acid and biological silages differ considerably. It should be kept in mind however, that the acid responsible for preservation is not the same in both cases, and the pH the silages should reach to be stable is determined by the pKa of the preservative acid (Raa

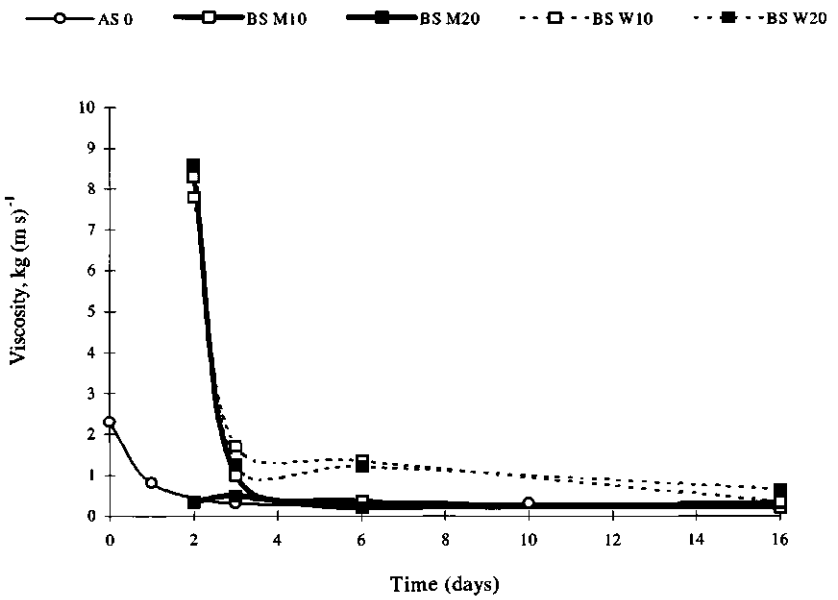

Fig. 1. Viscosity changes in blue whiting (*Micromesistius poutassou* Risso) ensiled by various methods.

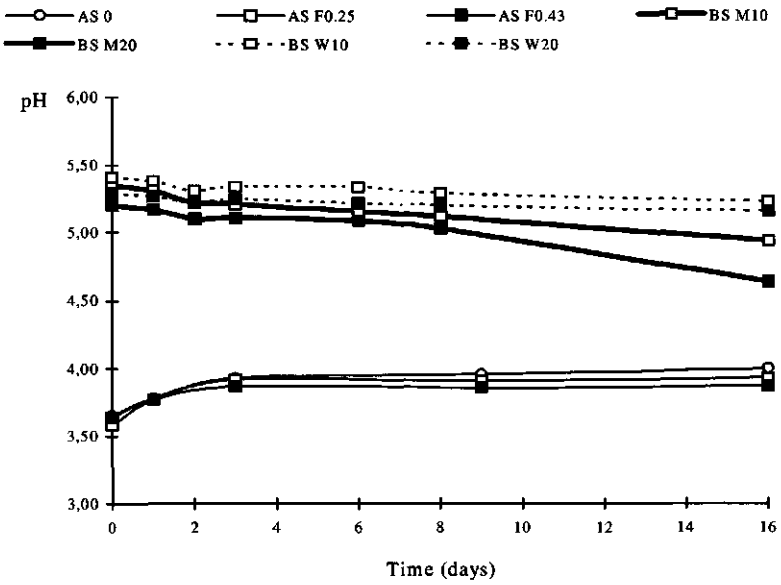

Fig. 2. pH changes in blue whiting (*Micromesistius poutassou* Risso) ensiled by various methods.

and Gildberg, 1982). The values reached in the acid silages in this experiment are within the range of acceptability (Raa and Gildberg, 1982). Final pH values ranging from 4.0 – 5.0 are recommended in the literature for biological silages (Ahmed and Mahendrakar, 1996a, Faid *et al.*, 1997). The values we obtained are too high to ensure long-term preservation. The high pH values may be due to the relatively low temperature used for fermentation and/or to inefficiency of the starter culture used. Several species of the genus *Lactobacillus* have temperature optima ranging from 30 to 40°C (Kandler and Weiss, 1984), so the use of higher fermentation temperatures could be beneficial. Also, lactic acid bacteria are known to vary in their ability to stabilise fish silage (van Wyk and Heydenrych, 1985); the selection of efficient starter cultures is a very important step for improving the keeping quality of biological fish silage.

Changes in the protein fraction

Protein solubilisation values, as expressed by the non-protein nitrogen content of the silages, are represented in Fig. 3. Protein solubilisation proceeded faster during the first two days (except for the biological silage with 20% added whey), but was still continuing at the end of the experiment.

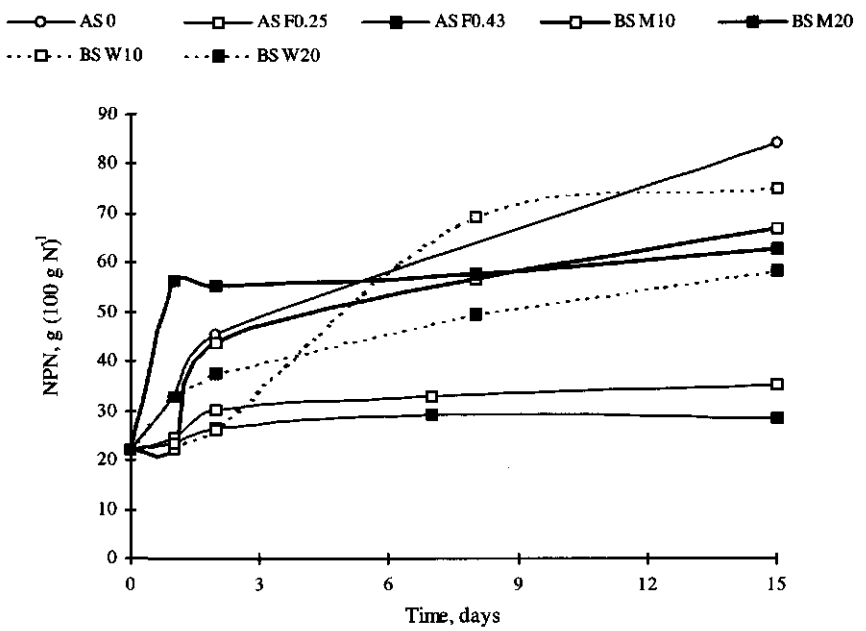

Fig. 3. Non-protein nitrogen changes in blue whiting (*Micromesistius poutassou* Risso) ensiled by various methods.

As expected, formaldehyde addition yielded remarkably low nitrogen solubilisation values. After 15 days of storage, the values obtained were 35 and 27% for the silages with 0.25 and 0.43% (w/w) of added formaldehyde, respectively. These values do not differ considerably from the initial concentration of non-protein nitrogen (about 24%). Ensiling by biological methods also afforded lower protein solubilisation values when compared with the acid ensilage without formaldehyde addition. Values ranging from 58 to 67% non-protein nitrogen were obtained for the biological silages, whereas the corresponding value for the acid silage prepared without formaldehyde addition was 84%. The silage prepared by adding 10% molasses yielded much higher nitrogen solubilisation values than the other biological silages (75%). The reasons for the lower levels of protein solubilisation in biological fish silages have not yet been established, but could be related to the different pH values, that could lead to differences in the activity of tissue proteases.

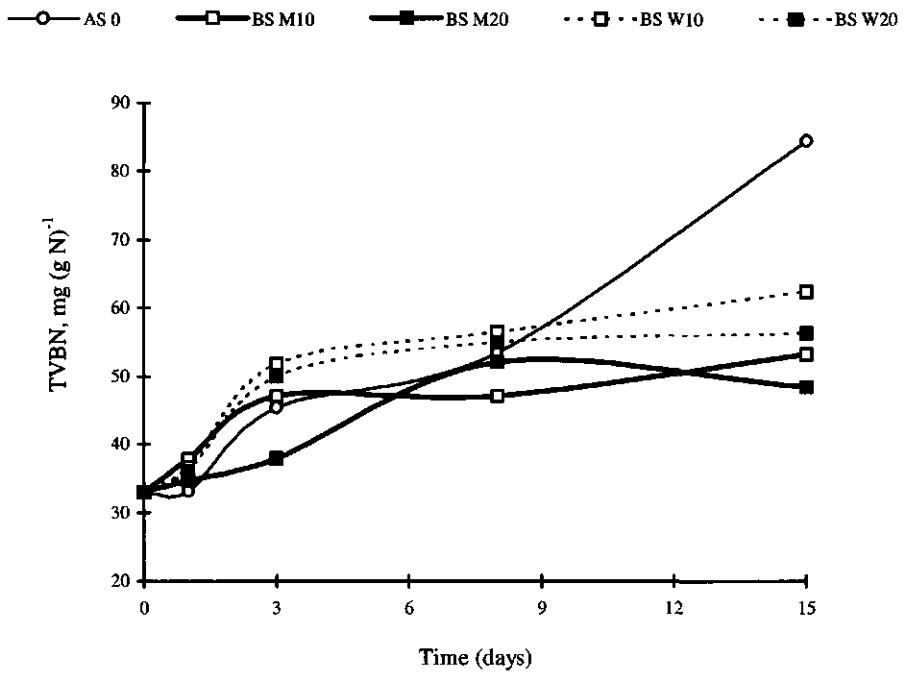

Fig. 4. Total volatile basic nitrogen changes in blue whiting (*Micromesistius poutassou* Risso) ensiled by various methods.

Fig. 4 represents the evolution of total volatile basic nitrogen (TVBN) in blue whiting ensiled by the acid and the biological methods. TVBN measures the amount of volatile bases formed from the solubilised nitrogen derivatives. Because the solubilisation values of the silages with added formaldehyde were considerably

low, total volatile nitrogen was not measured for these trials. After 15 days, the acid silage with no added formaldehyde had much higher levels of TVBN (84 mg N 100 g^{-1} sample) than the biological silages (48 - 62 mg N 100 g^{-1} sample). Since it leads to much lower formation of TVBN, biological ensilage can be considered as advantageous when compared with the acid procedure. The formation of higher contents of TVBN may lead to a reduction in the content of amino acids that could have negative consequences on the nutritive value of the silage.

The amino acid composition of these samples after 15 days of storage did not show considerable differences when compared with the amino acid composition of fresh blue whiting (table 3). The only exception was alanine in the case of the acid silage without added formaldehyde. It may be concluded that the source of the volatile nitrogen in the various types of silages tested might not be the same. It is proposed that, in biological silages, a small-scale deamination of the amino acids must have occurred, while in the acid silage bulk degradation of cystine may have led to the formation of a considerable amount of ammonia and/or volatile amines. While small-scale deamination of the amino acids is unavoidable and may not affect the nutritive value of the silages, heavy losses in one or a few essential amino acids will change substantially both the composition of the silage and its nutritional value (Haaland and Njaa, 1989).

Table 3. Concentrations of different amino acids (g amino acid 16 g^{-1} N) in blue whiting (*Micromesistius poutassou* Risso), fresh and ensiled by acid and biological methods.

Amino acid	Fresh fish	AS 0*	BS M10*	BS M20*	BS W10*	BS W20*
Aspartic acid	8.132	8.101	7.655	7.113	8.234	6.354
Threonine	3.647	3.598	3.247	2.817	3.781	2.890
Serine	3.383	3.692	3.211	2.878	3.591	2.843
Glutamic acid	13.210	13.219	11.493	10.426	13.402	9.969
Proline	3.096	3.083	2.289	2.678	3.226	2.559
Glycine	4.725	5.479	4.401	4.487	4.263	3.811
Cystine	0.766	0.142	0.620	0.643	0.526	0.520
Alanine	5.275	5.450	4.648	4.487	4.985	3.780
Valine	4.449	4.278	3.880	3.617	4.380	3.362
Methionine	2.581	2.532	2.225	1.904	2.372	1.764
Isoleucine	3.988	3.787	3.373	2.965	3.803	2.992
Leucine	6.713	6.533	5.739	5.183	6.730	5.039
Tyrosine	2.725	2.822	2.394	1.835	2.672	1.732
Phenylalanine	3.515	3.473	3.092	2.574	3.365	2.764
Histidine	2.198	2.089	1.803	1.400	2.000	1.276
Lysine	6.814	7.207	6.063	5.400	7.066	5.079
Arginine	4.515	ND	6.331	5.061	5.372	ND

* For abbreviations, see Table 1.
ND = not determined.

It should be noted that the acid hydrolysis method used for the determination of amino acids leads to a partial destruction of cystine. All values shown in table 3 for this amino acid are lower than its actual concentration in the silages. However, because this affects equally all of the results obtained for cystine, the comparison between different types of silages is still possible.

Lipid oxidation

The changes in peroxide values are depicted in Fig. 5. Although formaldehyde has been claimed to inhibit lipid oxidation (Haard *et al.*, 1985), the oils obtained from silages with added formaldehyde had substantially higher peroxide values than the oils from acid silage without added formaldehyde or from biological silages. Formaldehyde was found to increase the decomposition of the lipid molecules into their monomers and to notably increase lipid oxidation by Ostyakova and Kosvina (1975), which is in agreement with these findings. It should also be mentioned that the initial peroxide values of the samples were already high (about 18 mEq kg^{-1} oil). The presence of relatively high levels of peroxides from the very beginning of the process contributes to a faster progression of the free radical chain reaction leading to increased peroxide formation, as pointed out by Hsieh and Kinsella (1989).

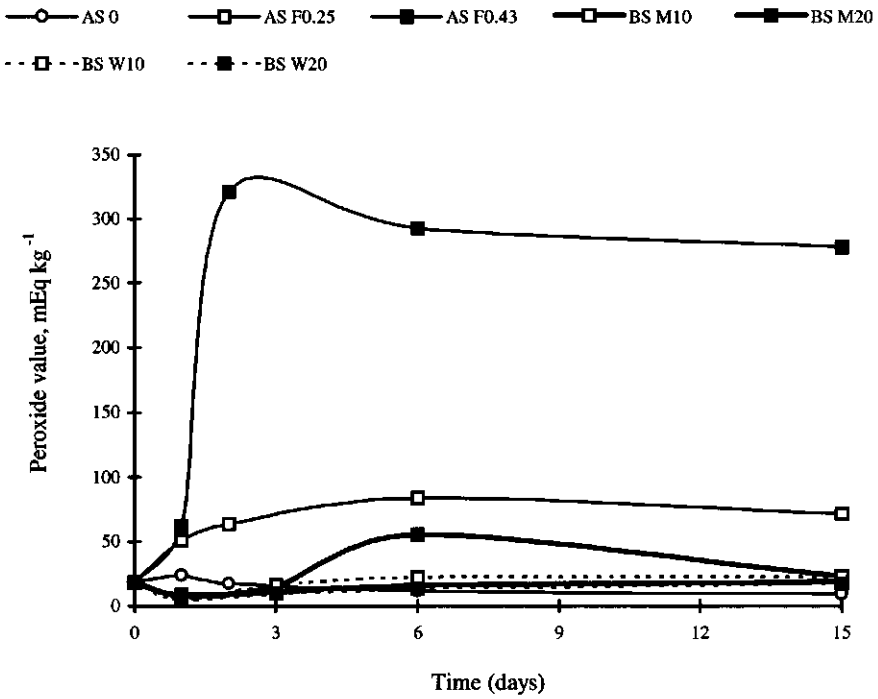

Fig. 5. Peroxide value changes in oil extracted from blue whiting (*Micromesistius poutassou* Risso) ensiled by various methods.

CONCLUSIONS

Although ensiling with formaldehyde resulted in a marked reduction in protein solubilisation, ensiling by biological methods seems to be a promising technique, as it led to lower peroxide values than ensiling with formaldehyde. It also yielded a considerable reduction in NPN and TVBN when compared with the current acid preservation technique. The biological procedure needs, however, further improvement leading to faster and more pronounced decreases in the pH values. The use of a higher fermentation temperature might be desirable and the choice of a suitable starter culture is of the utmost importance.

Chapter 3

Optimisation of fermentation conditions for ensiling blue-jack mackerel (*Trachurus picturatus* Bowdich)

ABSTRACT

The success of fish silage production is affected by a number of factors. In the present work, fermentation temperature, type of starter and potential additives were studied with the aim of improving the efficiency of the biological ensilage of fish.

Acid silages had stable pH values and low emission of volatile nitrogen. However, they displayed high final Non-Protein-Nitrogen (NPN) values, high numbers of fungi and were less homogenous than the biological silages prepared from the same material. Ensiling by the biological method appears to be more advantageous.

Starter cultures were selected among eleven lactic acid bacteria (LAB) isolates on the basis of their fast growth and acidification rates, ability to ferment sucrose and lactose, as well as growth in the presence of 4% NaCl and at ambient temperature. The performance of two selected cultures, *Lactobacillus plantarum* 009 and *Pediococcus pentosaceus* 007, as single-strain starters or as a mixed-culture starter, was evaluated in blue-jack mackerel/sucrose/water pastes (78:12:10 w/w/w) ensiled at 37°C or at ambient temperature. The results obtained show that temperature has a marked effect on the pH decrease, starter growth and control of undesirable microorganisms. All silages fermented at 37°C reached acceptable pH values within 48-72 h. Coliform and fungal counts were lower than at ambient temperature. *Pediococcus pentosaceus* displayed the lowest maximum LAB populations at both temperatures. The use of the mixed inoculum under testing brought no beneficial effects.

The addition of 2% NaCl to biological silages gave the best results. The pH decreased faster and reached lower values, NPN values were significantly lower than those in biological silages without added NaCl, and the TVBN values were somewhat lower. Adding 4% NaCl had adverse effects on the outcome of the ensilage, yielding decreased LAB growth, delayed pH decrease, proliferation of coliforms and fungi. The addition of organic acids (formic and/or propionic) at the

time of ensiling or after the 3-day incubation period brought no advantages. Although the pH decrease was fast enough, LAB growth was hindered, fungi proliferated and NPN values were increased.

INTRODUCTION

Biological fish silages are regarded as stable when their pH decreases below 4.5 (Lassén, 1995c). Considerable deterioration can occur while the pH is still above this value. Excessive proteolysis can be a problem, leading to the formation of volatile nitrogen compounds, offensive off-odours, considerable amounts of gas and decreased nutritional value (Raa and Gildberg, 1982). The critical period during which the pH of the silages is above 4.5 must be kept as short as possible. According to Lindgren and Pleje (1983), the pH decrease to values below 4.5 must be completed within 48-72 h to ensure silage stability.

Lactic acid bacteria (LAB), in very small numbers, are part of the natural microflora of fish (Raa *et al.*, 1983). However, inoculating the fish pastes intended for fish silage production with a suitable culture of LAB is a current practice to accelerate the fermentation (Sainclivier, 1985). A starter LAB culture must fulfil requisites such as: ability to utilise the added carbohydrate(s) by the homofermentative pathway in order to produce as much lactic acid as possible and to avoid gas production (Mackie *et al.*, 1971), fast growth at the intended temperature range, ability to compete against the fish native microflora (Adams *et al.*, 1987), and ability to decrease the pH of the silage as fast as possible to values close to 4.0 in order to inhibit quickly the contaminant microflora (Niinivaara *et al.*, 1964).

To avoid negative changes within the silages, action can also be exerted on factors that minimise the duration of the critical period and/or ensure the protection of the silages until the pH decrease is completed, such as incubation temperature or incorporation of additives that inhibit undesirable microorganisms. Gram-negative bacteria belonging to the genera *Pseudomonas* and to the *Acinetobacter-Moraxella* group were found to predominate among the microflora of deteriorating fish (Jackson *et al.*, 1997). Since these bacteria are regarded as sensitive to NaCl, this salt can be used to protect the silages during the early stages of fermentation and to ensure their subsequent preservation. The buffering capacity of fish pastes is especially high for pH 5-6 (Adams *et al.*, 1987). Therefore, considerable deterioration may occur before a safe pH has been achieved (Owens and Mendoza, 1985). Decreasing the initial pH of the silages by adding organic acids can complement LAB action during this initial period. Furthermore, organic acids may have a synergistic action towards NaCl (Ingram and Kitchell, 1967). Combinations of NaCl and low initial pH could lead to stronger inhibition of undesirable microorganisms. Mould growth can occur on the silage surfaces, leading to deterioration and eventually to mycotoxin formation. Propionic acid has been used

to control mould growth on the exposed surfaces of fish silages (Johnsen and Skrede, 1981, Strøm and Eggum, 1981, Lindgren and Pleje, 1983).

The work presented in this chapter aims at the optimisation of fermentation conditions including starter LAB cultures, incubation temperature, addition of NaCl and organic acids. The quality and stability of the products obtained when ensiling blue-jack mackerel by biological and acid methods were compared.

MATERIALS AND METHODS

Bacterial cultures

Eleven isolates of LAB available at the Department of Agricultural Science, University of the Azores, Azores, Portugal, were used: *Lactobacillus plantarum* 005, 006, 011, 012 (silage starters, obtained from the Wageningen Agricultural University collection), *Lb. plantarum* 009, 010, *Lb. casei* 008 (from tempeh soak water, obtained from the Wageningen Agricultural University collection), *Lb. delbrückii* CUC-I, *Lactobacillus* sp. LPS-20, LPCD-8 (from olives, obtained from the Technical University of Lisbon collection), and *Pediococcus pentosaceus* 007 (from fermented meat, obtained from the Wageningen Agricultural University collection). LAB isolates were maintained in MRS agar (MRS medium with 15g l^{-1} added Bacto Agar, Pronadisa, 1056, Hispanlab, Madrid, Spain) as stab cultures, at 4°C. Overnight (12-16 h) cultures were obtained prior to use by subculturing three times in MRS medium (Merck, 1066, Darmstadt, Germany).

Properties of the isolates

Growth at 35°C was assessed by measuring the optical density (O. D.) at λ=620 nm of MRS cultures of the eleven isolates. Sterile MRS medium was used as a negative control. The pH was measured directly in samples of the broth cultures with a combined glass calomel electrode. The LAB strains were tested for their ability to utilise sucrose and lactose in modified MRS with chlorophenol red as indicator (Collins *et al.*, 1989). The ability to grow in the presence of 4% NaCl and the growth at ambient temperature (approx. 17°C) were determined in MRS medium. All tests were performed in triplicate.

Fish silage trials

Fish. Fresh blue-jack mackerel (*Trachurus picturatus* Bowdich 1825) were purchased at the local market in Angra do Heroísmo, Azores, Portugal and immediately used for silage preparation. This species was chosen due to its abundance and low price in the local market.

Effect of the incubation temperature and of three types of starter cultures. Whole blue-jack mackerel were ground in a KENWOOD meat grinder

through a 4-mm die. To the resulting pastes, sucrose and water were added to a final content of 12 and 10%, respectively. Water was added with the purpose of facilitating homogenisation. The fish/sugar paste was thoroughly homogenised with a spatula and 300-g aliquots were placed into 500-ml glass jars with screw caps. To each jar, 1% (w/w) of starter was added. Eight jars were inoculated with 12 - 16 h old MRS cultures of *Lb. plantarum* 009, 8 jars with *P. pentosaceus* 007 and the remaining eight with a mixture (1/1 v/v) of both cultures. After inoculation, the pastes were thoroughly mixed. Half of the jars were placed at ambient temperature (approx. 17°C), the others were incubated at 37°C for 9 days.

Samples were drawn for pH measurement and for microbiological analysis. Following vigorous mixing with a spatula, 20 g of ensiled paste were aseptically collected at each sampling time. Plate counts of the following microorganisms were performed: LAB (MRS Agar), Coliforms (Violet Red Bile Agar, Difco, 0012-01-5, Detroit, MI), fungi (acidified Potato Dextrose Agar, Oxoid, CM 139, Basingstoke, UK) and aerobic sporeforming bacteria (Plate Count agar, Difco, 0479-01-1, Detroit, MI) after inactivation of the vegetative cells at 80°C during 5 minutes. All tests were performed in quadruplicate.

Table 1. Approximate proportions for the preparation of acid and biological fish silages from blue-jack mackerel (*Trachurus picturatus* Bowdich, 1825) with *Lb. plantarum* 009 as starter at 37°C.

Designation	Composition	Proportions (on a weight basis)
AS	Ground raw fish +water + formic acid + propionic acid	86.25/10/3/0.75
BS0	Ground raw fish + sucrose + water + inoculum	77/12/10/1
BS2	Ground raw fish + sucrose + water + NaCl + inoculum	75/12/10/2/1
BS4	Ground raw fish + sucrose + water + NaCl + inoculum	73/12/10/4/1
BS2-5.1	Ground raw fish + sucrose + water + NaCl + inoculum[1]	75/12/10/2/1
BS2-p0.75	Ground raw fish + sucrose + water + NaCl + inoculum + propionic acid[2]	74.25/12/10/2/1/0.75

[1] The pH of these silages was adjusted to 5.1 immediately after preparation.
[2] Propionic acid was added at the beginning of the storage period, i. e., after 3 days of incubation at 37°C.

Effect of NaCl and organic acid addition. Fish/sucrose pastes (12% sucrose/10% water w/w) were prepared as above and 0, 2 or 4% NaCl (w/w fish/sucrose paste) was added. Sugar and NaCl were added as an aqueous solution, to facilitate homogenisation. Another batch was acidified to pH 5.0 with a mixture

of formic and propionic acid at the proportion of 4:1 (pre-acidified biological silages). A batch of acid fish silage was also prepared by adding 3% (w/w) of formic acid and 0.75% (w/w) of propionic acid to the fish paste (no sucrose or NaCl added). All silages were placed in an incubator at 37°C for 3 days and subsequently stored at room temperature (approx. 17°C) for another 19 days. To one of the non-acidified silage batches, 0.75% (w/w) propionic acid was added at the beginning of the storage period (post-acidified biological silages). Table 1 summarises the formulation of the different types of silages prepared for this experiment.

Chemical composition. Moisture, organic nitrogen and ash were determined by standard procedures (AOAC, 1990). Non-protein nitrogen was determined by a micro-Kjeldahl procedure in the clear extract obtained when 25 g of silage were homogenised for 5 min with an UltraTurrax homogeniser in 150 ml of 7% trichloroacetic acid and filtered through Whatman No. 1 paper (Batista *et al.*, 1987). Total volatile basic nitrogen was determined by the Conway microdiffusion method (Anon., 1988).

Yield of supernatant. In order to monitor the formation of a liquid layer in the silages, 15 g samples were drawn from each silage, after thorough mixing with a spatula and centrifuged at 25 000 g for 30 min at 12°C in a refrigerated centrifuge (DAMON, B-20A, IEC Division). The supernatant was discarded and the tubes were weighed. The supernatant weight was calculated by difference. The yield of supernatant (YS) was calculated according to the formula:

$$YS\ (\%) = (\text{weight of supernatant/initial weight of the sample}) \times 100.$$

Microbiological analysis and pH. Samples were drawn for assessing the pH and performing microbiological analyses as described above.

Statistical methods

Equations were fitted to the growth and pH data obtained during the first experiment by linear regression (least squares method). Comparison among cultures was made on the basis of the slope of the lines obtained. The results obtained in all experiments were submitted to ANOVA according to Gomez and Gomez (1983). Separation of the means was performed by the Duncan test (Duncan, 1955).

RESULTS AND DISCUSSION

Screening LAB cultures

The results of this group of tests are presented in Table 2. *Lb. plantarum* 010 had a significantly ($p<0.05$) faster growth than all others. However, it could

not ferment sucrose and lactose and it did not display growth in the presence of 4% NaCl, so it was regarded as not suitable for our purposes. *Lb. plantarum* 009, *Lb. plantarum* 006, *P. pentosaceus* 007, *Lb. casei* 008, *Lb. plantarum* 009, *Lb. plantarum* 011 and *Lb. delbrueckii* CUC-I were all significantly ($p<0.05$) faster in growth than the two remaining isolates. Among the lactobacilli cultures in this group, *Lb. plantarum* 011 and *Lb. plantarum* 009 had adequate metabolic properties, although 011 had little growth at ambient temperature. For this reason, and because 011 was significantly ($p<0.05$) slower than all other cultures in acidifying the medium, *Lb. plantarum* 009 was selected for further testing. Although this isolate fulfils all of the considered criteria, the *Lb. plantarum* species is regarded as producing acid slowly while the pH of the medium is above 5.0 (Woolford, 1972). It would be beneficial to include in the inoculum, besides *Lb. plantarum*, another species capable of fast acidification in the pH interval 5.0-6.5. For this purpose, *P. pentosaceus* would be suitable, since this species has a higher

Table 2. Characteristics of 11 LAB intended for fish silage starters: growth rate, acidification rate, lactose (Lact.) and sucrose (Sucr.) utilisation, growth in MRS medium with 4% NaCl (all at 37°C during 24 h), and growth at ambient temperature (approx. 17°C).

Isolates	Growth rate (h^{-1})	Acidification rate (h^{-1})	Sucr.	Lact.	NaCl	Growth at ambient temp.
Lb. plantarum 005	0.520 ± 0.03^{a}*	0.278 ± 0.011^{a}	+[1]	-[2]	+	-
Lb. plantarum 006	0.419 ± 0.019^{abc}	0.334 ± 0.005^{b}	+	+	+	-
P. pentosaceus 007	0.542 ± 0.046^{a}	0.368 ± 0.028^{c}	+	-	+	+
Lb. casei 008	0.448 ± 0.016^{abc}	0.229 ± 0.020^{d}	+	-	-	(+)[3]
Lb. plantarum 009	0.512 ± 0.029^{a}	0.228 ± 0.007^{d}	+	+	+	+
Lb. plantarum 010	0.761 ± 0.040^{d}	0.280 ± 0.007^{a}	-	-	-	(+)
Lb. plantarum 011	0.446 ± 0.055^{abc}	0.199 ± 0.017^{e}	+	+	+	(+)
Lb. plantarum 012	0.307 ± 0.040^{bc}	0.250 ± 0.009^{d}	+	+	+	(+)
Lb. delbrueckii CUC-I	0.507 ± 0.068^{ab}	0.228 ± 0.019^{d}	+	-	+	+
Lactobacillus sp. LPS-20	0.324 ± 0.012^{bc}	0.164 ± 0.011^{f}	+	-	+	+
Lactobacillus sp. LPCD-8	0.304 ± 0.007^{c}	0.191 ± 0.002^{ef}	-	-	+	+

[1] The pH of these silages was adjusted to 5.1 immediately after preparation.
[2] Propionic acid was added at the beginning of the storage period, i. e., after 3 days of incubation at 37°C.

optimum pH interval (6.0 - 6.5, Kandler and Weiss, 1984) than lactobacilli do. *P. pentosaceus* 007 was selected, although it could not utilise lactose. This culture was significantly faster than all others in acidifying the medium.

Effect of the temperature and inoculum type

Table 3 presents the variation in pH, LAB, coliforms and fungi in silages corresponding to the different inocula and temperatures. Temperature had a major

Table 3. Microbial populations (in log CFU g^{-1}) and pH values in blue-jack mackerel (*Trachurus picturatus* Bowdich 1825) submitted to ensilage at two temperatures with three types of inoculum.

Incubation temperature	Time (days)	*Lactobacillus plantarum* 009				*Pediococcus pentosaceus* 007				*L. plantarum* 009 + *P. pentosaceus* 007 (1:1)			
		pH	LAB	Coliforms	Fungi	pH	LAB	Coliforms	Fungi	pH	LAB	Coliforms	Fungi
37°C	0	6.75	7.26±0.07	1.00	3.31	6.78	7.47±0.33	1.32	7.36	6.80	7.35±0.08	1.00	6.60
	1	4.93	9.68±0.33	5.11	8.26	5.00	8.78±0.68	3.91	8.49	5.00	8.70±0.59	4.83	8.27
	2	4.45	9.53±0.05	4.05	2.28	4.73	8.95±0.60	4.57	5.85	4.58	9.56±0.11	4.65	4.55
	3	4.25	9.38±0.20	<1.0	<1.0	4.10	8.72±0.19	<1.0	<1.0	4.23	9.22±0.01	<1.0	4.38
	7	3.90	7.53±0.10	<1.0	<1.0	3.88	6.89±0.11	<1.0	<1.0	3.80	7.22±0.02	<1.0	6.75
Ambient	0	6.83	7.19±0.02	1.32	3.04	6.80	7.24±0.11	1.80	1.00	6.78	7.00±0.01	1.00	6.66
	1	6.03	7.69±0.18	4.91	8.26	6.00	8.52±0.04	4.83	8.57	6.00	9.24±0.14	4.84	8.15
	2	5.00	8.78±0.03	4.66	7.56	5.03	8.41±0.21	4.83	7.28	5.00	8.81±0.16	4.67	6.43
	3	4.88	8.36±0.05	4.18	6.07	4.95	8.46±0.07	4.77	6.69	4.91	8.46±0.01	4.27	0.00
	7	4.50	8.05±0.04	4.29	4.27	4.75	7.91±0.04	4.38	4.18	4.55	8.04±0.01	3.50	0.00

The numbers represent the averages ± standard deviations of four samples.

effect on the pH values of the tested silages. All silages kept at 37°C had reached pH values below 4.5 within 3 days of incubation, whereas silages kept at ambient temperature (approx. 17°C) did not reach low enough values within 7 days to ensure preservation. Ahmed and Mahendrakar (1996a) also obtained a more rapid fall in pH during the fermentation of tropical fish viscera at 37°C than at ambient temperature (26±2°C). Differences among inoculum types were not significant ($p<0.05$).

The initial LAB counts did not differ significantly ($p<0.05$) among the various silages and were ca. 10^7 CFU g^{-1}. At 37°C, the *Lactobacillus* inoculum had reached its maximum population after 24 h of incubation, but it took 48 h for the mixed inoculum and *Pediococcus* to reach its maximum count. At ambient temperature, *Pediococcus* and the mixed inoculum reached maximum population levels after 24 h of incubation and *Lb. plantarum* only after 48 h. The maximum levels attained at room temperature were always lower than the corresponding values at 37°C. At the end of the experimental period, silages incubated at 37°C had significantly ($p<0.01$) lower LAB counts than those kept at ambient temperature due to a faster decrease in LAB numbers. Silages inoculated with *Lb. plantarum* and incubated at 37°C had higher LAB counts than those inoculated with *P. pentosaceus* and incubated at the same temperature. It was observed that in silages kept at 37°C coliforms, moulds and yeasts were no longer detectable from the third day of ensilage on. Fermentation led to low pH values and, presumably, to the accumulation of other antibacterial products of LAB, resulting in inhibition of undesirable microorganisms. No aerobic spores were detected in any of the ensilages, not even in those that did not reach pH values low enough for preservation (data not shown).

Effect of NaCl and organic acids

Biological fish silages prepared in this experiment had 75.3-78.2% moisture, 4.2-4.5% ash and 1.7-1.8% organic nitrogen. The corresponding values for the acid silages were 83.0%, 3.5% and 1.7%, respectively. The initial ash content in blue-jack mackerel was 2.5% (data not shown).

The pH values and microbial counts of the different silages throughout the experiment are shown in Table 4. Addition of 2% NaCl to biological silages led to a faster decrease in pH values. Silages with 2% NaCl reached the lowest pH values after three days of ensilage (3.6-3.7). These values are at the lowest limit for lactobacilli growth (Kandler and Weiss, 1984). Adding 4% NaCl to the silages, on the opposite, caused a delay in the pH decrease and led to somewhat higher final pH values. Pre-acidifying to pH 5.1 or adding 0.75% propionic acid after the first three days did not considerably affect the pH decrease. Acid silages had stable, acceptable pH values throughout the whole experiment. As expected (Adams *et al.*, 1987), NaCl addition led to a significant reduction in LAB growth. It is, however, interesting that silages with 2% NaCl added had the lowest pH values, in spite of

not reaching the highest LAB populations. From the third day of ensilage onward, coliforms decreased to non-detectable levels in silages with 0 or 2% NaCl. In silages with 4% NaCl, these bacterial populations increased. When 4% NaCl were

Table 4. Microbial populations, pH and yield of supernatant values in blue-jack mackerel (*Trachurus picturatus* Bowdich 1825) submitted to ensilage by different methods, at 37°C, with *Lb. plantarum* 009 as starter.

		Time (days)						
		0	1	2	3	7	14	21
AS	pH	3.8	3.6	3.8	3.7	3.8	3.8	3.8
	LAB	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	Colif.	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	Fungi	1.00±0.00	1.00±0.01	1.00±0.00	1.55±0.02	2.39±0.09	1.92±0.05	1.49±0.05
	YS	49.9±0.4	ND	ND	58.6±0.3	62.8±0.4	68.7±0.7	71.4±0.5
BS0	pH	6.7	5.4	4.4	3.9	3.9	4.1	4.1
	LAB	5.35±0.01	6.63±0.13	7.63±0.07	9.64±0.02	10.15±0.66	8.96±0.01	8.61±0.02
	Colif.	1.62±0.06	4.62±0.04	1.16±0.21	<1.0	<1.0	<1.0	<1.0
	Fungi	1.34±0.05	2.30±0.04	3.13±0.07	1.33±0.07	<1.0	<1.0	<1.0
	YS	21.7±0.9	ND	ND	65.2±0.9	65.1±0.6	65.5±0.9	66.2±0.8
BS2	pH	6.6	4.8	3.8	3.6	3.9	3.9	3.9
	LAB	5.22±0.04	6.68±0.02	7.05±0.12	7.96±0.02	9.93±0.66	7.56±0.01	6.95±0.02
	Colif.	0.35±0.09	3.49±0.02	0.15±0.01	<1.0	<1.0	<1.0	<1.0
	Fungi	1.44±0.11	2.75±0.15	4.96±0.11	1.31±0.09	<1.0	<1.0	<1.0
	YS	0.1±0.00	ND	ND	62.6±0.8	62.4±0.9	62.5±0.3	63.9±0.7
BS4	pH	6.6	6.0	4.6	4.0	4.0	4.0	4.1
	LAB	5.40±0.09	5.92±0.24	6.45±0.19	8.64±0.01	8.69±0.11	8.33±0.03	8.06±0.04
	Colif.	0.85±0.11	1.36±0.01	2.39±0.05	5.75±0.01	5.76±0.07	5.41±0.11	5.43±0.18
	Fungi	0.82±0.07	1.17±0.17	4.00±0.26	6.78±0.71	6.94±0.31	7.85±0.60	7.71±0.87
	YS	0.0±0.0	ND	ND	58.8±0.9	60.7±0.7	60.4±0.7	60.2±0.8
BS2-5.1	pH	5.1	4.8	3.7	3.7	3.8	3.8	4.0
	LAB	5.30±0.40	5.82±0.40	5.91±0.30	5.92±0.32	5.27±0.02	5.01±0.09	4.72±0.07
	Colif.	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	Fungi	1.00±0.01	1.00±0.02	1.00±0.00	1.64±0.00	1.96±0.17	1.34±0.17	1.70±0.13
	YS	60.4±0.3	ND	ND	77.0±0.4	76.7±0.3	77.5±0.4	76.8±0.3
BS2-0.75	pH	6.5	5.0	4.2	3.7	4.0	4.0	4.0
	LAB	5.32±0.06	6.21±0.06	6.16±0.07	6.10±0.06	5.70±0.06	5.02±0.07	4.85±0.16
	Colif.	0.50±0.08	1.94±0.02	<1.0	<1.0	<1.0	<1.0	<1.0
	Fungi	3.17±0.13	3.18±0.10	2.50±0.18	1.94±0.16	2.76±0.09	3.79±0.36	3.54±0.21
	YS	0.1±0.0	ND	ND	74.1±0.8	72.6±0.9	74.4±1.2	74.6±0.5

ND = not determined.

See Table 1 for abbreviations.

The sd values for pH (not shown) range between 0.0 and 0.1.

added to the fish pastes, they became tougher in consistency and more difficult to homogenise. This might have led to pockets of untreated fish that provided support for the growth of undesirable microorganisms. Similarly to what had been observed with the coliforms, addition of 4% NaCl led to increased fungal counts. The addition of formic and/or propionic acid had a strong inhibitory effect upon LAB

and coliforms. Lower LAB counts were obtained immediately after propionic acid addition and homogenisation (BS2-0.75, third day of ensilage). However, fungi could still be found after the third day of ensilage only in acid-treated silages. It is interesting to note that fungal growth was not a problem in silages with high LAB populations, which could be explained by the antifungal properties attributed to LAB (Gourama and Bullerman, 1995). Fungi have often been described as posing problems in otherwise well-preserved fish silages (Lindgren and Pleje, 1983, Ahmed and Mahendrakar, 1996a). No aerobic spores were detected in the silages during the 22 days of this experiment (data not shown).

Table 4 also shows the yield of supernatant values obtained in the silages resulting from the different treatments. Supernatant formation has been attributed to proteolysis (Backhoff 1976). Particularly the results obtained at t=0 show that acid and salt addition affects the water binding properties of the fish muscle protein, leading to the formation of different amounts of supernatant. Comparing the results obtained at t=0 for BS2, BS2-5.1, BS2-0.75 and AS, it can be seen that acid addition caused an immediate release of supernatant. The two silages in this group that were not acidified by direct acid addition at t=0 yielded no supernatant upon centrifugation, whereas directly acidified silages formed abundant supernatant. The isoelectric pH of fish myosin is 4.8-6.2 (Lin and Park, 1998). The initial pH values of the non-acidified biological silages, BS0, were higher than the isoelectric pH of fish myosin. Correspondingly, the observed initial supernatant formation was low. Non-acidified silages with added NaCl (BS2 and BS4) however, did not produce supernatant at t=0. Salt concentrations lower than 8% lead to increased water binding by myofibrillar proteins (Wheaton and Lawson, 1985).

BS2-5.1 and AS, directly acidified to pH=5.1 and pH=3.8 respectively, at t=0 formed abundant supernatant upon centrifugation. The pH of BS2-5.1 was within the range of values for the isoelectric pH range of fish myosin. The pH of AS is considerably lower than the isoelectric point of myosin and water binding properties will gradually increase again at such decreasing pH values. Correspondingly, supernatant formation was lower in AS than in BS2-5.1. Increased supernatant formation was also observed in BS2-0.75 at the time of propionic acid addition (3rd day).

In biological silages, subsequent supernatant formation was significantly ($p<0.05$) faster than in the acid fish silages and it was halted after 3 days of incubation. At that time, the yield of supernatant was about 60-70%. Significant differences ($p<0.01$) were observed among treatments after three days of incubation. Silages with NaCl still had significantly ($p<0.01$) lower levels of supernatant formation than the silages without NaCl. Acid silages or biological silages to which organic acids had been added had higher yields of supernatant throughout the experimental period. Pre-acidified biological silages (pH 5.1) exuded to a significantly ($p<0.05$) greater extent than all other types of silage. The

acid silage and biological silages to which acid had been added exuded significantly ($p<0.05$) faster. On the contrary, NaCl addition decreased the yield of supernatant.

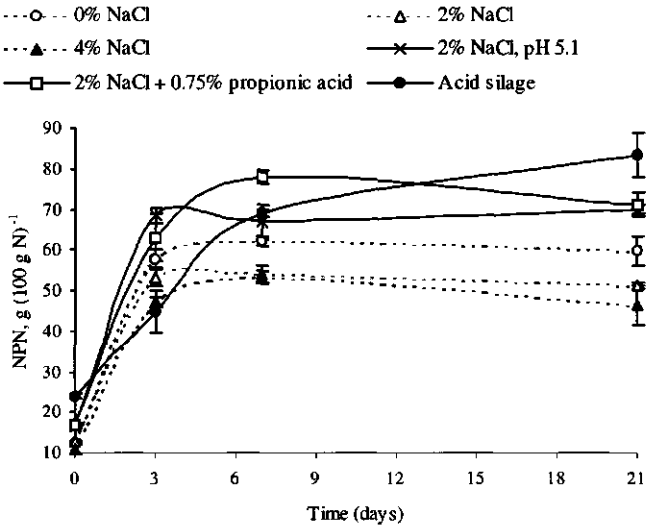

Fig. 1. NPN values in blue-jack mackerel (*Trachurus picturatus* Bowdich 1825) submitted to ensilage by different methods. (See Table 1 for abbreviations; the values depicted are the averages of three determinations; the bars represent the standard deviations).

The NPN variation is represented in Fig. 1. At the relatively high fermentation temperature and low pH values prevailing during the ensilage of fish, the activity of proteolytic enzymes is high (Raa and Gildberg, 1982, Haard *et al.*, 1985), resulting in high concentrations of 7% TCA-soluble nitrogen. It was observed that, from the third day of incubation on, the NPN concentration stabilised or even decreased, suggesting that the proteolytic action had been halted. This could be due to inactivation of proteolytic enzymes for pH values below 4. Another possible explanation is the existence of a protein fraction that is resistant to degradation (Hall *et al.*, 1985a and b). The latter explanation seems more likely since the acid silages, which displayed pH values below 4 right from the start, also autolysed. NPN contents varied differently in biological and acid silages. In biological silages, proteolysis proceeded quickly up to the third day of ensilage and stabilised from then on in most cases. In the case of acid silages, although proteolysis progressed significantly ($p<0.05$) slower at the beginning, soluble nitrogen compounds were formed at a considerable rate until the end of the experiment. This may be related to the different pH dynamics in both types of silage. In the case of biological silages, the period during which most of the

proteolysis process takes place corresponds to the period in which the pH of the silages is close to that indicated in literature as optimum for the action of the proteolytic enzymes (Raa and Gildberg, 1976, Lindgren and Pleje, 1983). Additionally, part of the NPN in biological silages is assimilated by the microflora, which could also contribute to final lower values than in the acid silage.

This hypothesis is reinforced by the pattern of NPN variation in biological silages with organic acids. All biological silages had lower NPN values than the acid silages. Adding 2 or 4% NaCl to the silages led to lower NPN values. The lower NPN values observed in biological silages with NaCl suggest that NaCl addition may have decreased the activity of proteolytic enzymes and/or the susceptibility of muscle proteins to proteolysis. The addition of organic acids led to somewhat higher NPN values. In the biological silages, NPN reached its maximum after 3-7 days. In the acid silage, NPN was still increasing, although at a lower rate, by the end of the experiment. About 80% of the nitrogen had been solubilised in the acid silages by the end of the experimental period. In biological silages, much lower values (ranging from 40 to 70%) were observed. Low NPN values are generally regarded as advantageous from the nutritional point of view (Espe *et al.*, 1992a).

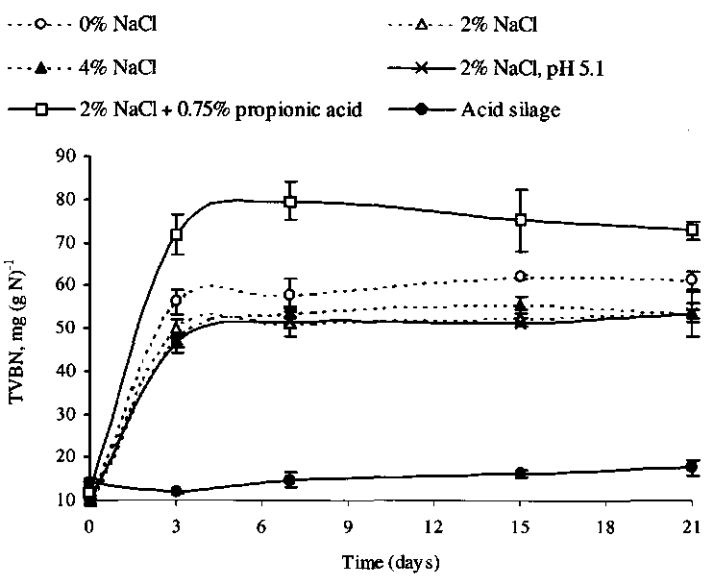

Fig. 2 Total volatile basic nitrogen values in blue-jack mackerel (*Trachurus picturatus* Bowdich 1825) submitted to ensilage by different methods. (See Table 1 for abbreviations; the values depicted are the averages of three determinations; the bars represent the standard deviations).

The results on total volatile basic nitrogen (TVBN) are shown in Fig. 2. Salt addition had a significant effect upon TVBN formation. Silages with 2 or 4% NaCl had significantly lower ($p<0.05$) TVBN values after 16 days of incubation than biological silages to which no NaCl had been added. Acid silages had significantly ($p<0.01$) lower TVBN values – 17.6 mg (g N)$^{-1}$ - after 21 days of incubation. The highest values corresponded to the non-acidified biological silages and to those silages with 0.75% propionic acid after three days of incubation. The maximum values obtained for these two types of silage were 61.1 and 72.8 mg (g N)$^{-1}$, respectively. This is comparable to the values obtained by other authors (Van Wyk and Heydenrych, 1985, Hassan and Heath, 1987). Acid addition at the beginning of the incubation period (pre-acidification to pH 5.1) did not lead to a significant ($p<0.01$) decrease on the formation of volatile basic nitrogen. The differences in TVBN values in the different types of silages can be explained by differences in microbial populations resulting from the treatments applied. Silages with low microbial populations (with salt or acid), had lower TVBN values than BS0 or BS2-0.75. As expected for the biological silages the period during which the most intense formation of these products occurred were the three initial days at 37°C. This did not occur, however, in the acid fish silages, in which the pH was kept at low values throughout the whole experiment. However, TVBN has a limited value as a quality criterion in well-preserved silages (stable pH). In this case, TVBN comes from the deamination of non-essential amino acids and the nutritional value of the products is not affected (Espe *et al.*, 1992b).

CONCLUSIONS

Lb. plantarum 009 was found to yield stable biological silages, with low NPN values, when a 3-day incubation period at 37° C was applied and 2% (w/w) NaCl were added. Acidification treatments were not beneficial. The acid process was found to yield silages that were more sensitive to fungal proliferation and had a higher degree of protein solubilisation than those produced by biological ensilage.

Supernatant formation in fish silage is not exclusively related to proteolysis, but it also reflects exudation due to loss of water binding capacity by the myofibrillar proteins.

Chapter 4

Lactic acid bacteria from naturally fermented mackerel: isolation, growth, acidification and antimicrobial activity

ABSTRACT

The preservation of fish or fish residues by biological ensilage is based upon fast growth, rapid acidification to below pH 4.5 and production of antimicrobial substances by the starter culture, which limit the growth of undesirable microorganisms and reduce the formation of toxic products such as biogenic amines.

Lactic acid bacteria (LAB) enrichment was performed in mackerel-sucrose-NaCl pastes (86:12:2) by back-slopping at two temperatures, 15 and 22°C. Seventy-seven LAB were isolated from these pastes, identified to the genus or to the species level and tested for their potential as fish silage starters by screening for fast growth and acidification at 15, 22 and 30°C and by testing their antimicrobial properties by a direct antagonism test. Six biogenic amine producing bacteria (*Lactobacillus sakei* 15.19, *Weissella hellenica* 15.32, *Enterococcus* sp. 22.12, *Lactobacillus büchneri* 2A, *Escherichia coli* B351 and *Morganella morganii* 6675), three other potential spoilage microorganisms (*Shewanella putrefaciens* A2, *Candida curvata* and *Candida lipolytica*) and five pathogens (*Listeria monocytogenes* Scott A, *Salmonella* sp., *E. coli* O157:H7 BJA2230, 9999-1 and 96004) were used as indicator strains. When appropriate, the inhibition mechanisms were tentatively determined. The effect of two temperatures (15 and 22°C) on the antimicrobial activity of the test strains was tested as well.

Natural fermentation at 15 or 22°C for 48 h did not lead to pH values below 4.5. *Lb. sakei* was the most frequently identified species from mackerel pastes incubated at 15°C, whereas at 22°C *Lactobacillus plantarum* was predominant. The fastest growing isolates at 30 and 22°C were obtained from fish pastes fermented at 22°C for 48h. All isolates grew very little at 15°C. The fastest acidifying isolates were obtained from 48-h old pastes. Acidification was considerably slower at 22 than at 30°C and no acidification could be observed at 15°C in the screening experiments. Ensiling at 15 and 22°C with these LAB cultures can pose problems due to slow acidification or growth.

All pathogens tested were inhibited by all prospective starter cultures studied by a general mechanism – acid production. Spoilage microorganisms such as *M. morganii*, *E. coli* and the biogenic amine producing strains of *W. hellenica*, *Lb. büchneri* and *Enterococcus* were inhibited by all isolates tested, predominantly due to non-dissociated organic acids. *Candida curvata* was inhibited by all of the test isolates due to the decrease in pH of the medium. *Candida lipolytica* could not be inhibited by any of the LAB cultures. All LAB displayed antimicrobial activity against *S. putrefaciens* as well, although the inhibition mechanisms could not be established in the present study. *Lb. sakei* 15.19 was the most resistant to inhibition by other LAB. At 30°C, only two of the tested isolates were able to inhibit its growth.

INTRODUCTION

Lactic acid bacteria (LAB) can be found on fish. They are not, however, predominant members of its microflora (Raa *et al.*, 1983). Thus, addition of a LAB culture to fish pastes intended for ensilage is used or recommended in most studies (Fagbenro and Jauncey, 1994a, Lássen, 1995c). Starter cultures intended for fish silage production must be able to inhibit pathogens and spoilage microorganisms in order to ensure a safe product.

At ambient temperature, motile pseudomonads are the specific spoilage microflora of aerobically stored fish. A large proportion of the flora on ambient-stored mackerel consists of *Shewanella putrefaciens*, indicating that this bacterium may also take part in the spoilage (Gram and Huss, 1996). *S. putrefaciens* is capable of anaerobic respiration using trimethylamine oxide (TMAO) and it can grow to 10^6 - 10^8 CFU g^{-1} under anaerobic conditions (Gram and Huss, 1996). It can be of concern as a spoilage microorganism in fish silage. Yeasts have been pointed out as one of the main problems during the storage of fish silage (Lindgren and Pleje, 1983). *Candida lipolytica* has been identified as one of the predominant spoilage microorganisms in fish silage (Levin and Witkowski, 1991).

Biogenic amines may pose a potential risk in fish silage. Ensiled fish contains a considerable amount of the free amino acids that constitute the precursors for biogenic amines such as histamine, tyramine, putrescine and cadaverine (Haard *et al.*, 1985). The low Eh reached during ensilage (below -550 mV, Lássen, 1995c) and the low pH due to LAB metabolic activities create favourable conditions for the action of amino acid decarboxylases (Beutling, 1992). Biogenic amine formation in food products is associated with the growth of decarboxylase-positive microorganisms (Silla-Santos, 2001). Biogenic amine production is widely spread among bacteria and has been reported to occur in many species that are commonly isolated from fish, such as *Morganella morganii* and *Escherichia coli* (Baranowski, 1985). It has also been described for some LAB species associated with food fermentation (Straub *et al.*, 1995).

It is important to ensure that fish silage is free from pathogens. *Listeria monocytogenes* has been associated with some fish products (Ben Embarek, 1994) and products such as fish meal can play a role in the spreading of *Salmonella* throughout the food chain (Raa and Gildberg, 1982). Acid resistant strains of the enterohaemorrhagic *E. coli* (*E. coli* O157:H7) have been reported (Benjamin and Datta, 1995, Conner and Kotrola, 1995) and could become a serious problem in acidified products such as fish silage.

The aim of the present work was to obtain LAB isolates and test their antimicrobial properties against pathogenic and spoilage microorganisms in fish silage. Special attention was paid to the inhibition of biogenic amine producing microorganisms, since they can pose a considerable risk in fish silage.

MATERIALS AND METHODS

Back-slopping

Whole, non-eviscerated, Atlantic mackerel (*Scomber scombrus* Linnaeus 1785) was purchased from a local retailer (Wageningen, The Netherlands) and ground in a food processor until a homogenous paste was obtained. Twelve per cent sucrose and 2% NaCl (w/w) were added, the resulting mixture was thoroughly homogenised using a spatula and divided into 150 g batches which were distributed in glass jars closed with a screw cap fitted with a water-column type air lock. Two jars were placed at 22°C and two at 30°C. Back-slopping was performed by preparing every day new pastes from a stock of frozen fish (freezing temperature of –20°C) and inoculating them with 10% (w/w) of a previously prepared, 24 h old fish paste. The pH was determined for each new batch at 0, 24 and 48 h.

Cultures and media

LAB strains were isolated from the above mentioned mackerel/sucrose/NaCl pastes (86:12:2, w/w/w), fermented without the addition of any starter and submitted to back-slopping at 15 and 22°C. After 18 back-slopping cycles, LAB were isolated by plating in MRS medium (Merck 10661, Darmstadt, Germany) with 2% potassium sorbate and 0.04% chlorophenol red (Stoffels *et al.*, 1992). All obtained LAB isolates were maintained at –20°C in MRS medium with 20% glycerol (Merck 04094, Darmstadt, Germany) and tempered overnight in MRS medium at 30, 22 and 15°C before use. All 77 isolates were tentatively identified according to Kandler and Weiss (1984) and Garvie (1984), by cell and colony morphology, Gram reaction, catalase test, oxidase test, utilisation of gluconate and ribose, growth at 45 and 15°C and gas production from glucose. Seven to nine isolates from each group defined by fermentation temperatures (15 or 22°C) and isolation time (24 or 48 h) were characterised by their carbohydrate fermentation pattern using the API-50CH or API 20 STREP kits (API System, Montalieu-Vercieu, France). The results obtained were analysed with the help of

IBIS software (Wijtzes *et al.*, 1997). LAB isolates with interesting characteristics (antimicrobial properties, biogenic amine production, histamine degradation, fast growth or acidification) were identified to the level of species by determining cell morphology, motility, spore formation, catalase, gas from glucose, gas from gluconate, growth at 15 and 45°C, acid from ribose, arabinose, xylose, rhamnose, mannitol, sorbitol, ribitol, glycerol, fructose, mannose, galactose, glucose, lactose, maltose, sucrose, trehalose, cellobiose, raffinose, melibiose, salicin and gluconate, NH_3 from arginine, configuration of lactic acid, presence of meso-diaminopimelic acid in cell hydrolysate, peptidoglycan type and 16S rDNA sequencing. One additional *Lactobacillus plantarum* test strain (*Lb. plantarum* 009) was obtained from the collection of Wageningen Agricultural University. Two yeast cultures were isolated from fresh mackerel in MEA medium (Oxoid CM59, Unipath, UK) and tentatively identified as *Candida lipolytica* and *Candida curvata*. Identification procedures for the yeast cultures were carried out using the API ID32-C gallery (API System, Montalieu-Vercieu, France). Yeast isolates were maintained in MEA medium at –20°C. Prior to their use in fermentation experiments, they were cultivated for 48h in MEA plates, plus 48h in MEB medium, at the respective fermentation temperatures.

Growth inhibition was assessed using isolates of pathogens and microorganisms involved in the deterioration of fish silage as indicator strains. These included biogenic amine producing bacteria, *Lactobacillus sakei* 15.19, *Weissella hellenica* 15.32, *Enterococcus* sp. 22.12 (this thesis, chapter 5) *Lactobacillus büchneri* 2A (Joosten and Northolt, 1987), *Escherichia coli* B351 (Joosten and Northolt, 1987) and *Morganella morganii* DSMZ 6675 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Spoilage-causing microorganisms tested were *Shewanella putrefaciens* A2 (Danish Ministry of Fisheries, Copenhagen, Denmark) and two yeast cultures isolated by us, *Candida curvata* and *Candida lipolytica*. Isolates of *L. monocytogenes* Scott A and *Salmonella* were obtained from the collection of Wageningen University, Wageningen, The Netherlands and three isolates of enterohaemorrhagic *Escherichia coli* (*E. coli* O157:H7, 95 BJA230, 9999-1, 96004) from the Food Inspection Department, Zutphen, The Netherlands. *L. monocytogenes*, *S. putrefaciens*, *M. morganii* and *E. coli* cultures were maintained in TSB medium (Oxoid, CM129, Unipath, UK) with 20% glycerol at –20°C and were pre-cultivated for 24 h at the intended fermentation temperatures in the same medium. A bacteriocin-producing strain of *Pediococcus acidilactici* (*P. acidilactici* PAC 1.0, Chikindas *et al.*, 1993) was used as control organism.

Assessment of antimicrobial activity

The inhibitory activity of 64 selected LAB was tested against the above mentioned indicator strains by a direct antagonism test (well assay technique, Bibek, 1992) at 30°C. Overnight cultures incubated at 30°C as well as culture

supernatants prepared by centrifuging 1 ml of the incubated broth culture at 18 900 × g, 4°C, for 10 min, were used. Remaining cells were excluded from the supernatant using 0.45µm disposable filters (disposable filter holders 462100, Schleicher & Schuell, Germany) as described by Schillinger and Lücke (1989). Control assays were performed using sterile MRS medium (negative control) and a MRS culture of *P. acidilactici* PAC 1.0 (positive control) to fill the wells. All tests were carried out in triplicate.

Mechanism of antibiosis

The mechanism of inhibition against *Lb. sakei* 15.19, a biogenic amine producing microorganism isolated from fermented mackerel/sucrose pastes (Dapkevicius *et al.*, 2000) was determined for a total of 64 selected LAB isolates. Resistance of the inhibitory principle to pronase E (Merck, 7433, Darmstadt, Germany, 10 mg ml^{-1} in 50 µM~~mol~~ ml^{-1} phosphate buffer, pH 7) was examined by the well assay test. Pronase was added at the concentration 0.25g l^{-1} to the test organism (Schillinger and Lücke, 1989). Five g l^{-1} of catalase (from bovine liver, C-3155 Sigma Chemical, USA) were added to culture supernatants, to determine if the antagonistic effect derived from H_2O_2 production (Schillinger and Lücke, 1989).

To determine whether acidification of the medium played a role in the inhibitory effect, all indicator strains were tested by the same well assay technique, but the wells were filled with sterile MRS broth of adjusted pH instead. Since biological fish silage is regarded as stable only when LAB starters are able to decrease its pH to 4.5 or less (Twiddy *et al.*, 1987), the pH of MRS broth was adjusted to this value by adding an aqueous solution of HCl (100 g l^{-1}). To determine the effect of non-dissociated molecules of weak organic acids (lactic and acetic acid) produced by the LAB isolates, the wells were filled with sodium acetate or sodium lactate solutions at two concentrations, 1 and 2% (Bibek and Daeschel, 1992). The pH of these solutions was adjusted to 6.0 by adding a 0.1 *M* aqueous solution of NaOH. All tests were carried out in triplicate.

Effect of temperature on the antimicrobial action against *Lb. sakei* 15.19 and *C. curvata*

To determine the effect of incubation temperatures of interest for fish silage production on the antimicrobial activity of selected LAB isolates against *Lb. sakei* 15.19 and *C. curvata*, the above described tests were carried out also at 15 and 22°C.

Screening for growth and acidification rate

Isolates that did not produce gas from glucose and that had not been found to decarboxylate amino acids (this thesis, chapter 5) were screened for their growth and acidification properties in a modified MRS medium in which sucrose replaced glucose and 0.5 g l^{-1} cysteine was added. Sucrose was used instead of glucose since

Chapter 4

Table 2. Identity of LAB isolated from naturally fermented mackerel / sucrose / NaCl pastes incubated at 15 and 22°C.

Isolate No.	LAB group	Identity to the species level	Identification procedure
		15°C, 24 h	
15.01[ab]	*Lactobacillus*, FH	*Lb. sakei*	Carbohydrate fermentation
15.03	*Lactobacillus*, FH	*Lb. bavaricus/alimentarius*	Carbohydrate fermentation
15.04[ab]	*Lactobacillus*, FH	*Lb. bavaricus/alimentarius*	Carbohydrate fermentation
15.05[b]	*Lactobacillus*, FH	*Lb. sakei*	DNA/DNA homology
15.08[ab]	*Lactobacillus*, FH	*Lb. alimentarius*	Carbohydrate fermentation
15.13	*Lactobacillus*, FH	*Lb. alimentarius*	Carbohydrate fermentation
15.18[ab]	*Lactobacillus*, FH	*Lb. sakei*	DNA/DNA homology
		15°C, 48h	
15.19	*Lactobacillus*, FH	*Lb. sakei*	DNA/DNA homology
15.27[ab]	*Lactobacillus*, FH	*Lb. alimentarius/bavaricus*	Carbohydrate fermentation
15.28	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
15.30[ab]	*Lactobacillus*, FH	*Lb. sakei*	Carbohydrate fermentation
15.32	*Leuconostoc* or related genus	*Weissella hellenica*	DNA/DNA homology
15.33	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
15.35	*Lactobacillus*, FH	*Lb. curvatus*	DNA/DNA homology
15.36[ab]	*Lactobacillus*, FH	*Lb. sakei*	DNA/DNA homology
15.39[ab]	*Lactobacillus*, FH	*Lb. sakei*	Carbohydrate fermentation
		22°C, 24 h	
22.01[ab]	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
22.02	*Lactobacillus*, FH	*Lb. bavaricus*	Carbohydrate fermentation
22.03	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
22.05[b]	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
22.06	*Lactococcus*	*L. lactis*	Carbohydrate fermentation
22.12	*Enterococcus*	*E. faecium* species group	DNA/DNA homology
22.16	*Lactobacillus*, FH	*Lb. alimentarius/bavaricus*	DNA/DNA homology
22.17	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
		22°C, 48 h	
22.19	*Leuconostoc* or related genus	*Lc. mesenteroides*	Carbohydrate fermentation
22.24[a]	*Lactobacillus*, FH	*Lb. curvatus*	DNA/DNA homology
22.25	*Lactobacillus*, FH	*Lb. sakei*	Carbohydrate fermentation
22.29[ab]	*Lactococcus*	*L. lactis*	Carbohydrate fermentation
22.30[a]	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation
22.37	*Lactobacillus*, FH	*Lb. plantarum*	Carbohydrate fermentation

Shown only the results of the isolates that were submitted to identification at species level. Double isolations may be present.

[a] Isolates that decreased the pH of MRS medium to values below 4.8 in 3 hours, at 30°C.

[b] Isolates that decreased the pH of MRS medium to values below 4.8 in 9 hours, at 22°C.

FH: facultative heterofermentative

Growth screening

Isolates that were found to produce biogenic amines (chapter 5) were excluded from the experimental scheme. The remaining 65 isolates were screened for their ability to grow rapidly in MRS containing sucrose instead of dextrose at 15, 22 and 30°C. The first two temperatures are of interest for fish silage production and the latter corresponds to the generally recognised optimum for LAB growth.

The results of growth screening are shown in Fig. 2 — 4. In general, the isolates grew much faster at 30°C. At 15°C, the Δ O. D. values obtained after 9 h were, on average, four times lower than the corresponding values obtained at 30°C. For all three temperatures, the fastest growing isolates were obtained from the pastes incubated at 22°C. Most of these fast-growing isolates were obtained from pastes that had been incubated for 48 h at 22°C. None of the *Lb. sakei* isolates was among the fastest growing strains at 30°C. *Lb. curvatus* (22.24) was among the fast-growing strains at all three temperatures. Growth at 15°C was very slow. This temperature is not likely to be appropriate for the ensilage of fish.

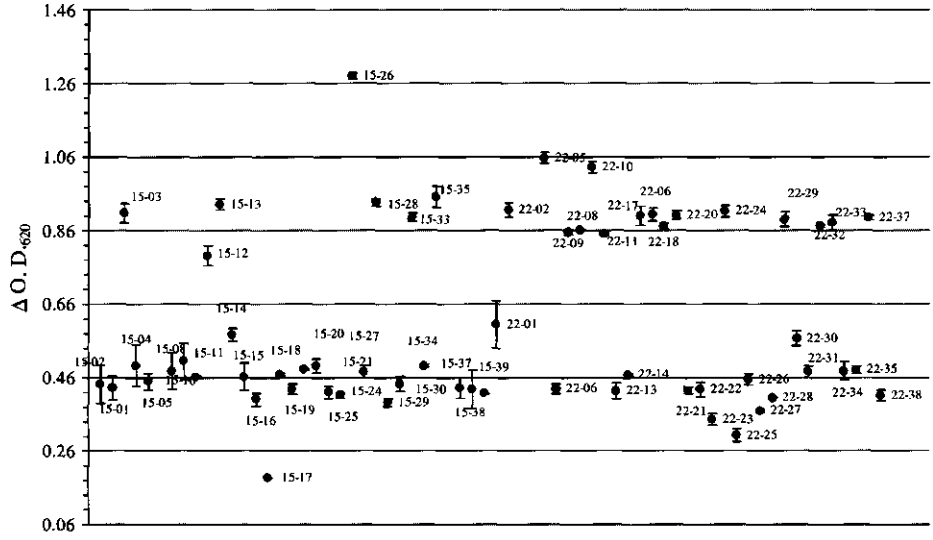

Fig. 2. Increases in optical density (λ=620 nm) after 18 h at 30°C of modified MRS medium inoculated with LAB isolates obtained from naturally fermented mackerel-sucrose-NaCl pastes. The points represent the averages of four determinations and the bars show the standard deviations. Isolate numbers are indicated next to the points.

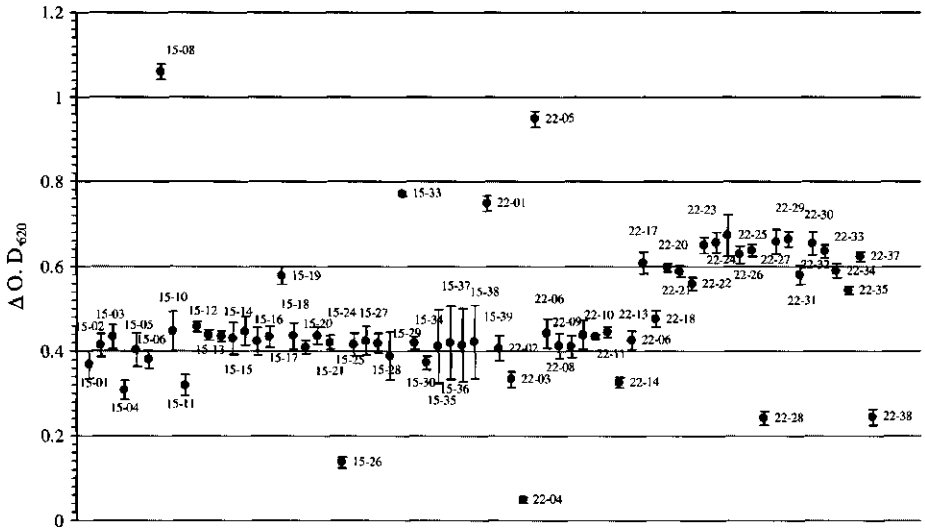

Fig. 3. Increases in optical density (λ=620 nm) after 18 h at 22°C of MRS medium inoculated with LAB isolates obtained from naturally fermented mackerel-sucrose-NaCl pastes. The points represent the averages of four determinations and the bars show the standard deviations. Isolate numbers are indicated next to the points.

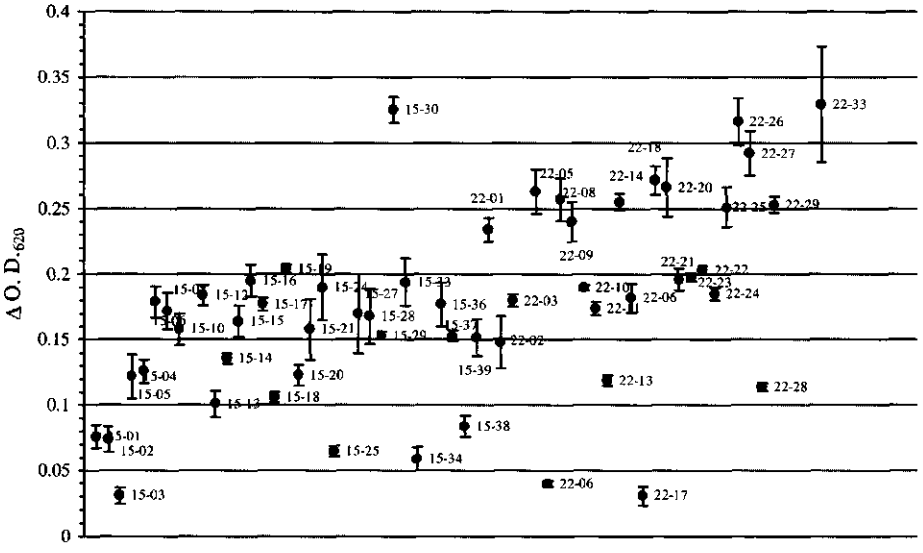

Fig. 4. Increases in optical density (λ=620 nm) after 18 h at 15°C of MRS medium inoculated with LAB isolates obtained from naturally fermented mackerel-sucrose-NaCl pastes. The points represent the averages of four determinations and the bars show the standard deviations. Isolate numbers are indicated next to the points.

Acidification screening

The fastest isolates to turn the indicator colour at 30 and 22°C are shown in Table 2. At 30°C, the first changes in colour of the medium were visible after 3 h of incubation. These were mostly isolates obtained from 48 h-old pastes. Most of these isolates were not among the fastest growing ones, except for *Lb. curvatus* 22.24 and *L. lactis* 22.29.

At 22°C, the first group of isolates to change the colour of the medium to yellow did so after 9 h of incubation. Acidification was considerably slower at this temperature than at 30°C. Ensiling fish at 22°C is likely to pose problems due to slow pH decrease. As previously observed for growth, most of the isolates that acidified the medium faster were obtained from 48 h-old naturally fermented pastes incubated at 15°C. They are practically the same as those that acidified the medium faster at 30°C. At 22°C, however, the number of isolates that grow fast and, simultaneously, lead to fast acidification, was smaller than at 30°C.

At 15°C, no changes were visible in the colour of the medium, even after incubation for 24 h. Acidification is likely to proceed very slowly at this temperature. For this reason, and because of the slow growth observed for all isolates, 15°C was not regarded as a suitable temperature for ensiling fish.

Antimicrobial activity

One of the most important properties required from the potential starters is the ability to predominate over the native microflora of the fish pastes, eliminating or greatly reducing the numbers of pathogenic and spoilage microorganisms. Microorganisms such as *S. putrefaciens*, *C. lipolytica*, *C. curvata*, *Salmonella* spp., *L. monocytogenes* and enterohaemorrhagic *E. coli* O157:H7 can survive or thrive under the conditions present in fish silage and compromise its safety and/or stability.

Due to the favourable conditions for the action of decarboxylase enzymes prevailing within fish silage, biogenic amine formation can become a problem. For this reason, LAB intended for fish silage starters should be able to inhibit the growth of biogenic amine producing microorganisms. In a parallel study, 13 out of the 77 LAB isolates obtained from naturally fermented mackerel pastes were found to produce one or more of the biogenic amines: histamine, tyramine, cadaverine and putrescine (this thesis, chapter 5). We used a number of these biogenic amine producing LAB cultures as indicator strains in direct antagonism tests along with collection strains of biogenic-amine producing *E. coli*, *M. morganii* and *Lb. büchneri*.

Two of the LAB isolates inhibited *Lb. sakei* 15.19, a biogenic amine producing strain, when tested at 30°C (Table 3). All of the LAB isolates inhibited the growth of the histamine producing bacterial isolates *M. morganii*, *E. coli* B351, *Lb. büchneri*, *W. hellenica* 15.32 and *Enterococcus* sp. 22.12 when tested at this temperature. The inhibition zones were relatively small and not clear, which indicates that inhibition was probably caused by lactic acid production. According to Daeschel (1992), organic acids, such as lactic and acetic acid, cause a gradient of

inhibition and therefore these somewhat diffuse inhibition zones, whereas substances such as bacteriocins and H_2O_2 give very sharp boundaries. Eleven strains produced inhibition zones of 1.0 cm or larger against the histamine producing *E. coli* B351 when whole cultures were used for the assay (Table 3). *Lactobacillus* sp.15.24 and *Enterococcus* sp. 22.29 displayed inhibitory effect when whole cells were used, but no inhibition was evident when using culture supernatants. In general, smaller inhibition zones were observed with culture supernatants than with whole cells. All of the LAB isolates were capable of inhibiting the growth of *C. curvata, S. putrefaciens, L. monocytogenes, Salmonella* and *E. coli* O157:H7, but no inhibition was observed when *C. lipolytica* was used as the target strain (data not shown).

Table 4 shows the results of the direct antagonism tests performed when

Table 3. Antimicrobial activity at 30°C of 12 LAB isolated from naturally-fermented mackerel, *Lactobacillus plantarum* 009, and one control strain (*Pediococcus acidilactici* PAC 1.0) against five biogenic amine producing bacteria.

Test strains	Indicator strains									
	Lb. sakei 15.19		*Lb. büchneri* B300		*W. hellenica* 15.32		*Enterococcus* 22.12		*E. coli* B351	
	W	S	W	S	W	S	W	S	W	S
15.03	-	-	+	+	+	+	+	+	1.0	0.
15.04	-	-	+	+	+	+	+	+	1.0	0.
15.24	-	-	+	+	+	+	+	+	1.0	-
15.25	-	-	+	+	1.1	1.1	+	+	+	+
15.29	-	-	+	+	1.1	1.1	+	+	+	+
15.31	-	-	+	+	+	+	+	+	1.0	0.
15.33	-	-	+	+	+	+	+	+	1.0	0.
15.35	0.9	0.9	+	+	0.9	+	1.8	1.8	1.0	0.
22.03	-	-	+	+	+	+	+	+	1.0	0.
22.19	-	-	1.8	1.6	1.1	1.3	+	+	+	+
22.24	-	-	+	+	0.9	+	1.2	1.0	+	+
22.29	-	-	+	+	+	+	+	+	1.0	-
Lb .plantarum 009	0.7	0.7	1.6	1.6	+	-	+	-	+	+
Control (*P. acidilactici* PAC 1.0)	1.2	1.2	1.0	1.0	-	-	1.1	1.1	1.0	1.0

The numbers represent the diameter of the inhibition zone in cm; - indicates absence of inhibition; W – whole culture; S – supernatant.

using supernatants and whole cultures of some LAB isolates against *L. sakei* 15.19.

These results show that the inhibitory effects were maintained, at all three temperatures tested, for the supernatant cultures of all isolates and of the control strain, indicating an extracellular metabolite as the responsible for the inhibitory effect.

Table 4. Growth inhibition of *Lb. sakei* 15.19 by four LAB isolates obtained from naturally fermented mackerel pastes and by strains of *Lb. plantarum* and *P. acidilactici* (control) at 15, 22 and 30°C.

Inhibiting strains	Temperature					
	15°C		22°C		30°C	
	W	S	W	S	W	S
Lb. curvatus 15.35	1.0	0.9	0.9	0.9	0.9	0.9
Enterococcus 15.25	-	-	1.0	1.1	-	-
Enterococcus 15.29	-	-	1.1	0.9	-	-
Lb. curvatus 22.24	-	-	1.0	0.8	-	-
Lb. plantarum	-	-	0.9	0.8	0.7	0.7
Control (*P. acidilactici*)	-	-	0.9	0.9	1.2	1.2

The numbers represent the diameter of the inhibition zone in cm; - indicates absence of inhibition; W – whole culture; S – supernatant.

Further experiments led to the conclusion that a general bacteriostatic effect of LAB, such as the production of organic acids, is probably responsible for the inhibition caused against *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. These target strains were inhibited when the agar wells were filled with medium (pH 4.5) or when the medium contained 1 or 2% of sodium lactate or sodium acetate, at pH 6.0 (Table 5). Precise diameters of inhibition could not be obtained for all samples, since weak organic acids create a gradient of inhibition resulting in a less sharply defined inhibition zone (Daeschel, 1992).

These results were as expected for *L. monocytogenes*, since the limit pH value allowing the initiation of its growth is around 4.5 (Piard and Desmazeaud, 1993). *Salmonella typhimurium* also has a minimum pH for growth of 4.5 (Jynshiun *et al.*, 1995). Strains of *E. coli* O157:H7 have, however, been found to be acid-resistant (Bibek, 1992, Abdoul-Raouf *et al.*, 1993). The possibility that mechanisms of antagonism other than acid production (e. g. hydrogen peroxide and bacteriocin production) may also be involved in the inhibitory effect of LAB against *L. monocytogenes* and *E. coli* O157:H7 cannot be ruled out. However, since the direct antagonism test does not allow further differentiation of combined effects when strong medium acidification is involved, no more tests were performed involving these bacteria.

Table 5. Inhibition of indicator strains *E. coli* O157:H7, *L. monocytogenes*, *C. curvata* and *C. lipolytica* in media with adjusted pH (4.5) or 1 or 2% sodium lactate or sodium acetate (pH adjusted to 6.0).

Inhibitory agents	*E. coli* O157:H7			*L. monocytogenes* Scott A			*C. curvata*		
	15°C	22°C	30°C	15°C	22°C	30°C	15°C	22°C	30°C
1% lactate	+	+	+	+	+	+	-	-	-
2% lactate	+	+	+	+	+	+	-	-	-
1% acetate	+	+	+	+	+	+	-	-	-
2% acetate	+	+	+	+	+	+	-	-	-
pH 4.5	1.5	1.5	1.7	1.5	1.7	1.6	0.6	0.8	0.6

The numbers represent the diameter of the inhibition zone in cm; - indicates absence of inhibition; W – whole culture; S – supernatant.

Acid production or pH decrease did not account for the antagonistic activity displayed by LAB isolates 15.35 (*Lb. curvatus*), 15.25 (*Enterococcus* sp.), 15.29 (*Enterococcus* sp.), 22.24 (*Lb. curvatus*) and *Lb. plantarum* against *Lb. sakei* 15.19 and by all of the LAB isolates against *S. putrefaciens*, since negative results were obtained when the above described tests were performed with these target bacteria. *C. curvata* was not inhibited by the salts, in solutions adjusted to pH 6.0, but was inhibited when the pH was adjusted to 4.5 by means of HCl addition (Table 5). The diameters of the inhibition zones obtained were smaller than those observed for *E. coli* O157:H7 and *L. monocytogenes*, indicating a weaker inhibitory effect. These results suggest that *C. curvata* is probably able to degrade the organic acids produced by LAB, but is not resistant to the pH decrease resulting from LAB growth.

The inhibitory effect of *Lb. curvatus* 15.35 disappeared when catalase was added to its supernatant culture (Table 6). This indicates H_2O_2 production as the mechanism responsible for the inhibitory effect. Such hypothesis is also confirmed by the fact that this effect was detectable at all three temperatures. The production of other inhibitory compounds such as bacteriocins is, usually, limited to a narrow temperature range (Jack *et al.*, 1995).

It is interesting to note that, at 30°C, the inhibitory effect of *Lb. plantarum* 009 disappeared when catalase was added. This did not occur at 22°C, although smaller inhibition diameters were observed at this temperature, when comparing with the supernatant tests. All culture supernatants of the remaining isolates maintained their antagonistic activity after the addition of catalase (Table 6).

When pronase E was added to the culture supernatants, isolates 15.35, 15.25 and 15.29 still showed positive results (Table 6). Isolate 22.24 (identified as *Lb. curvatus*) lost its inhibitory effect, which was maintained if the supernatants were submitted instead to 100°C for 20 minutes. This indicates that a bacteriocin-

like substance produced by the isolate could be causing the inhibitory effect (Schillinger and Lücke, 1989, Daeschel, 1992). Furthermore, the results obtained for the control strain (a bacteriocin producer) were similar.

Table 6. Inhibition of *Lb. sakei* 15.19 by supernatants obtained from cultures of *Lb. curvatus* 15.35, *Enterococcus* 15.25, *Enterococcus* 15.29, *L. curvatus* 22.24, *L. plantarum* and *P. acidilactici* (control strain) at 15, 22 and 30°C, when catalase and pronase E have been added (5 mg ml^{-1} of culture supernatant and 0.25 mg ml^{-1} of culture supernatant, respectively).

Inhibitory isolates	Catalase			Pronase E			100°C – 20 min			Control		
	Temperature, °C											
	15	22	30	15	22	30	15	22	30	15	22	30
Lb. curvatus 15.35	-	-	-	0.9	0.9	0.9	Nt	Nt	Nt	0.9	0.9	0.9
Enterococcus 15.25	-	1.1	-	-	1.1	-	Nt	Nt	Nt	-	1.1	-
Enterococcus 15.29	-	0.9	-	-	0.9	-	Nt	Nt	Nt	-	0.9	-
Lb. curvatus 22.24	-	1.8	-	-	-	-	Nt	0.7	Nt	-	0.8	-
Lb. plantarum 009	-	0.5	-	-	0.3	0.7	Nt	0.8	Nt	-	0.8	0.7
Control strain (*P. acidilactici* PAC 1.0)	-	0.8	1.0	-	-	-	-	0.8	0.9	-	0.9	1.2

The culture supernatants of *Lb. curvatus* 22.24, *Lb. plantarum and P. acidilactici* were heated at 100°C for 20 min to test heat-stability of the metabolites produced.
A control (no heat treatment, no enzyme addition) is also included.
The numbers represent the diameter of the inhibition zone in cm; - indicates absence of inhibition; + indicates the presence of a diffuse inhibition zone; Nt – not tested.

Lb. plantarum 009 partially lost its inhibitory effect at 22°C when pronase E was added, showing a minor remaining inhibition zone diameter when compared to the results obtained with the culture supernatant at the same temperature (Table 6). The full effect was recovered if the culture supernatant was submitted to 100°C for 20 minutes. On the other hand, the inhibitory effect verified at 30°C was not affected by the addition of pronase E. This suggests that different mechanisms of inhibition are produced by this strain, depending on the growth temperature. At 30°C, H_2O_2 production could be the only mechanism responsible for inhibition, while at 22°C only a partial inhibitory effect can be ascribed to such mechanism. A bacteriocin-like substance may be another cause for the observed inhibition, which

is in accordance with the knowledge that the production of bacteriocins is limited to a narrow temperature range, while H_2O_2 production is not (Daeschel and Penner, 1992, Piard and Desmazeaud, 1993, Jack *et al.*, 1995).

Isolates 15.25 and 15.29 maintained their inhibitory effect after pronase E had been added (Table 6). To the extent of the tests performed, this may indicate that these isolates produce an antibacterial metabolite of non-protein nature. *Lb. curvatus* 15.35 also maintained its inhibitory effect after pronase E had been added, confirming that the sole mechanism of antagonism involved was H_2O_2 production.

Temperature seems to play an important role in the presence or absence of inhibitory effect against *Lb. sakei* 15.19, since there was generally a stronger inhibition at 22°C than at 15 or 30°C. The control strain did not show inhibitory effect against *Lb. sakei* 15.19 at 15°C, presenting a more intense effect at 30°C than at 22°C. Since the control strain and the indicator strain are both LAB and the inhibitory agent was sensitive to pronase E, it is reasonable to conclude that the inhibitory effect obtained with the control strain is due to the production of pediocin PA-1. These results are in agreement with the finding that bacteriocin production is dependent on growth temperature, and that it may be activated or intensified when bacteria are under stress conditions (Jack *et al.*, 1995).

CONCLUSIONS

Natural fermentation of fish residues did not lead to sufficiently low pH values rapidly enough to ensure preservation. Thus, the use of a starter culture is essential. Fifteen of the isolates enriched at 15°C were identified to the species level. Seven of these isolates were ascribed to the *Lb. sakei* species. Fourteen isolates enriched at 22°C were also identified to the species level. Six of these isolates were *Lb. plantarum*. The fastest growing isolates at 30 and 22°C, as indicated by higher increases in O. D. after 9 h incubation, were obtained from fish pastes fermented at 22°C for 48 h. Growth at 15°C was very reduced, so this temperature was not regarded as suitable for the ensilage of fish. The fastest acidifying isolates at 30°C had been obtained from 48-h old pastes incubated at 15°C. Acidification was rather slower at 22 than at 30°C and at 15°C it was too slow to be observed within the short interval required. Ensiling at 22°C may also pose problems because although most LAB isolates grew well at 22°C, acidification was slower.

Pathogens such as *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7 should not pose problems during the ensilage of fish, because all of the LAB tested could inhibit them by a general mechanism - acid production. Spoilage microorganisms such as *M. morganii*, *E. coli*, and the biogenic-amine producing strains of *W. hellenica*, *Lb. büchneri* and *Enterococcus* spp. were also inhibited by all isolates, predominantly due to acid production. All of the LAB isolates inhibited *C. curvata* due to the decrease in pH of the medium resulting from their metabolic activities. All of the LAB displayed antimicrobial activity against *S. putrefaciens* as

well, although the inhibition mechanism could not be established. Of the bacterial indicator strains, *Lb. sakei* 15.19 proved to be the most difficult to inhibit. At 30°C, only two of the indicator bacteria were able to inhibit its growth. At 22°C, a higher number of isolates had an inhibitory effect on *Lb .sakei* 15.19. This strain, which was isolated from naturally fermented mackerel pastes, can produce biogenic amines and is, thus, undesirable as it can compromise the toxicological safety of fish silage. A careful choice of starter cultures and ensilage conditions is required to keep this type of contaminant under control.

C. lipolytica, one of the yeast species involved in the spoilage of fish silage, could not be inhibited by any of the LAB tested. A different approach, comprising the choice of adequate ensilage additives, may help keeping this microorganism under control. This is also in line with earlier findings that *C. lipolytica* can be a dominant spoilage organism in fish silage (Levin and Witkowski, 1991).

The results obtained in the present work show that fish silage has the potential to be a microbiologically safe product, since all of the pathogens tested were inhibited by all of the prospective starter cultures. Biogenic amine production by non-starter LAB can however pose risks and means of reducing it should be investigated.

Chapter 5

Biogenic amine formation and degradation by potential fish silage starter microorganisms

ABSTRACT

During the preservation of fish by ensilage with lactic acid bacteria, the pH value of the pastes decreases to below 4.5. This pH decrease is partly responsible for preservation. Decreased pH values and relatively low oxygen concentrations within the silage facilitate decarboxylase activity. Biogenic amines may constitute a potential risk in this kind of product since their precursor amino acids are present in fish silage. It is important that the LAB strains chosen for starters do not produce biogenic amines. Some bacteria, among which some LAB species, are able to degrade these metabolites by means of amino oxidases. This could be of interest for fish silage production, to control biogenic amine build-up in this product.

Seventy-seven LAB cultures isolated from fish pastes submitted to natural fermentation at two temperatures (15 and 22°C) and selected combinations of these isolates were examined for histamine, tyramine, cadaverine and putrescine production. Seventeen per cent of the isolates tested were found to produce one or more of these biogenic amines. The behaviour of diamine oxidase was tested under the conditions present in fish silage. Addition of 12% sucrose or 2% NaCl did not affect histamine degradation. Addition of 0.05% cysteine decreased histamine degradation. Degradation occurred at all temperatures tested (15, 22 and 30°C), but not at pH 4.5. Forty-eight potential fish silage starters were tested for histamine degradation in MRS broth containing 0.005 g l^{-1} histamine and incubated at 30°C. Indications were found that five of these isolates could degrade as much as 20-56% of the histamine added to the medium within 30 h, when used as pure cultures. No histamine degradation was observed with combinations of cultures. Histamine degradation (50-54%) by two of these isolates was also observed in ensiled fish slurry.

INTRODUCTION

Ensiled fish contains a considerable amount of the free amino acids that constitute the precursors for biogenic amines such as histamine, tyramine,

Enes Dapkevicius, M. L., Nout, M. J. R., Rombouts, F. M., Houben, J. H., and Wymenga, W. 2000. *Int. J. Food Microbiol.*, 57: 107-114.

putrescine and cadaverine (Haard *et al.*, 1985). Biogenic amines are formed by decarboxylation of their precursor amino acids, as a result of the action of either endogenous amino acid decarboxylase activity (Halász *et al.*, 1994) or by the growth of decarboxylase positive microorganisms (Halász *et al.*, 1994, Silla-Santos, 2001). The low pH (below 4.5 after 2 days of fermentation) and the physical characteristics of fish silage that lead to a low oxygen concentration within the ensiled fish are favourable for the action of amino acid decarboxylases (Beutling, 1992). Thus, biogenic amines may pose a potential risk in fish silage. These compounds are toxic for livestock, causing liver damage and decreasing the performance of the animals (Krizek, 1991). In susceptible humans, they can lead to a variety of cutaneous, gastrointestinal, haemodynamic and neurological symptoms (Taylor, 1986).

Biogenic amines can be degraded by amine oxidases. Diamines, such as histamine and putrescine, are degraded by diamine oxidase (DAO). The activity of these enzymes is maximum under neutral to alkaline conditions, and oxygen is necessary for their action (Beutling, 1992). DAO can provide a means of controlling histamine accumulation during the first days of ensilage, while the pH is high enough and some oxygen is still available within the product. Little is known about histamine degradation by LAB, but Voigt and Eitenmiller (1978) and Leuschner *et al.* (1998) found DAO activity among some dairy and meat isolates, respectively.

The aims of the present work were to select a starter culture for the production of safe fish silage and to assess the ability of diamine oxidase to degrade histamine under the conditions prevailing in this product. LAB isolates were screened for the production of biogenic amines. The effect of different conditions on the amount of histamine degraded by DAO was studied and LAB isolates were tested for DAO activity in broth and in ensiled fish slurry.

MATERIALS AND METHODS

Biogenic amine production by LAB

Seventy-seven LAB isolates obtained from naturally fermented pastes prepared from mackerel (*Scomber scombrus* Linnaeus 1785) and sucrose (22:3, w/w) incubated at 15 and 22°C were screened for their ability to produce biogenic amines by decarboxylation of the corresponding amino acids (histidine, tyrosine, lysine and ornithine). Histidine, tyrosine, lysine and ornithine degradation was tested in a pH-based differential medium for the detection of biogenic amine formation, the Modified Moeller Broth (Joosten and Northolt, 1987). Histamine formation was confirmed by qualitative thin-layer chromatography, according to Lieber and Taylor (1978), using silica-gel plates (F_{254}, 20×20cm, thickness of layer: 0.25 mm; Merck no. 5715, Darmstadt, Germany). Ten-microlitre aliquots of Modified Moeller Broth inoculated with the bacterial cultures and incubated at

30°C for 48 h were spotted onto the plates. Samples of Modified Moeller Broth containing added histamine were also spotted, as a control. The samples were eluted with methanol:ammonia (20:1). After drying, the plates were developed with ninhydrine spray reagent (300 mg ninhydrine, 100 ml n-butanol, 3 ml glacial acetic acid). Histamine was detected as violet-grey spots (Rf=0.74). The detection level for this method was 0.01 g l^{-1}.

Functionality of DAO

The functionality of this enzyme was assessed in a model system consisting of a solution of 0.05 g l^{-1} histamine dihydrochloride (Acros Organics, 15062-0050, Geel, Belgium) in phosphate buffer (0.5M, pH 7) to which 633.5 units l^{-1} of DAO (diamine oxidase, EC 1.4.3.6, from porcine liver, 0.14 units per mg, Sigma, D-7867, St. Louis, U. S. A.) were added. Unless stated otherwise, the reaction mixture was incubated at 37°C. Several conditions were tested, viz.: (a) pH 4.5 achieved by the addition of lactic acid (racemic mixture); (b) micro-aerobic conditions, achieved by adding 0.05 g l^{-1} cysteine to the culture medium prior to sterilisation and overlaying the reaction mixture with sterile paraffin oil; (c) effect of the usual additives intended for use in fish silage: sucrose; (12%, w/w) and NaCl (2%, w/w); and (d) different temperatures (37, 30, 22, and 15°C).

Samples were taken at time 0, 0.5, 1, 3, and 5 h and the reaction mixture was rapidly heated in a boiling water bath for 30 min to inactivate the enzyme. The inactivated samples were submitted to an extraction procedure according to Shore (1971) and histamine was quantified by a spectrofluorimetric method based on the reaction of histamine with *o*-phthalaldehyde (Shore, 1971).

Degradation of histamine by LAB in a model system

Forty-eight LAB cultures isolated from naturally fermented fish pastes were tested for their ability to degrade histamine in a model system, Diamine Oxidase Assay Broth, consisting of 1 g l^{-1} glucose, 3 g l^{-1} yeast extract, 3 g l^{-1} tryptone, 5 g l^{-1} NaCl, 1 g l^{-1} Tween 80, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.038 g l^{-1} $MnSO_4 \cdot H_2O$ g l^{-1}, 0.08 g l^{-1} $FeSO_4 \cdot 7H_2O$, 0.05 g l^{-1} histamine dihydrochloride, pH 6.0. The isolates had been selected for their good growth, acidification and antimicrobial properties in paralel studies (this thesis, chapter 4). Mixed cultures consisting of *Lactobacillus curvatus* 22.24 and various isolates of *Lactobacillus sakei* (15.05, 15.18, 15.36 and 15.39) were tested for histamine degradation in Diamine Oxidase Assay Broth. Histamine concentration was determined by the spectrofluorimetric method of Shore (1971). Samples were taken at time 0 and after 30 hours of incubation at 30°C. When apparent, histamine degradation was confirmed by quantitative HPLC analysis of the histamine concentration. The model system and incubation conditions were the same as above, with the exception of added histamine dihydrochloride (0.075 g l^{-1}). The method for HPLC

determination of histamine was based on Luten *et al.* (1991) and Gouygou *et al.* (1987).

Sample aliquots of 159 µl were derivatised in a plastic tube protected from light by adding 1.9 ml of distilled water and 0.4 ml 1 N NaOH. This mixture was allowed to stand for 1 minute and then 100 µl of an *o*-phthalaldehyde methanolic solution (1 g *o*-phthalaldehyde l^{-1} methanol) was added. The tube was shaken vigorously, the reaction was allowed to proceed for 4 min and then stopped by adding 0.2 ml of 3 N HCl. The derivatised sample solution was centrifuged for 15 minutes at 8000 rpm, filtered through 0.2 µm millipore syringe filters (Schleicher & Schuell, FP 030/AS, Ref. 462960, 's Hertogenbosch, The Netherlands) and analysed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Separation was carried out on a C_{18} column (Spherisorb 2.5 µm ODS; 25 cm×4.6mm internal diameter; Chrompack, Middelburg, The Netherlands). 20 µl of injected sample mixture was eluted isocratically during 20 min with a mixture of acetonitrile (40%, HPLC-grade, LAB-Scan, C2502, Dublin, Ireland) and 50 mM NaH_2PO_4 solution in millipore water (60%). The flow rate was 0.70 ml min^{-1}. Detection was done with a continuous flow fluorescence meter (Spectra System Series, Thermo Separation Products, FL 3000, Riviera Beach, Florida, U.S.A.) with excitation at λ=350 nm and emission at λ=450 nm. All determinations were carried out in triplicate.

Degradation of histamine by LAB in ensiled fish slurry

Atlantic mackerel was purchased form a local retailer (Wageningen, The Netherlands) and ground in a meat grinder with a 4 mm die. Each batch of silage consisted of 100 g ground mackerel to which 100 ml of an aqueous solution containing 24 g l^{-1} sucrose, 4 g l^{-1} NaCl and 2 g l^{-1} histamine dihydrochloride were added. The resulting mixture was thoroughly homogenised using a spatula and 2% (v/w) of an 18-hour MRS broth (Oxoid, CM 359, Basingstoke, U.K.) culture of the designated LAB isolate was added. For the DAO experiment, 10780 units kg^{-1} DAO were added as well. Cultures 15.18 and 15.36 (both *Lb. sakei*) were used as inoculum in the fish experiment, since they had been found to degrade histamine in the model system. Fish slurries with added DAO were inoculated with a non-histamine degrading bacterium (*Lb. curvatus* 22.24). A control slurry was inoculated with isolate 22.24 only, to determine histamine autodegradation during ensilage. After thorough mixing with a spatula, the fish pastes were distributed in glass jars closed with a screw cap fitted with a water-column type air lock. Incubation was at 30°C for 4 days. Samples were aseptically withdrawn for pH measurement and spectrofluorimetric histamine determination.

RESULTS AND DISCUSSION

The results of the tests for amino acid decarboxylation are summarised in Table. 1. Production of more than one of the assessed biogenic amines was common among the isolates. Seventeen percent of the screened isolates produced one or more of the biogenic amines. This shows that attention must be paid to biogenic amine production when selecting bacterial strains for fish silage production. Most of the biogenic amine positive isolates were obligatory heterofermentative lactobacilli belonging to the species *Lb. sakei* and *Lb. curvatus* (data not shown).

Table 1. Amino acid degradation by 13 lactic acid bacteria isolated from naturally fermented mackerel/sucrose (88:12, w/w) pastes.

Code and identity of the isolates	His	Lys	Orn	Tyr
15.07 (*Leuconostoc* or related genus)	-	+	-	-
15.09 (*Lactobacillus* sp., FH)	+	-	-	-
15.19 (*Lb. sakei*)	+	-	-	+
15.22 (*Leuconostoc* or related genus)	-	-	+	+
15.23 (*Lactobacillus* sp., FH)	-	-	-	+
15.31 (*Lactobacillus* sp., FH)	+	+	-	-
15.32 (*Weissella hellenica*)	+	-	-	-
22.04 (*Leuconostoc* or related genus)	+	-	-	+
22.07 (*Lactobacillus* sp., FH)	-	+	+	-
22.12 (*E. faecium* species group)	+	-	+	+
22.15 (*Lactobacillus* sp., OH)	+	+	-	-
22.19 (*Leuconostoc mesenteroides*)	+	-	-	+
22.36 (*Leuconostoc* or related genus)	-	-	-	+

His=histidine, histamine precursor, Lys=lysine, cadaverine precursor, Orn=ornithine, putrescine precursor, Tyr=tyrosine, tyramine precursor.
FH=facultative heterofermenter, OH=obligatory heterofermenter.

To obtain a deeper insight into its potential for histamine degradation in fish silage, the effect of added DAO was investigated in a buffered model system simulating the main conditions prevailing in this product. Estimations of DAO activity in the literature have been based on determinations of oxygen and histamine consumption, aldehyde, ammonia or hydrogen peroxide formation (Roscoe and Kupfer, 1972). In this experiment, histamine degradation resulting from DAO activity was determined by measuring the decrease in histamine content.

The effect of temperature on histamine degradation was noticeable (Fig. 1). The highest degradation rate was observed at 37°C, but at 22 and 15°C degradation was still considerable. These results are in agreement with the characteristics described for diamine oxidase (Schomburg and Stephan, 1993). DAO has its temperature optimum at 37°C and it is active within a range of 20-63°C, retaining about 50% of its maximum activity at 20°C. The pH of the model system was adjusted to 4.5 to assess the pH sensitivity of DAO. This pH value corresponds to the final pH achieved during the ensilage of fish. Oxygen diffusion to the interior of the fermenting fish pastes is difficult, resulting in reduced oxygen availability within the fish mass. To simulate micro-aerobic conditions, cysteine was added to the test solution and the reaction mixture was covered with sterile paraffin oil before incubating. NaCl and sucrose were chosen as additives for the production of fish silage. Model systems with adequate amounts of these compounds were also prepared, to determine whether their presence interferes with DAO. The results of this set of experiments are shown in Fig. 2.

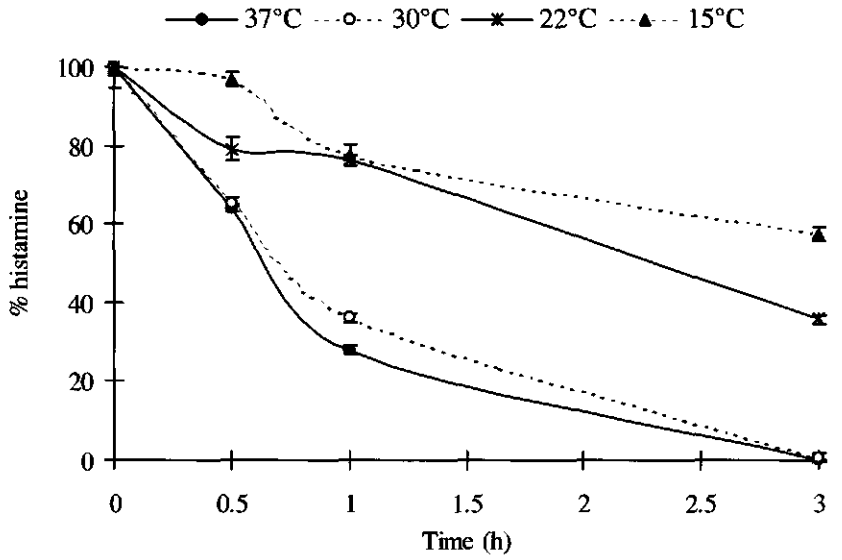

Fig. 1. Effect of temperature on the degradation of histamine by diamine oxidase in phosphate buffer (pH 7.0). The bars represent the standard deviations (n=3).

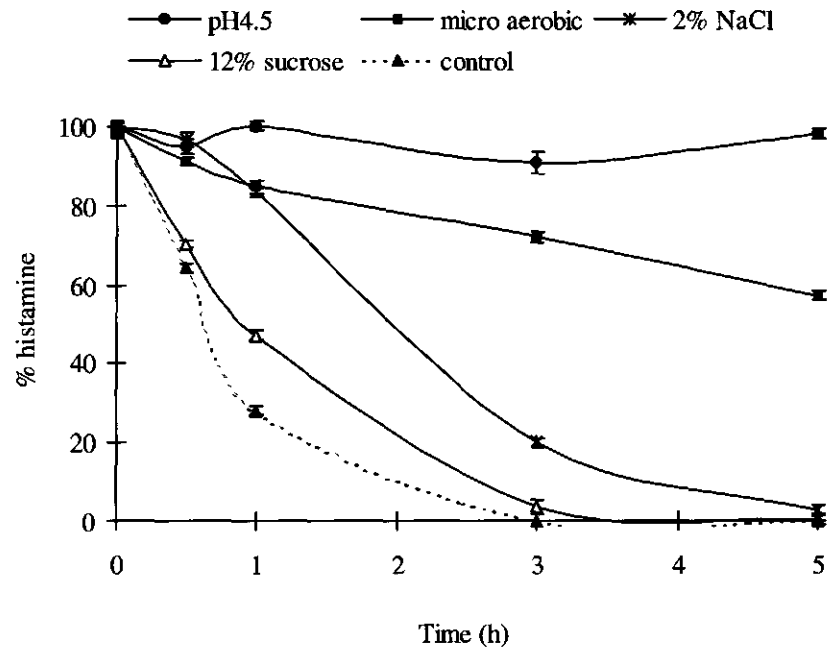

Fig. 2. Histamine degradation by diamine oxidase in phosphate buffer (pH 7.0). Effect of pH 4.5, micro-aerobic conditions (pH 7.0) and the addition of 2% NaCl or 12% sucrose (pH 7.0).The bars represent the standard deviations (n=3).

Decreasing the pH to 4.5 proved to be the most limiting factor. This was not unexpected, since DAO is known to have a pH optimum of 7.0 and a pH range of 5 – 10 (Raa and Gildberg, 1982). Furthermore, a precipitate was formed in the reaction mixture at pH 4.5, indicating denaturation of the enzyme. However, fish silage acidifies gradually, and will only reach pH 4.5 after 2 days of fermentation. Initially, the pH is within the range that facilitates DAO activity. During this initial period, bacterial contaminants present in the fish are still active and their decarboxylating activity can take place. Although decarboxylase enzymes have their optimum activity at low pH values, they can also be active at higher pH. The pH optimum of the L-histidine decarboxylase from one strain of *Oenococcus oeni (Leuconstoc oenos)* was found to be 4.8, both for cell suspensions and cell-free extracts. The enzyme still showed activity at pH 7.2 and at pH values close to 3.5 (Rollan *et al.*, 1995). Thus, the presence of DAO could be of value to prevent build-up of histamine during the early stages of ensilage. During fermentation, the majority of Gram-negative bacteria disappears due to microbial competition and acidity. In silages that reached a pH below 4.5, such bacteria would not contribute to biogenic amine production. However, non-starter LAB can become a problem at the later stages of ensilage, since the low pH attained will not inhibit their growth

completely. In this case, another approach should be used. The use of starter cultures that have antimicrobial properties against such contaminants could reduce the risk of biogenic amine formation during the later stages of ensilage. Micro-aerobic conditions had a limiting effect on the degradation of histamine. However, it is possible that the little amount of oxygen left within the fish mass during ensilage would be enough to allow for some decrease in the level of histamine in the silage. As it can be seen in Fig. 2, almost one half of the histamine added was degraded within a 5-hour period under micro-aerobic conditions. The addition of NaCl or sucrose showed only a minor effect on the functionality of DAO. Initially, histamine degradation was hindered by the presence of NaCl.

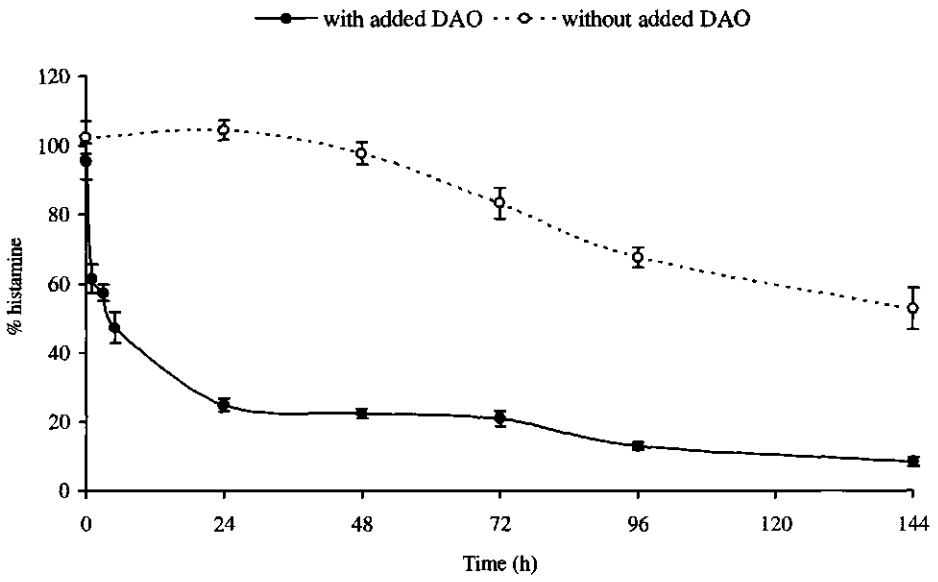

Fig. 3. Histamine degradation by diamine oxidase in a fish slurry (pH 6.40) incubated at 30°C.The bars represent the standard deviations (n=3).

Little is known about histamine degradation by bacteria. Kumagai *et al.* (1969) described some monoamine oxidase activity in the species *Sarcina lutea*. Voigt and Eitenmiler (1978), Beutling (1992) and Leuschner *et al.* (1998) investigated bacterial DAO activity in a rich medium containing several nitrogen sources. In the present study, histamine degradation was initially investigated in modified MRS medium. Further experiments were performed in ensiled mackerel slurry. The results of these experiments are shown in Fig. 3 and Table 1. Histamine autodegraded in the ensiled fish slurry as it did in the model system. Histamine concentration remained stable for the first 72 h and decreased to 80% of the initial concentration after 96 h of ensilage (Fig. 3). DAO degraded histamine to about

20% of its initial concentration. Its inactivation after 24 h may be related to the decrease in pH values within the ensiled fish slurries. Fig. 3 shows that DAO was active during the early stages of ensilage and that it can be used to reduce the histamine content in fish pastes.

The effect of different LAB isolates on the histamine concentration and pH in the model system and in fish slurry silage are shown in Table 2. Four isolates (15.05, 15.18, 15.36 and 15.39, all *Lb. sakei*) were found to degrade histamine in the model system. These results were confirmed when monitoring histamine degradation by RP-HPLC. Additionally, RP-HPLC assessment indicated that one more isolate, *Lb. curvatus* 15.35, was able to degrade histamine (Fig. 4). Although it showed good results in the model system, *Lb. sakei* 15.05 was not used for the degradation experiment in ensiled fish slurry because it does not yield stable silages. *Lb. sakei* 15.39 was not used because it gave similar results to 15.36 in the model system experiment. None of the combinations of isolates tested showed histamine degradation under the conditions of our experiment.

Table 2. Degraded histamine and pH values in MRS broth with 50 mg l^{-1} histamine (starting pH 6.49) and in ensiled fish slurry with 10mg l^{-1} histamine (starting pH 6.40) after 30 and 96 h of incubation at 30°C with lactic acid bacteria isolates or diamine oxidase.

	Model system		Ensiled fish slurry	
	Degraded histamine (g $100g^{-1}$)	Final pH	Degraded histamine (g $100g^{-1}$)	Final pH
Lb. sakei 15.05	56.18±4.28	3.72	ND	ND
Lb. sakei 15.18	35.81±4.53	3.75	49.85±3.60	4.35
Lb. sakei 15.36	28.67±4.75	3.78	53.63±2.54	4.42
Lb. sakei 15.39	19.61±3.15	3.74	ND	ND
DAO	99.49±0.01	7.00	86.20±5.99	4.50
Control	16.36±3.15	6.49	33.64±1.81	4.51

The results of isolates that were not found to degrade histamine are not shown.
The numbers represent the averages ± standard deviations of three values.
ND=not determined.

The pH decrease in fish slurry silage is much slower than in the buffered model system, therefore, more time was allowed in the fish experiment than in the model system (96 and 30 h, respectively). Nevertheless, the pH of the ensiled fish slurries did not reach the very low values achieved in the model system (Table 1). This may be due to the difference in buffering capacity between both systems. DAO was less efficient in the fish slurry silage than in the model system (Table 1) perhaps due to the gradual decrease in pH in the first. However, a higher degradation could be observed for the LAB in the silage than in the model system.

The different pH decrease patterns might provide an explanation for this. Histamine autodegradation was comparable in both cases.

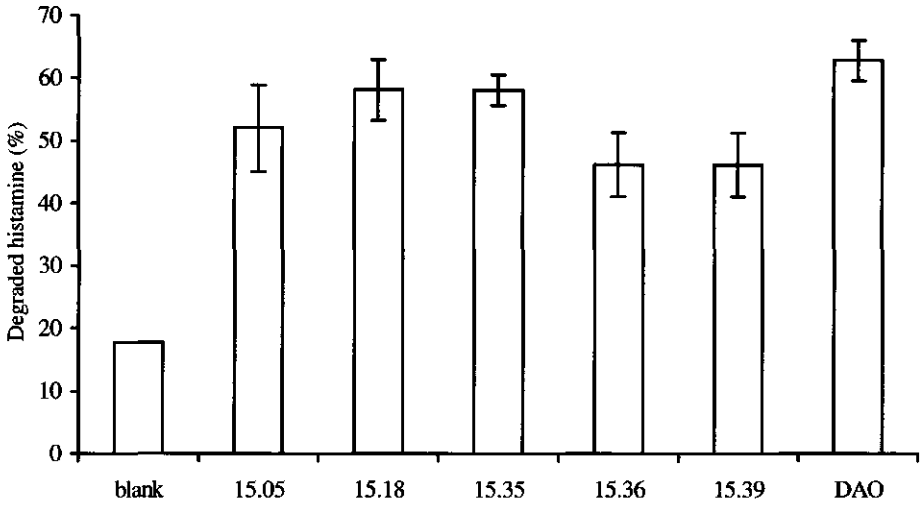

Fig. 4. Histamine degradation measured by RP-HPLC in Diamine Oxidase Assay Broth with added histamine (750 mg ml^{-1}, pH 6.49) after 24 h of incubation at 30°C with different lactic acid bacteria isolates and diamine oxidase (DAO). The bars represent the standard deviations (n=3).

CONCLUSIONS

Attention should be paid to biogenic amine production by LAB intended for fish silage starters. A considerable proportion of the LAB isolated from fish display such specific decarboxylase activity and may cause problems in the later stages of the ensilage. Ideally, fish silage starters should be able to inhibit undesirable non-starter LAB.

The results obtained showed that DAO is able to degrade histamine during the early stages of fish silage fermentation, when the pH is still above 4.5 and there is some oxygen left within the fish mass. Indications were found that four of the 48 LAB isolates could degrade histamine in a model system. Histamine degradation by two of these isolates was also observed in the ensiled fish slurry. Their use for fish silage preparation could reduce the risks posed by this amine.

Chapter 6

Growth, acidification and competitive properties of potential fish silage starter strains in pure cultures and as combinations

ABSTRACT

Six pure cultures, found to possess good growth, antimicrobial properties, fast acidification or ability to degrade histamine in previous studies (*Lactobacillus curvatus* 15.35 and 22.24, *Lactobacillus sakei* 15.05, 15.18, and 15.39, *Lactobacillus plantarum* 009) and six combinations of the aforementioned *Lb. curvatus* and *Lb. sakei* strains (15.35+15.05, 15.35+15.18, 15.35+15.39, 22.24+15.05, 22.24+15.18, and 22.24+15.39) were tested for their growth and acidification properties in MRS broth at 30°C, with the objective of determining their suitability as potential starters for the ensilage of fish. Growth was monitored by plate counts at regular time intervals in MRS medium (pure cultures) and in a modified MRS broth, containing trehalose instead of sucrose (differential enumeration of *Lb. sakei* isolates in the combinations). Growth parameters were calculated from these data, using the Gompertz model. Titratable acidity and pH were also monitored in the pure cultures and combinations.

Lb. curvatus 15.35 showed a bactericidal effect on the 3 *Lb. sakei* cultures. It was therefore regarded as not suitable for use in combinations. The antibacterial properties of this isolate could, however, be interesting when used as a single-strain inoculant. The growth of *Lb. curvatus* 22.24 was inhibited in culture combinations with *Lb. sakei*. Furthermore, culture combinations led to slower acidification and were regarded as not suitable for mixed-culture fermentation. Among the pure cultures tested, *Lb. curvatus* 15.35 and *Lb. sakei* 15.39 displayed high growth and pH decrease rates, high maximum populations and short lag phases and were selected for further testing.

Cooked Atlantic mackerel slurries with 12% (w/w) sucrose and 2% (w/w) NaCl added were ensiled using *Lb. curvatus* 15.35 or *Lb. sakei* 15.39 cultures as inoculants, with or without the addition of *E. coli* NIZO B351 (a histamine-

producing bacterium) at the beginning of the experiment. Fermentation temperature was 30°C. Under these conditions, *Lb. curvatus* 15.35 yielded a pH value below 4.5 within 72 h, while it took *Lb. sakei* 15.39 13 days to yield such pH values, which are low enough to ensure silage preservation. In challenge experiments with *E. coli* NIZO B351, growth and pH decrease by both strains studied were decreased. Fermentation with these isolates was not successful in suppressing the challenge by *E. coli*. *Lb. curvatus* 15.35 was regarded as suitable for the ensilage of fish, but the use of higher fermentation temperatures might be required for more effective acidification.

INTRODUCTION

The competitiveness of a microorganism has been mentioned as a prerequisite for its application as a starter in food fermentations in the presence of more or less competitive intrinsic microflora (Dossman *et al.*, 1996). The success of fish preservation by lactic acid fermentation depends on quickly achieving low pH values and successfully inhibiting the growth of deterioration microorganisms. Attaining pH values below 4.5 within 2–3 days is regarded as indispensable to ensure the stability of fish preserved by this method (Van Wyk and Heydenrych, 1985). A quick growth of the starter LAB is another desirable property, as it influences their ability to prevail over undesirable members of the intrinsic fish microflora (Adams *et al.*, 1987).

General criteria that should be met by starter cultures have been defined by Buckenhüskes (1993). They include safety considerations (lack of pathogenic or toxigenic activity), technological effectiveness (capacity to predominate over the spontaneous microflora, ability to perform the required metabolic activity) and economical aspects. In the case of fish silage, degradation of amino acids to the corresponding biogenic amines is a potential risk, and by consequence, lack of decarboxylase enzymes is a safety requisite for prospective fish silage starter cultures. The low pH (below 4.5 after 2 days of fermentation) and the physical characteristics of fish silage that lead to a low oxygen concentration within the ensiled fish are favourable for the action of amino acid decarboxylases (Beutling, 1992). Therefore, the formation of biogenic amines is likely to take place in fish silage.

Nowadays, starter and protective cultures are added to food as defined, single- or multi-strain cultures. The latter are preferred when single strains fail to accomplish the task (Geisen and Holzapfel, 1996). Multiple-strain cultures are currently used in the production of fermented foods of animal origin, such as dairy products and fermented meats (Buckenhüskes, 1993). Often, the starter cultures used in the production of fish silage were single-strain preparations (Fagbenro and Jauncey, 1998, Hammoumi *et al.*, 1998). However, some works used combinations of strains in the preparation of fish silage (Faid *et al.*, 1994, 1997).

In this chapter, growth and acidification properties of a number of prospective LAB starter strains and their combinations are studied, with the aim of selecting a culture or combination that would maximise the quality and safety of fish silage.

The use of combinations among the selected *Lb. curvatus* and *Lb. sakei* cultures aimed to make use of the desirable properties of both types of isolates. Combinations of *Lb. curvatus* 15.35 and *Lb. sakei* 15.05, 15.18 or 15.39 *and Lb. curvatus* 22.24 and *Lb. sakei* 15.05, 15.18 and 15.39 were studied in MRS medium at 30°C. The growth and acidification potential of the most promising starters were tested in cooked Atlantic mackerel slurry.

MATERIALS AND METHODS

LAB cultures

Five isolates were selected for growth and acidification, antimicrobial activity (this thesis, chapter 4), absence of amino acid decarboxylase activity, and ability to degrade histamine (this thesis, chapter 5). Two *Lb. curvatus* (15.35 and 22.24) and three *Lb. sakei* isolates (15.05, 15.18 and 15.39) were selected on this basis. *Lb. curvatus* 15.35 was chosen due to its significantly higher Δ O.D. at 30°C and its broad antimicrobial activity. *Lb. curvatus* 22.24 had significantly ($p<0.05$) higher Δ O.D. at all temperatures tested and belonged to the group of isolates that acidified the medium to pH 4.8 or below within 3 h, at 30°C. It also displayed considerable antimicrobial activity (this thesis, chapter 4). *Lb. sakei* 15.05, 15.18, 15.39 were chosen because of their ability to degrade histamine (Dapkevicius *et al.*, 2000). Furthermore, *Lb. sakei* 15.18 and 15.39 acidified the medium quickly at 30°C. None of the selected isolates were found to decarboxylate amino acids (this thesis, chapter 5). Amino acid decarboxylation is an undesirable property in bacteria that are intended for use as starter cultures in the food industry, since the resulting biogenic amines can constitute a health hazard both for humans (Taylor, 1986) and animals (Krizek, 1991). The use of combinations among the selected *Lb. curvatus* and *Lb. sakei* cultures was envisaged to make use of the desirable properties of both types of isolates. Combinations of *Lb. curvatus* 15.35 and *Lb. sakei* 15.05, 15.18 or 15.39 *and Lb. curvatus* 22.24 and *Lb. sakei* 15.05, 15.18 and 15.39 were studied in MRS medium at 30°C. All these combinations were found to be compatible by the cross-inoculation technique.

Lactobacillus plantarum 009, obtained from the collection of Wageningen University, was included for comparison. This strain had been previously used in the preparation of fish silage (this thesis, chapter 3).

Testing for compatibility among combinations of LAB strains

Combinations of *Lb. curvatus* 15.35 and *Lb. sakei* 15.05, 15.18 or 15.39 *and Lb. curvatus* 22.24 and *Lb. sakei* 15.05, 15.18 and 15.39 were tested for compatibility by the cross inoculation technique (Rogers and Bevan, 1978).

Growth and acidification testing

Growth and acidification experiments were performed in MRS medium (Merck, 10611, Darmstadt, Germany). When necessary, the pH of the medium was adjusted to 6.0 by adding 4 N NaOH or HCl. Portions of 250-ml medium were stored overnight at the study temperature and subsequently inoculated to a level of about 10^4 CFU ml^{-1} with an overnight (12-16 h) culture of each of the strains and combinations. After that, portions of 10-ml were aseptically dispensed into sterile culture tubes. The tubes were immediately placed in an incubator at 30°C. Scombroid fish was successfully ensiled at 37°C with *Lactobacillus plantarum* 009 (this thesis, chapter 3). This temperature, however, is the optimum growth temperature for many pathogens. Lower fermentation temperatures may also be desirable from the economical point of view. A temperature of 30°C was chosen for the present study because it would still allow for good growth of the starter LAB. Immediately after dispensing and at regular time intervals, twelve tubes from each strain or combination were sacrificed, for measurement of viable count, pH and titratable acidity (four tubes each).

The total number of LAB was counted in MRS agar (Oxoid, CM 361, Basingstoke, UK) and differential counts on a differential medium that we developed to distinguish the growth of the two strains in each mixture. This medium was based on differences in carbohydrate fermentation patterns among strains. It was a modified MRS agar in which trehalose replaced glucose, with added pH indicator (bromocresol purple, pH 5.2-6.8). Trehalose-positive strains (*Lb. sakei*) developed yellow colonies, while trehalose-negative strains(*Lb. curvatus*) had pinpoint, colourless colonies.

Fish silage experiments

Fish silage was prepared with Atlantic mackerel (*Scomber scombrus* Linnaeus 1875) obtained from a local fish distributor. The fish was eviscerated and cooked for 5 minutes. The cooked fish was allowed to cool, ground using a meat-grinder with 6-mm die-openings (complete grinder previously sterilised by autoclaving) and was mixed with a solution which consisted of 10.0 g NaCl and 60.0 g sucrose per 100 ml distilled water in the ratio of 60:20, yielding final concentrations of 2% NaCl and 12% sucrose (w/w) in the resulting cooked fish slurries (this thesis, chapter 3). This mixture was divided into four 1.5 kg batches before inoculating with 1.5% (w/w) of actively growing starter strains (two each with *Lb. curvatus* 15.35 and *Lb. sakei* 15.39). An actively growing culture of a

histamine producing *E. coli* strain (NIZO B351, Joosten and Northolt, 1987) was added (0.1 % v/v) to one batch of each type of starter strain to simulate microbiological contamination of the silage. After homogenisation by vigorous mixing with a spatula, the slurries were placed in glass jars with a screw cap to which a water-column type air lock had been fitted. The slurries were incubated anaerobically at 30°C for 13 days. Determination of pH, titratable acidity and microbiological counts were after 0, 24, 48, 72, 144 and 312 hours, in triplicate samples.

The starter strains were pre-cultured in MRS medium at 30° C for 12 – 16 h. *E. coli* NIZO B351 was cultured in Tryptone Soya Broth (TSB; Oxoid CM 129, Unipath, Basingstoke, UK) at 37°C before use. Titratable acidity (as % lactic acid) and pH were determined according to Hassan and Heath (1986). Viable counts of total aerobic mesophiles, LAB and *E. coli* were determined during fermentation. Counts of total aerobic mesophiles were done using MPCA (Modified Plate Count Agar, Houtsma *et al.*, 1993), which supports LAB better than Plate Count Agar. Incubation was at 30°C for 72 h. Lactic acid bacteria were counted in MRS agar (incubated at 30°C for 72 h) and *E. coli* in Violet Red Bile Agar (Difco 0112-01-5; Detroit, MI) incubated at 37° C for 20 – 24 h.

Modelling

Fitting of the growth and pH decrease curves was carried out using the modified Gompertz equation, and nonlinear regression with a Marquardt algorithm, according to Zwietering *et al.* (1990). Bacterial counts were expressed as log (CFU g^{-1}) as a function of time (h). Values for lag time λ (h), maximum specific growth rate μ (h^{-1}), final population level A and their 95% confidence intervals were calculated. Similarly, pH decrease lag time (λ_{pH}), maximum pH decrease rate (μ_{pH}), final pH decrease level (A_{pH}), were calculated from $|pH_t - pH_0|$, in which pH_t = pH value obtained at each sampling time and pH_0 = initial pH value.

Statistical analysis

The results obtained in all experiments were submitted to ANOVA (Gomez and Gomez, 1983). Separation of the means was performed by the Duncan test (Duncan, 1955).

RESULTS AND DISCUSSION

Tables 1 and 2 present the growth data. Table 1 indicates bactericidal action of *Lb. curvatus* 15.35 against all *Lb. sakei* strains. This corresponds with the previous finding that *Lb. curvatus* 15.35 inhibited the growth of biogenic-amine producing *Lb. sakei* 15.19. Inhibition was attributed to hydrogen peroxide production (this thesis, chapter 4). Since no growth could be observed after 12-24 h and a reduction in CFU g^{-1} was evident from 10 hours on, no growth parameters

were calculated for the *Lb. sakei* strains in combinations involving *Lb. curvatus* 15.35. *Lb. curvatus* 15.35 cannot be used in mixed culture fermentation with *Lb. sakei* due to its bactericidal action against the latter.

However, its bactericidal action may be of interest when used as a pure culture inoculum. The antibacterial activity of *Lb. curvatus* 15.35 against *Lb. sakei*

Table 1. Growth of *Lb. sakei* 15.05, 15.18 and 15.39 as pure cultures and in combination with *Lb. curvatus* 15.35 in MRS medium at 30°C (log CFU g^{-1}). The numbers represent the averages ± standard deviations of four samples.

ime (h)	*Lb. Sakei* 15.05 (pure cult.)	*Lb. sakei* 15.05+ *Lb. curvatus* 15.35	*Lb. sakei* 15.18 (pure cult.)	*Lb. sakei* 15.18+ *b. curvatus* 15.35	*Lb. sakei* 15.39 (pure cult.)	*Lb. sakei* 15.39+ *Lb. curvatus* 15.35	*Lb. curvatus* 15.35 (pure cult.)
0	4.60±0.06	4.70±0.01	4.72±0.03	4.82±0.05	4.57±0.08	4.53±0.08	4.68±0.06
2	4.83±0.03	4.74±0.05	5.43±0.41	5.09±0.08	5.00±0.20	4.53±0.06	4.86±0.33
4	5.55±0.03	5.55±0.04	5.91±0.08	5.73±0.61	5.86±0.01	5.54±0.01	5.28±0.09
8	7.26±0.11	7.25±0.02	7.51±0.01	7.30±0.25	7.59±0.01	6.63±0.31	7.12±0.03
10	8.04±0.01	6.54±0.04	8.16±0.08	7.42±0.03	8.03±0.01	5.80±0.16	7.68±0.11
12	8.47±0.07	<1.0*	8.68±0.07	7.05±0.01	8.54±0.04	<1.0*	8.16±0.20
24	8.53±0.04	<1.0*	8.77±0.04	<1.0*	8.73±0.03	<1.0*	8.57±0.08

* Lowest dilution tested: 10^{-1}.

could not be detected by the cross-inoculation technique. Thus, this method is regarded as unsuitable for our purposes.

Lb. curvatus 22.24 had no bactericidal effect against the *Lb. sakei* (Table 2). No significant ($p<0.05$) effect on growth was observed when *Lb. sakei* 15.05 was grown together with *Lb. curvatus* 22.24. For *Lb. sakei* 15.18, combination with *Lb. curvatus* 22.24 resulted in significantly ($p<0.05$) lower maximum population and reduced growth rate. *Lb. sakei* 15.39 had a significantly lower maximum population and significantly ($p<0.05$) longer lag phase when grown together with *Lb. curvatus* 22.24 than in pure culture.

The growth of *Lb. curvatus* 15.35 and 22.24 is also affected by other LAB isolates in the combinations (Table 2). *Lb. curvatus* 15.35 showed significantly ($p<0.05$) shorter lag times in the combinations than in pure culture. Its growth was inhibited in combination with *Lb. sakei* 15.05 and 15.18. With *Lb. sakei* 15.05 a significant ($p<0.05$) reduction in growth rate was observed, whereas a lower maximum population was obtained with *Lb. sakei* 15.18. In combination with 15.39, growth stimulation seemed to occur, with significantly ($p<0.05$) higher maximum population and growth rate than in pure culture. *Lb. curvatus* 22.24 showed significantly ($p<0.05$) lower growth rates in all combinations tested,

compared with the growth rate in pure culture. Furthermore, it reached significantly ($p<0.05$) lower maximum populations in combination with *Lb. sakei* 15.05 and 15.39. Growth in combination with *Lb. sakei* 15.18 resulted in significantly ($p<0.05$) higher maximum population and significantly ($p<0.05$) shorter lag time than in pure culture.

Table 2. Comparison of growth parameters (A, μ and λ) of *Lb. sakei* (15.05, 15.18 and 15.39) and *Lb. curvatus* (15.35 and 2.24) pure cultures and combinations, in MRS medium, at 30°C. Growth parameters for *Lb. plantarum* strain 009 were also included for comparison purposes. The numbers shown are the averages ± standard deviations of 4 samples. Within each column, averages marked with different letters are significantly ($p<0.05$) different.

Cultures	A (CFU g^{-1})	μ (h^{-1})	λ (h)
Lb. sakei 15.05	3.99 ± 0.12^{a}	0.522 ± 0.025^{ab}	2.40 ± 0.17^{a}
Lb. sakei 15.05 (+*Lb. curvatus* 22.24)	3.98 ± 0.08^{a}	0.533 ± 0.017^{b}	2.48 ± 0.15^{ab}
Lb. sakei 15.18	4.19 ± 0.03^{b}	0.456 ± 0.043^{c}	1.48 ± 0.25^{cd}
Lb. sakei 15.18 (+*Lb. curvatus* 22.24)	3.19 ± 0.09^{c}	0.335 ± 0.017^{d}	1.05 ± 0.29^{c}
Lb. sakei 15.39	4.20 ± 0.08^{b}	0.505 ± 0.021^{ab}	1.47 ± 0.30^{cd}
Lb. sakei 15.39 (+*Lb. curvatus* 22.24)	3.90 ± 0.08^{a}	0.496 ± 0.008^{a}	2.19 ± 0.35^{ae}
Lb. curvatus 15.35	3.92 ± 0.15^{a}	0.476 ± 0.011^{c}	2.76 ± 0.16^{b}
Lb. curvatus 15.35 (+*Lb. sakei* 15.05)	3.85 ± 0.07^{a}	0.291 ± 0.009^{e}	1.28 ± 0.08^{c}
Lb. curvatus 15.35 (+*Lb. sakei* 15.18)	4.33 ± 0.11^{b}	0.506 ± 0.049^{ab}	0.70 ± 0.04^{f}
Lb. curvatus 15.35 (+*Lb. sakei* 15.39)	4.33 ± 0.06^{b}	0.506 ± 0.010^{ab}	0.70 ± 0.07^{f}
Lb. curvatus 22.24	3.89 ± 0.08^{a}	0.447 ± 0.023^{c}	1.68 ± 0.34^{d}
Lb. curvatus 22.24 (+*Lb. sakei* 15.05)	3.24 ± 0.28^{c}	0.332 ± 0.009^{d}	2.49 ± 0.58^{ab}
Lb. curvatus 22.24 (+*Lb. sakei* 15.18)	5.01 ± 0.81^{d}	0.332 ± 0.051^{def}	0.00 ± 0.00^{g}
Lb. curvatus 22.24 (+*Lb. sakei* 15.39)	3.71 ± 0.08^{e}	0.370 ± 0.016^{fg}	1.77 ± 0.11^{de}
Lb. plantarum 009	4.64 ± 0.20^{d}	0.389 ± 0.005^{g}	2.32 ± 0.15^{a}

On the basis of these growth data, the combinations were less effective than the pure cultures. Combinations with *Lb. curvatus* 15.35 led to the elimination of *Lb. sakei* within 12 – 24 h. Although *Lb. curvatus* 22.24 had little effect on Lb. *sakei*, it slowed down in combination with the other strains.

Table 2 also allows the comparison among the pure cultures. *Lb. curvatus* (15.35 and 22.24) had the lowest final population levels. These strains differed significantly ($p<0.05$) in lag times (15. 35 has the longest), but not in growth rates. *Lb. sakei* (15.05, 15.18 and 15.39) had significantly ($p<0.05$) higher final population levels than *Lb. curvatus*, but significantly ($p<0.05$) lower than *Lb. plantarum* 009. *Lb. sakei* 15.05 and 15.39 had the highest growth rates, *Lb. sakei* 15.18 had significantly ($p<0.05$) lower μ than the other two *Lb. sakei* strains. *Lb. sakei* 15.18 and 15.39 had much longer lag times than *Lb. sakei* 15.05. *Lb.*

plantarum 009 had significantly ($p<0.05$) higher A value than all other studied strains, the lowest μ value and a long lag time. In previous experiments (this thesis, chapter 3), the estimated growth rate of *Lb. plantarum* 009, in the same medium, at 35°C, was 0.512±0.029, which may indicate that the growth optimum for this strain may be considerably higher than 30°C.

Table 3 shows the pH and titratable acidity values, over a 24-h period, of pure cultures of *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum* and several combinations. Culture combinations did not bring beneficial effects from the point of view of acidification. In all cases, the titratable acidity obtained was lower, the pH decrease slower and the final pH higher when combinations were compared with the corresponding pure cultures.

Table 3. pH and titratable acidity of pure cultures of *Lb. curvatus* (15.35, 22.24), *Lb. sakei* (15.05, 15.18, 15.39) and combinations of *Lb. curvatus* and *Lb. sakei* cultures. The numbers represent the average of four samples. Standard deviation values (not shown) range from 0.00 – 0.01 and from 0.00 to 0.02 for pH and titratable acidity, respectively.

Cultures	Time (h)					Time (h)				
	0	4	8	12	24	0	4	8	12	24
	pH					Titratable acidity (%)				
Lb. sakei 15.05	6.00	5.86	5.64	5.39	4.68	0.00	0.02	0.08	0.20	0.68
Lb. sakei 15.18	6.00	5.78	5.38	4.77	4.13	0.00	0.04	0.11	0.29	0.68
Lb. curvatus 15.35	6.00	5.98	5.92	4.52	4.34	0.00	0.00	0.14	0.68	0.83
Lb. sakei 15.39	6.00	5.78	5.41	4.76	4.09	0.00	0.03	0.11	0.29	0.71
Lb. curvatus 22.24	6.00	6.00	5.90	5.38	4.51	0.00	0.04	0.1	0.17	0.68
Lb. plantarum 009	6.00	6.00	5.93	5.82	4.84	0.00	0.00	0.00	0.02	0.44
Lb. curvatus 22.24 + *Lb. sakei* 15.05	6.00	5.94	5.73	5.41	4.67	0.00	0.01	0.04	0.12	0.66
Lb. curvatus 22.24 + *Lb. sakei* 15.18	6.00	5.90	5.74	5.38	4.62	0.00	0.03	0.07	0.20	0.70
Lb. curvatus 22.24 + *Lb. sakei* 15.39	6.00	5.92	5.77	5.45	4.81	0.00	0.03	0.07	0.17	0.56

Table 4 shows that growth in combinations resulted in the lowest pH decrease rates, lower final pH decrease values and longer pH decrease lag times than those observed for the *Lb. sakei* strains grown as pure cultures. *Lb. plantarum* 009 had the highest final pH decrease, a low pH decrease rate and the longest pH decrease lag time. Similarly to what had been observed for growth, the pH decrease rate of this strain at 35°C (0.228 h^{-1}, this thesis, chapter 3) was considerably higher than the corresponding value for 30°C (0.111 h^{-1}, this thesis, chapter 3). *Lb.*

curvatus strains had the lowest final pH decrease lag time, the highest pH decrease rate and relatively long pH decrease lag times. *Lb. sakei* had lower final pH decrease values than *Lb. curvatus* and the shortest pH decrease lag times among all

Table 4. Comparison of pH decrease parameters (A_{pH}, μ_{pH} and λ_{pH}) of *Lb. sakei* and *Lb. curvatus* , in pure culture or as combinations, in MRS medium, at 30°C. The pH decrease parameters for *Lb. plantarum* were included for comparison purposes. The numbers shown are the average of 4 samples. Standard deviation values (not shown) range from 0.00 – 0.02 for all parameters. Within each column, averages marked with different letters are significantly ($p<0.05$) different.

Cultures	A_{pH}	μ_{pH} (h^{-1})	λ_{pH} (h)
Lb. curvatus 15.35	1.66^{a}	0.186^{a}	4.96^{a}
Lb. curvatus 22.24	1.54^{b}	0.156^{b}	8.22^{b}
Lb. sakei 15.05	1.69^{a}	0.072^{c}	3.39^{c}
Lb. sakei 15.18	1.97^{c}	0.142^{d}	3.32^{c}
Lb. sakei 15.39	2.02^{d}	0.145^{d}	3.53^{d}
Lb. plantarum 009	3.90^{e}	0.111^{e}	13.55^{e}
Lb. curvatus 22.24 + *Lb. sakei* 15.18	1.66^{a}	0.086^{f}	4.95^{a}
Lb. curvatus 22.24 + *Lb. sakei* 15.05	1.56^{b}	0.086^{f}	5.12^{f}
Lb. curvatus 22.24 + *Lb. sakei* 15.39	1.39^{f}	0.077^{g}	4.97^{a}

studied isolates. *Lb. sakei* 15.05 had the lowest pH decrease rate of all studied strains.

Because of the poor performance of the mixtures, single pure cultures were used in the subsequent fish silage experiments. Mackerel was cooked prior to ensiling with the objective of achieving a strong reduction in its intrinsic bacterial population and thus reduce bacterial competition with the starter. *E. coli* was added to assess the competitiveness of the strains under study. *Lb. curvatus* 15.35 and *Lb. sakei* 15.39 were used as starters in this experiment. These isolates were selected for their high μ values for growth and pH, high A values and short lag phases. *Lb. sakei* 15.05 also had a high growth rate, but it had a long lag phase and reached significantly ($p<0.05$) lower maximum population than the selected isolates. Additionally, *Lb. curvatus* 15.35 inhibited several potential bacterial contaminants tested at 30°C due to H_2O_2 production, whereas *Lb. sakei* 15.39 did not inhibit the deteriorating LAB strains tested and did not inhibit the *E coli* strain added in this experiment (this thesis, chapter 4). Table 5 shows the results of the mackerel ensilage experiments. The fastest pH decrease and titratable acidity formation were obtained in silages prepared with *Lb. curvatus* 15.35 inoculum. These silages also had the fastest LAB growth and reached the highest LAB population levels. In silages prepared from *Lb. sakei* 15.39 the growth, pH decrease and titratable acidity

formation were considerably slower, maximum numbers were lower and final pH values were higher No coliform growth was detected in VRB Agar medium throughout the experiment in silages inoculated exclusively with the *Lactobacillus* cultures under study. Comparing LAB numbers and the numbers of microorganisms that grew on MPC Agar, it is clear that LAB were the predominant

Table 5. Titratable acidity, pH, MPCA, MRS and VRB counts in ensiled, cooked Atlantic mackerel slurries with 12% (w/w) sucrose and 2% (w/w) NaCl prepared with 15.35 and 15.39 as starters and incubated at 30° C. The numbers represent the averages±standard deviations of three samples.

ests	Bacterial cultures added	Time (h)					
		0	24	48	72	144	312
LAB (log CFU g^{-1})	*Lb. curvatus* 15.35	6.68±0.10	8.53±0.03	8.82±0.05	8.95±0.02	8.57±0.11	7.88±0.09
	15.35 and *E. coli*	6.70±0.05	8.43±0.08	8.79±0.07	8.83±0.01	8.61±0.12	7.77±0.05
	Lb. sakei 15.39	7.18±0.02	8.27±0.14	8.62±0.14	8.81±0.73	8.38±0.34	8.00±0.03
	15.39 and *E. coli*	7.20±0.01	8.00±0.01	8.06±0.15	8.30±0.07	8.51±0.07	8.10±0.02
MPCA (log CFU g^{-1})	*Lb. curvatus* 15.35	6.84±0.03	8.63±0.10	8.83±0.06	8.97±0.07	8.66±0.05	7.84±0.05
	15.35 and *E. coli*	6.78±0.03	8.56±0.08	8.84±0.01	8.87±0.05	8.70±0.08	8.12±0.07
	Lb. sakei 15.39	7.23±0.05	8.01±0.02	8.03±0.09	8.15±0.12	8.52±0.04	8.20±0.03
	15.39 and *E. coli*	7.25±0.01	8.18±0.15	8.36±0.03	8.32±0.13	8.47±0.09	8.67±0.04
VRBA (log CFU g^{-1})	*Lb. curvatus* 15.35	<1.0*	<1.0*	<1.0*	<1.0*	<1.0*	<1.0*
	15.35 and *E. coli*	6.33±0.20	6.88±0.13	6.79±0.18	6.61±0.05	6.15±0.10	3.22±0.38
	Lb. sakei 15.39	<1.0*	<1.0*	<1.0*	<1.0*	<1.0*	<1.0*
	15.39 and *E. coli*	5.93±0.01	6.15±0.06	6.17±0.16	6.31±0.25	6.25±0.04	4.33±0.13
pH	*Lb. curvatus* 15.35	6.21±0.01	5.26±0.02	4.69±0.01	4.42±0.02	4.32±0.02	4.26±0.03
	15.35 and *E. coli*	6.20±0.02	5.46±0.04	4.77±0.02	4.47±0.02	4.29±0.02	4.28±0.03
	Lb. sakei 15.39	6.46±0.04	6.05±0.01	5.68±0.01	5.34±0.02	4.77±0.01	4.36±0.03
	15.39 and *E. coli*	6.47±0.02	6.20±0.03	5.99±0.06	5.35±0.12	5.09±0.03	4.35±0.04
Titratable acidity %	*Lb. curvatus* 15.35	0.38±0.01	0.77±0.01	1.08±0.02	1.19±0.01	1.40±0.02	1.53±0.03
	15.35 and *E. coli*	0.38±0.01	0.63±0.01	0.98±0.02	1.15±0.01	1.34±0.01	1.47±0.02
	Lb. sakei 15.39	0.28±0.01	0.40±0.06	0.48±0.01	0.59±0.01	0.84±0.06	1.17±0.02
	15.39 and *E. coli*	0.26±0.01	0.41±0.03	0.44±0.01	0.48±0.01	0.70±0.02	1.19±0.03

* Lowest dilution tested: 10^{-1}.

microorganisms in these silages. Both silages had a fresh, acidic smell and showed no sensorial signs of putrefaction throughout the experiment.

Challenge by simultaneous inoculation with *E. coli* brought about delays in growth and pH decrease. This negative effect was more pronounced in silages

prepared with *Lb. sakei* 15.39 than with *Lb. curvatus* 15.35. A putrid smell indicated that spoilage had occurred in both silages with added *E. coli* by the end of the experimental period. The *E. coli* numbers showed some decrease only when the pH was well below 4.5 and the titratable acidity had reached values above 1%, which is in accordance with earlier observations in broth systems (chapter 4, this thesis). *E. coli* populations of $\geq 10^6$ CFU g^{-1} were present in the contaminated silages for 6 days. Fermentation with *Lb. curvatus* 15.35 or *Lb. sakei* 15.39 was not successful in suppressing the challenge by *E. coli*.

Growth and acidification parameters were also calculated for the fish slurry ensilage experiments. Table 6 presents the calculated growth and acidification parameters for Lb. *curvatus* 15.35, *Lb. sakei* 15.39, and, for comparison purposes, *Lb. plantarum* 009 (data taken from Chapter 3).

Table 6. Growth and acidification parameters of *Lb. curvatus* and *Lb. sakei*, grown at 30°C, in Atlantic mackerel slurries (with 12% sucrose and 2% NaCl), with or without added *E. coli*. Parameters calculated for *Lb. plantarum* 009 during growth in ensiled blue-jack mackerel (with 12% sucrose and 2% NaCl) are included for comparison. The numbers represent the averages±standard deviations of three samples. Within each row, averages marked with different letters are significantly ($p<0.05$) different.

	Parameters	*Lb. curv.* 15.35	*Lb. curv.* 15.35 + *E. coli*	*Lb. sakei* 15.39	*Lb. sakei* 15.39 + *E. coli*	*Lb. plantarum* 009
Growth	A (log CFU g^{-1})	2.21±0.10^a	2.12±0.07^a	1.57±0.1^b	1.30±0.10^c	4.99^d
	μ (h^{-1})	0.114±0.013^a	0.110±0.005^a	0.056±0.008^b	0.020±0.009^c	0.043±0.001^d
	λ (h)	4.63±0.25^a	5.56±0.73^{ad}	2.82±0.24^b	3.85±0.24^c	5.72±0.83^d
Acidific-ation	A_{pH}	1.90±0.01^b	1.90±0.03^b	2.05±0.02^a	2.09±0.28^a	2.92±0.08^c
	μ_{pH} (h^{-1})	0.041±0.001^c	0.037±0.001^d	0.016±0.000^a	0.012±0.001^b	0.144±0.000^e
	λ_{pH} (h)	2.42±0.05^a	4.68±0.31^b	2.44±0.02^a	2.42±0.05^a	4.52±0.83^b

Both *Lb. curvatus* 15.35 and *Lb. sakei* 15.39 grew significantly ($p<0.05$) better and displayed higher acidification rates in MRS broth than in fish slurry. The A_{pH} and λ_{pH} values observed in the fish slurries were similar to those obtained in MRS broth. Inclusion of *E. coli* in the fish slurries resulted in significantly ($p<0.05$) poorer growth of *Lb. sakei* 15.39, but had no significant ($p<0.05$) effect on the growth of *Lb. curvatus* 15.35. It led to longer pH decrease lag times for both isolates, but had little effect on the other acidification parameters.

When cultivated in fish slurries, *Lb. curvatus* 15.35 had significantly higher A and μ values, but λ was longer than that of *Lb. sakei* 15.39. In terms of acidification, *Lb. curvatus* 15.35 led to lower final pH decrease values, but had higher acidification rate and the pH decrease lag time was shorter.

Lb. plantarum 009 displayed much higher A values (1 – 3 log cycles higher) when growing in fish paste at 37°C, than those obtained for *Lb. curvatus* 15.35 or *Lb. sakei* 15.39, but the growth rate of *Lb. plantarum* 009 was lower and its lag time was longer. It displayed much higher acidification rate than *Lb. curvatus* and *Lb. sakei*, but the acidification lag time observed was longer.

LAB growth and acidification were less efficient in this experiment than in a previous study in which raw, uneviscerated blue-jack mackerel was ensiled at 37°C using *Lb. plantarum* 009 at the same sucrose and NaCl concentrations. In the experiment with *Lb. plantarum* 009, the critical pH (<4.5) was attained 1 day earlier than with *Lb. curvatus* 15.35 and 11 days earlier than with *Lb. sakei* 15.39. Although *Lb. sakei* and *Lb. curvatus* have been reported as growing well and giving rapid acidification in salmon fermentation at 12°C, with 5g kg^{-1} added sucrose (Morzel *et al.*, 1997), *Lb. plantarum* appeared more suitable for the production of fish silage (Beal *et al.*, 1989, Vignolo *et al.*, 1988). Comparisons with literature data are limited by the fact that different fish species were used. Fish composition affects the buffering capacity (Hassan and Heath, 1986), influencing the pH resulting from the acidification process. Furthermore, different fermentation temperatures influence acidification, and play an important role in the success of fish silage (this thesis, chapter 3). We regarded the temperature used in this experiment as optimum for the growth of most *Lactobacillus* species. However, maximum acidification has been reported to take place at higher temperatures than maximum growth for different LAB species (Beal *et al.*, 1989, Vignolo *et al.*, 1988). In view of this, the effect of temperature on acidification and growth by potential fish silage starters will be studied in detail in the next chapter.

The two types of silages that reached pH ≤4.5 within a reasonable period were prepared with *Lb. curvatus* 15.35, as well as the silages previously prepared with *Lb. plantarum* 009. These silages were characterised by high maximum LAB populations and fast pH decrease rates.

CONCLUSIONS

Under the conditions we tested, single cultures grew better and decreased faster the pH of the model system. For this reason, the envisaged culture combinations are not regarded as suitable starters for biological fish silage.

None of the cultures tested in this chapter (*Lb. curvatus* 15.35 and *Lb. sakei* 15.39) were able to suppress the challenge posed by simultaneous high inoculum of *E. coli* in fish slurries. However, they might be effective against lower levels of contaminants or contamination at later stages of ensilage.

The effect of temperature on starter growth and acidification needs to be studied, since the temperature used in these experiments may not be the optimum for these strains, under the conditions prevailing in the ensilage of fish.

Chapter 7

Effects of temperature, redox potential, sodium chloride, and potassium sorbate on growth and acidification of potential fish silage starter lactic acid bacteria in MRS medium

ABSTRACT

Growth and acidification properties of seven potential fish silage starter cultures (*Lb. sakei* 15.05, 15.18, 15.36, 15.39, *Lb. curvatus* 15.35, 22.24 and *Lb. plantarum* 009) were studied in different model systems. The culture media used were ten different modifications of the MRS medium (MRS medium, MRS + 2 or 4% NaCl, MRS + 0.1 or 0.2% potassium sorbate, MRS + 0.05% cysteine, MRS + 0.05% cysteine+2 or 4% NaCl and MRS + 0.05% cysteine + 0.1 or 0.2% K-sorbate). Four incubation temperatures, 30, 35, 37 and 40°C, were used for each strain and medium formulation. Growth was measured as the increase in absorbance and acidification as the pH decrease of the medium. Data obtained were used to calculate growth and acidification parameters (μ and λ for growth, ΔpH after 48 h, pH decrease rate and pH lag time).

Significant ($p<0.01$) interaction was observed among strain, temperature, cysteine and sodium chloride or potassium sorbate. Therefore, the technological ensilage conditions have to be optimised for each particular strain. In all cases, temperatures of 35 – 37°C should be used in order to ensure a fast stabilisation of the silages. Cysteine inhibited growth or acidification by *Lb. sakei* 15.05 and 15.36. Two-percent NaCl can be used with some strains, such as *Lb. plantarum* 009, but 4% NaCl considerably inhibited growth and acidification by most strains. Both K-sorbate concentrations inhibited growth and acidification by all strains. The seven strains performed differently in the various media formulations. *Lb. plantarum* 009 and *Lb. curvatus* 15.35 seem particularly suitable for usage as biological fish silage starters. The former had the shortest pH decrease lag times (<1 h), while the latter displayed the best growth characteristics.

INTRODUCTION

Biological fish silage (BFS) is regarded as a safe product. Fermentation by lactic acid bacteria (LAB) ensures that organoleptic and nutritional properties are maintained during the fermentation process and that microbial contamination is kept under control. It often is the only hurdle encountered by microbial contaminants in the biological ensilage of fish. Thus, it is evident that only fast growth and acidification by the starter bacteria can ensure that quality and safety requirements will be met. Therefore, an adequate choice of LAB fermentation conditions, such as temperature and eventual additional hurdles (chemical preservatives), is essential for the success of ensilage.

From the point of view of bacterial growth, a temperature as close as possible to the optimum for the potential starter strain(s) would be the most desirable. Bacteria belonging to the genus *Lactobacillus* grow optimally at temperatures within the range 30–40°C (Kandler and Weiss, 1984). Maximum acidification may, however, occur at higher temperatures than the optimum for growth (Beal *et al.*, 1989). In most cases, the temperatures at which fish silage is fermented lay within this range (Fagbenro and Bello-Olusoji, 1997, Fagbenro and Jauncey, 1998). Some attempts at using temperatures below 26°C have been made (Faid *et al.* 1997, Dapkevicius *et al.*, 1998, Hammoumi *et al.*, 1998), but they met, in general, with limited success. At these temperatures, acidification proceeded slower and pH 4.5 was not reached within 2–3 days. A notable exception to this was a group of studies on the ensilage of tropical freshwater fish viscera at ambient temperature. This substrate already had a very low initial pH and did not require the addition of a starter. The pH value of 4.5 was, in this case, reached within 30 h (Ahmed and Mahendrakar, 1996a, c and d). Ensiling salmon viscera with the addition of a starter culture also provided a very fast pH decrease, to 4.5 within 2 days, at 22 – 25°C (Dong *et al.*, 1993). Also, co-ensiling fish waste with cereals led to a rapid pH decrease, to values below 4.5 within 30 h, at 24°C (Lindgren and Pleje, 1983).

For the sake of simplicity and economy, usage of chemical preservatives is relatively uncommon in biological ensilage. In some cases, sodium chloride has been used (Ahmed *et al.*, 1996, Ahmed and Mahendrakar, 1996b) or recommended (Raa and Gildberg, 1982, Lupín, 1983) with the aim of controlling gas formation. In a previous work (this thesis, chapter 4), the addition of 2% NaCl brought about several positive effects:

- Non-protein nitrogen (NPN) formation was decreased, which is in agreement with the results of Fagbenro and Jauncey (1993b).
- Lower total volatile basic nitrogen (TVBN) values were reached.
- Lower pH values were reached, in spite of the lower LAB numbers obtained.

Potassium sorbate is sometimes added to BFS with the aim of controlling fungal growth (Lindgren and Pleje, 1983, Ahmed *et al.*, 1996). It has been found to

be more efficient than propionate as an antimycotic agent (Lindgren and Pleje, 1983). In spite of being primarily used to control fungal growth, sorbates also exhibit some antibacterial activity. With some exceptions (Costilow *et al.*, 1955, Hamden *et al.*, 1971, Restaino *et al.*, 1981), LAB are resistant to sorbates. Inhibition of biogenic amine formation by potassium sorbate, as reported by Shalaby and Abd El-Rahman (1995), could be an additional reason to include it in BFS formulations. Synthetic (Dong *et al.*, 1993, Ahmed and Mahendrakar, 1996a,b, and c) and natural antioxidants (Fagbenro and Jauncey, 1994a, Fagbenro and Jauncey, 1998) have also been applied to BFS with the aim of retarding oxidation of the highly susceptible fish lipids. This type of additives is, however, not dealt with in the present work.

Fish silage is a rather reducing substrate, in which very low redox potential (Eh) values become prevalent within the ensiled mass after a relatively short period (< -500 mV in 1 day, Lássen, 1995b). It is important to take this into account when ascertaining the effect of other factors on growth and acidification by potential fish starter LAB. Cysteine is commonly used to decrease Eh values to *ca.* –200 mV in model systems (Garbutt, 1997, Dave and Shah, 1998).

Studies on the effects of the above mentioned factors (temperature, Eh and chemical preservatives) on growth and acidification by potential fish silage starter cultures have not been published. They are, however, of great importance for understanding the behaviour of LAB in the fish silage environment and for optimising the quality and safety of the final product. The objectives of the present work were to study the effects of temperature, potassium sorbate, NaCl and cysteine addition on growth and pH decrease by potential fish silage starter microorganisms.

MATERIALS AND METHODS

LAB cultures

Growth properties and pH decrease were assessed for seven previously isolated potential starter microorganisms, *Lactobacillus sakei* 15.05, 15.18, 15.36, 15.39, *Lactobacillus curvatus* 15.35, 22.24 (this thesis, chapter 4), and one *Lactobacillus plantarum* strain (009) that had been successfully used to ensile blue-jack mackerel (this thesis, chapter 3).

Growth and acidification testing

Growth and acidification experiments for LAB were performed in ten MRS medium (Merck, 10611, Darmstadt, Germany) modifications, at 30, 35, 37 or 40°C, as follows:

- MRS medium
- MRS medium with 2% or 4% (w/w) NaCl added

- MRS medium with 0.1 or 0. 2% (w/w) K-sorbate added
- MRS with 0.05% (w/w) cysteine added
- MRS with 0.05% (w/w) cysteine and 2 or 4% NaCl added
- MRS with 0.05% (w/w) cysteine and 0.1 or 2% K-sorbate added.

When necessary, the pH of the media was adjusted to 6.0 by adding 4 N NaOH or HCl. Portions of 250 ml broth were stored for 16 – 18h at the required temperature (30, 35, 37, or 40°C) and subsequently inoculated to a level of about 10^4 CFU ml^{-1} with a 16 – 18h culture of each of the strains. After that, portions of 10 ml were aseptically dispensed into sterile culture tubes and placed in an incubator at the required temperature (30, 35, 37 or 40°C), for 48 h. Directly after dispensing and at regular time intervals, two tubes from each strain and broth were sacrificed for pH determination. Growth was monitored by following the increase in optical density (O. D.) at 620 nm in a Microtiter Reader (340 ATTC, SLT-Labinstruments, Austria). The wells in the microtiter reader plates were filled with 150µl of medium inoculated with the desired strain until O. D. 0.079±0.006 (corresponding to 10^4 CFU ml^{-1}) and covered with 50µl sterile mineral oil. Four wells were filled for each strain and each medium. Incubation at the desired temperature (30, 35, 37 or 40°C) was done in the microtiter reader, using the thermostatic device of the apparatus. Measurements were done every 30 min over a 24-h period. Samples were automatically agitated by the apparatus before each measurement

Modelling

Modelling of the growth and pH decrease curves was carried out with the modified Gompertz equation, which was fitted to the data by nonlinear regression with a Marquardt algorithm, according to Zwietering *et al.* (1990). Bacterial counts were expressed as O. D. as a function of time (h). Values for lag time λ (h), maximum specific growth rate μ (ΔO. D. h^{-1}), maximum OD reached *A* and their 95% confidence intervals were calculated in this way. Similarly, pH decrease lag time, λ_{pH} (h), maximum pH decrease rate (μ_{pH}), and final pH decrease level (ΔpH_{48}) were calculated from $|pH_i - pH_0|$, in which pH_0=pH value obtained at t=0 and pH_i=pH value obtained at each reading time.

Statistical analysis

The results obtained in all experiments were submitted to ANOVA (Gomez and Gomez, 1983). Separation of the means was performed by the Duncan test (Duncan, 1951).

RESULTS AND DISCUSSION

Tables 1 – 7 present the growth and acidification parameters calculated from the obtained experimental data for each of the strains under study. The maximum population levels (*A* values) are not shown and will not be discussed under the scope of the present work. High optical density values cannot be used because photometric determinations of bacterial cell densities show a poor correlation with actual numbers of living cells.

As shown in Tables 1 – 7, the studied factors affected the various strains in different ways. For easier reading, the data are also presented under the form of graphs (Figures 1 – 9).

Table 1. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus sakei* 15.05 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.085 ± 0.005^{Aa}	0.172 ± 0.008^{Ba}	0.189 ± 0.011^{Ba}	0.130 ± 0.008^{Ca}
+2% NaCl	0.082 ± 0.002^{Aa}	0.111 ± 0.006^{Bb}	0.114 ± 0.005^{Bb}	0.084 ± 0.002^{Abd}
+4% NaCl	0.059 ± 0.003^{Ab}	0.077 ± 0.001^{Bb}	0.082 ± 0.011^{Bc}	0.061 ± 0.010^{Ac}
+0.1% K sorbate	0.060 ± 0.006^{Ab}	0.126 ± 0.006^{Bd}	0.142 ± 0.009^{Cd}	0.087 ± 0.001^{Db}
+0.2% K sorbate	0.042 ± 0.004^{Ac}	0.105 ± 0.003^{Bb}	0.122 ± 0.018^{Bb}	0.087 ± 0.002^{Cbd}
+0.05% cysteine	0.028 ± 0.003^{Ad}	$0.086\pm0.^{009Bd}$	0.093 ± 0.009^{be}	0.079 ± 0.006^{Bd}
+0.05% cysteine+2% NaCl	0.020 ± 0.001^{Ae}	$0.065\pm0.^{001Bc}$	0.076 ± 0.015^{Bf}	0.022 ± 0.004^{ae}
+0.05% cysteine+4% NaCl	0.021 ± 0.005^{Ade}	0.028 ± 0.005^{ABf}	0.032 ± 0.005^{Bg}	0.016 ± 0.002^{Af}
+0.05% cysteine +0.1% K-sorbate	0.018 ± 0.004^{Ae}	0.055 ± 0.002^{Bg}	0.066 ± 0.008^{Bh}	0.046 ± 0.006^{Cgc}
+0.05% cysteine +0.2% K-sorbate	0.012 ± 0.000^{Af}	0.049 ± 0.004^{Bg}	0.059 ± 0.005^{Ci}	0.042 ± 0.002^{Dg}
	λ (h)			
MRS	2.709 ± 0.098^{Aa}	2.656 ± 0.070^{Ba}	1.733 ± 0.033^{Ca}	2.037 ± 0.083^{Da}
+2% NaCl	3.457 ± 0.044^{Ab}	2.686 ± 0.026^{Ba}	1.887 ± 0.040^{Cb}	2.278 ± 0.045^{Ab}
+4% NaCl	3.993 ± 0.040^{Ac}	3.985 ± 0.038^{Ab}	1.996 ± 0.230^{Cc}	3.274 ± 0.297^{Cc}
+0.1% K sorbate	3.447 ± 0.042^{Ab}	2.484 ± 0.096^{Bc}	2.139 ± 0.049^{Ca}	2.584 ± 0.246^{Dd}
+0.2% K sorbate	3.936 ± 0.068^{Ac}	2.502 ± 0.042^{Bc}	2.206 ± 0.171^{Ca}	2.464 ± 0.179^{Ae}
+0.05% cysteine	4.073 ± 0.141^{Ac}	3.346 ± 0.388^{Bd}	2.816 ± 0.072^{Cd}	2.920 ± 0.234^{Cbe}
+0.05% cysteine+2% NaCl	6.560 ± 0.359^{Ad}	4.136 ± 0.794^{Bbd}	2.878 ± 0.366^{Cd}	5.371 ± 0.084^{Cbg}

Table 1. (continuation)

+0.05% cysteine+4% NaCl	11.283±0.201^{Ae}	5.154±0.509^{Be}	4.184±0.497^{Ce}	5.154±0.335^{Cf}
+0.05% cysteine +0.1% K-sorbate	8.008±0.001^{Af}	4.018±0.001^{Bbd}	3.017±0.008^{Cf}	6.040±0.010^{Ca}
+0.05% cysteine +0.2% K-sorbate	8.010±0.021^{Af}	4.990±0.632^{Be}	3.152±0.100^{Cd}	6.762±0.119^{Dg}
	ΔpH_{48}			
MRS	1.88	1.90	1.91	1.89
+2% NaCl	1.84	1.75	1.76	1.72
+4% NaCl	1.71	1.68	1.64	1.59
+0.1% K-sorbate	1.78	1.79	1.78	1.75
+0.2% K-sorbate	1.71	1.74	1.75	1.64
+0.05% cysteine	1.85	1.77	1.78	1.70
+0.05% cysteine+2% NaCl	1.76	1.68	1.57	1.54
+0.05% cysteine+4% NaCl	1.55	1.48	1.46	1.43
+0.05% cysteine +0.1% K-sorbate	1.60	1.62	1.59	1.54
+0.05% cysteine +0.2% K-sorbate	1.71	1.69	1.67	1.60
	μ_{pH} (h^{-1})			
MRS	0.176	0.194	0.204	0.225
+2% NaCl	0.196	0.209	0.218	0.249
+4% NaCl	0.191	0.202	0.208	0.245
+0.1% K-sorbate	0.166	0.174	0.196	0.222
+0.2% K-sorbate	0.120	0.145	0.175	0.197
+0.05% cysteine	0.175	0.181	0.196	0.222
+0.05% cysteine+2% NaCl	0.178	0.186	0.218	0.230
+0.05% cysteine+4% NaCl	0.102	0.175	0.197	0.224
+0.05% cysteine +0.1% K-sorbate	0.164	0.171	0.195	0.220
+0.05% cysteine +0.2% K-sorbate	0.117	0.136	0.171	0.196
	λpH (h)			
MRS	4.917	3.525	1.940	2.127
+2% NaCl	5.892	4.222	3.736	4.318
+4% NaCl	6.713	6.189	6.017	6.497
+0.1% K-sorbate	5.564	4.032	3.740	5.313
+0.2% K-sorbate	6.637	4.262	4.083	4.612
+0.05% cysteine	6.595	3.571	3.114	4.573
+0.05% cysteine+2% NaCl	6.695	4.318	4.002	6.686
+0.05% cysteine+4% NaCl	8.662	6.946	6.713	6.848
+0.05% cysteine +0.1% K-sorbate	6.702	4.441	4.092	6.625
+0.05% cysteine +0.2% K-sorbate	7.430	4.786	4.182	6.922

Table 2. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus sakei* 15.18 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.139 ± 0.004^{Aa}	0.146 ± 0.012^{Aa}	0.134 ± 0.002^{Aa}	0.131 ± 0.004^{Aa}
+2% NaCl	0.140 ± 0.003^{Aa}	0.146 ± 0.006^{Aa}	0.133 ± 0.004^{Ba}	0.096 ± 0.005^{Cb}
+4% NaCl	0.076 ± 0.002^{Ab}	0.097 ± 0.005^{Bb}	0.082 ± 0.007^{Ab}	0.056 ± 0.011^{Cc}
+0.1% K sorbate	0.075 ± 0.002^{Ab}	0.140 ± 0.012^{Ba}	0.099 ± 0.006^{Cc}	0.033 ± 0.003^{Dd}
+0.2% K sorbate	0.072 ± 0.003^{Ab}	0.120 ± 0.007^{Bc}	0.096 ± 0.004^{Cc}	0.015 ± 0.005^{De}
+0.05% cysteine	0.183 ± 0.005^{Ac}	0.195 ± 0.006^{Bd}	0.144 ± 0.011^{Ca}	0.132 ± 0.011^{Da}
+0.05% cysteine+2% NaCl	0.189 ± 0.024^{Ac}	0.191 ± 0.004^{Ad}	0.188 ± 0.010^{Ad}	0.149 ± 0.003^{Bf}
+0.05% cysteine+4% NaCl	0.096 ± 0.007^{Ad}	0.117 ± 0.018^{Ab}	0.114 ± 0.017^{Ac}	0.102 ± 0.011^{Ab}
+0.05% cysteine +0.1% K-sorbate	0.159 ± 0.005^{Ac}	0.159 ± 0.024^{Aa}	0.135 ± 0.002^{Aa}	0.099 ± 0.008^{Bb}
+0.05% cysteine +0.2% K-sorbate	0.150 ± 0.005^{Ae}	0.154 ± 0.005^{Aa}	0.144 ± 0.011^{Aa}	0.112 ± 0.009^{Bb}
	λ (h)			
MRS	2.963 ± 0.077^{Aa}	1.845 ± 0.202^{Ba}	2.480 ± 0.070^{Ca}	4.153 ± 0.184^{Da}
+2% NaCl	4.798 ± 0.236^{Ab}	3.489 ± 0.072^{Bb}	3.791 ± 0.142^{Bb}	6.583 ± 0.151^{Cb}
+4% NaCl	6.467 ± 0.463^{Ac}	4.895 ± 0.268^{Bc}	6.027 ± 0.159^{Ac}	9.040 ± 0.423^{Cc}
+0.1% K sorbate	4.407 ± 0.030^{Ad}	3.078 ± 0.071^{Bd}	3.958 ± 0.082^{Cb}	4.377 ± 0.034^{Ad}
+0.2% K sorbate	5.120 ± 0.198^{Ab}	4.200 ± 0.213^{Be}	4.676 ± 0.109^{Cd}	5.400 ± 0.209^{Ae}
+0.05% cysteine	2.906 ± 0.102^{Aa}	2.223 ± 0.214^{Ba}	2.253 ± 0.070^{Be}	3.153 ± 0.082^{Cf}
+0.05% cysteine+2% NaCl	4.126 ± 0.036^{Ae}	2.385 ± 0.127^{Bf}	3.036 ± 0.062^{Cf}	4.076 ± 0.114^{Aa}
+0.05% cysteine+4% NaCl	5.133 ± 0.153^{Ab}	3.475 ± 0.087^{Bbg}	4.926 ± 0.064^{Ag}	5.773 ± 0.187^{Ce}
+0.05% cysteine +0.1% K-sorbate	4.156 ± 0.120^{Ae}	3.153 ± 0.086^{Bd}	3.475 ± 0.083^{Ch}	4.037 ± 0.067^{Aa}
+0.05% cysteine +0.2% K-sorbate	4.965 ± 0.191^{Ab}	3.354 ± 0.058^{Bg}	4.459 ± 0.140^{Cd}	5.540 ± 0.128^{Dg}
	ΔpH_{48}			
MRS	1.71	1.64	1.59	1.83
+2% NaCl	1.55	1.45	1.37	1.85
+4% NaCl	1.48	1.33	1.34	1.78
+0.1% K sorbate	1.53	1.41	1.34	1.58
+0.2% K sorbate	1.69	1.62	1.38	1.61
+0.05% cysteine	1.58	1.52	1.50	1.53
+0.05% cysteine+2% NaCl	1.59	1.43	1.38	1.86
+0.05% cysteine+4% NaCl	1.42	1.27	1.28	1.51
+0.05% cysteine +0.1% K-sorbate	1.44	1.40	1.31	1.40
+0.05% cysteine +0.2% K-sorbate	1.44	1.32	1.07	1.36

Table 2. (continuation)

	μ_{pH} (h^{-1})			
MRS	0.101	0.179	0.202	0.070
+2% NaCl	0.170	0.186	0.207	0.056
+4% NaCl	0.069	0.156	0.180	0.051
+0.1% K sorbate	0.192	0.208	0.217	0.160
+0.2% K sorbate	0.115	0.153	0.195	0.055
+0.05% cysteine	0.159	0.186	0.226	0.099
+0.05% cysteine+2% NaCl	0.172	0.198	0.279	0.137
+0.05% cysteine+4% NaCl	0.070	0.170	0.198	0.047
+0.05% cysteine +0.1% K-sorbate	0.139	0.204	0.215	0.081
+0.05% cysteine +0.2% K-sorbate	0.068	0.067	0.069	0.041
	λpH (h)			
MRS	5.518	4.457	4.526	9.731
+2% NaCl	5.653	5.665	6.285	10.831
+4% NaCl	7.704	7.633	7.814	14.154
+0.1% K sorbate	7.461	6.954	7.445	28.312
+0.2% K sorbate	9.306	6.980	8.220	41.597
+0.05% cysteine	5.479	1.280	2.137	6.571
+0.05% cysteine+2% NaCl	5.590	4.919	5.120	10.673
+0.05% cysteine+4% NaCl	7.615	5.799	7.750	26.011
+0.05% cysteine +0.1% K-sorbate	7.751	7.110	8.525	29.084
+0.05% cysteine +0.2% K-sorbate	9.386	7.724	8.883	45.271

Table 3. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus sakei* 15.36 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	**Growth temperature (°C)**			
	30	**35**	**37**	**40**
	μ (ΔO. D. h^{-1})			
MRS	0.064 ± 0.002^{Aa}	0.253 ± 0.021^{Ba}	0.192 ± 0.030^{Ca}	0.183 ± 0.042^{Cad}
+2% NaCl	0.057 ± 0.001^{Ab}	0.191 ± 0.001^{Bb}	0.161 ± 0.007^{Cb}	0.139 ± 0.005^{Da}
+4% NaCl	0.056 ± 0.008^{Aab}	0.127 ± 0.012^{Bce}	0.117 ± 0.011^{Bcd}	0.104 ± 0.018^{Cb}
+0.1% K sorbate	0.012 ± 0.005^{Ac}	0.154 ± 0.004^{Bd}	0.134 ± 0.006^{Bc}	0.060 ± 0.004^{Cc}
+0.2% K sorbate	0.012 ± 0.005^{Ac}	0.133 ± 0.004^{Bc}	0.155 ± 0.003^{Cb}	0.059 ± 0.004^{Dc}
+0.05% cysteine	0.043 ± 0.001^{Ad}	0.220 ± 0.012^{Ba}	0.174 ± 0.014^{Ca}	0.175 ± 0.021^{Cd}
+0.05% cysteine+2% NaCl	0.020 ± 0.005^{Acg}	0.188 ± 0.005^{Bb}	0.166 ± 0.008^{Cb}	0.115 ± 0.004^{Db}
+0.05% cysteine+4% NaCl	0.002 ± 0.000^{Ae}	0.107 ± 0.011^{Be}	0.101 ± 0.010^{Bd}	0.103 ± 0.018^{Bb}

Table 3. (continuation)

+0.05% cysteine +0.1% K-sorbate	0.030±0.002^{Af}	0.170±0.011^{Bf}	0.170±0.005^{Bd}	0.093±0.011^{Cb}
+0.05% cysteine +0.2% K-sorbate	0.029±0.010^{Ag}	0.139±0.004^{Bc}	0.149±0.003^{Ce}	0.042±0.004^{Ae}
	λ (h)			
MRS	4.156±0.185^{Aac}	2.563±0.120^{Ba}	3.261±0.175^{Ca}	3.639±0.103^{Da}
+2% NaCl	4.401±0.066^{Aa}	3.201±0.121^{Bb}	4.086±0.044^{Cb}	4.398±0.036^{Ab}
+4% NaCl	7.715±0.221^{Ab}	4.926±0.257^{Bc}	6.785±0.219^{Cc}	6.785±0.463^{Cc}
+0.1% K-sorbate	3.926±0.105^{Ac}	2.513±0.086^{Ba}	3.094±0.045^{Ca}	3.274±0.073^{Dd}
+0.2% K-sorbate	4.051±0.052^{Ac}	2.513±0.086^{Ba}	3.201±0.121^{Ca}	4.087±0.112^{Ae}
+0.05% cysteine	4.784±0.086^{Ad}	2.668±0.165^{Ba}	4.289±0.153^{Cd}	4.289±0.139^{Cbe}
+0.05% cysteine+2% NaCl	6.076±0.730^{Ae}	3.844±0.174^{Bd}	4.564±0.261^{Cd}	4.564±0.219^{Cbg}
+0.05% cysteine+4% NaCl	45.780±3.352^{Af}	5.025±0.316^{Bc}	5.694±0.207^{Ce}	5.694±0.327^{Cf}
+0.05% cysteine +0.1% K-sorbate	6.821±0.045^{Ag}	3.094±0.045^{Bb}	3.709±0.118^{Cf}	3.598±0.128^{Ca}
+0.05% cysteine +0.2% K-sorbate	8.602±0.113^{Ah}	3.770±0.053^{Bd}	4.398±0.004^{Cd}	4.596±0.150^{Dg}
	ΔpH_{48}			
MRS	1.76	1.79	1.82	1.70
+2% NaCl	1.65	1.73	1.73	1.56
+4% NaCl	1.41	1.52	1.70	1.26
+0.1% K-sorbate	1.67	1.70	1.73	1.51
+0.2% K-sorbate	1.60	1.58	1.73	1.48
+0.05% cysteine	1.70	1.76	1.78	1.74
+0.05% cysteine+2% NaCl	1.64	1.59	1.62	1.49
+0.05% cysteine+4% NaCl	1.52	1.48	1.49	1.46
+0.05% cysteine +0.1% K-sorbate	1.65	1.52	1.54	1.33
+0.05% cysteine +0.2% K-sorbate	1.54	1.57	1.63	1.33
	μ_{pH} (h^{-1})			
MRS	0.074	0.122	0.138	0.158
+2% NaCl	0.076	0.112	0.117	0.131
+4% NaCl	0.063	0.075	0.100	0.129
+0.1% K-sorbate	0.076	0.080	0.129	0.133
+0.2% K-sorbate	0.071	0.075	0.096	0.117
+0.05% cysteine	0.123	0.158	0.195	0.210
+0.05% cysteine+2% NaCl	0.144	0.187	0.219	0.229
+0.05% cysteine+4% NaCl	0.091	0.098	0.110	0.170
+0.05% cysteine +0.1% K-sorbate	0.115	0.149	0.157	0.158
+0.05% cysteine +0.2% K-sorbate	0.088	0.096	0.149	0.155
	λpH (h)			
MRS	4.107	2.209	2.953	3.248
+2% NaCl	5.046	3.391	3.584	3.954

Table 3. (continuation)

+4% NaCl	8.743	4.791	5.661	6.655
+0.1% K sorbate	5.535	3.584	3.828	4.625
+0.2% K sorbate	6.216	4.135	4.935	6.138
+0.05% cysteine	4.832	2.813	3.179	4.364
+0.05% cysteine+2% NaCl	5.577	3.558	4.652	4.865
+0.05% cysteine+4% NaCl	9.590	6.315	6.651	6.828
+0.05% cysteine +0.1% K-sorbate	5.376	4.336	5.016	6.087
+0.05% cysteine +0.2% K-sorbate	6.315	4.935	5.186	6.216

Table 4. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus sakei* 15.39 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	**Growth temperature (°C)**			
	30	**35**	**37**	**40**
	μ (ΔO. D. h^{-1})			
MRS	0.162 ± 0.002^{Aa}	0.209 ± 0.004^{Ba}	0.178 ± 0.018^{Aa}	0.158 ± 0.015^{Aa}
+2% NaCl	0.109 ± 0.002^{Ab}	0.180 ± 0.009^{Bb}	0.174 ± 0.008^{Ba}	0.109 ± 0.007^{Ba}
+4% NaCl	0.067 ± 0.003^{Ac}	0.113 ± 0.007^{Bc}	0.113 ± 0.006^{Bb}	0.090 ± 0.020^{Bbd}
+0.1% K sorbate	0.063 ± 0.005^{Ac}	0.166 ± 0.014^{Bb}	0.180 ± 0.024^{Bac}	0.100 ± 0.003^{Ba}
+0.2% K sorbate	0.024 ± 0.005^{Ad}	0.130 ± 0.015^{Bdc}	0.145 ± 0.018^{Bbc}	0.101 ± 0.010^{Bc}
+0.05% cysteine	0.175 ± 0.006^{Aa}	0.206 ± 0.007^{Ba}	0.170 ± 0.006^{Bd}	0.152 ± 0.008^{Aa}
+0.05% cysteine+2% NaCl	0.093 ± 0.010^{Ae}	0.177 ± 0.004^{Bb}	0.166 ± 0.008^{Ba}	0.127 ± 0.008^{Ba}
+0.05% cysteine+4% NaCl	0.058 ± 0.005^{Ac}	0.118 ± 0.009^{Bcd}	0.105 ± 0.018^{Bb}	0.104 ± 0.014^{Bb}
+0.05% cysteine +0.1% K-sorbate	0.081 ± 0.005^{Ae}	0.175 ± 0.017^{Bb}	0.180 ± 0.024^{Ba}	0.090 ± 0.002^{Ba}
+0.05% cysteine +0.2% K-sorbate	0.048 ± 0.002^{Af}	0.139 ± 0.007^{Be}	0.127 ± 0.005^{Bc}	0.089 ± 0.003^{Cd}
	λ (h)			
MRS	3.546 ± 0.158^{Aa}	2.237 ± 0.097^{Ba}	2.811 ± 0.212^{Ca}	3.630 ± 0.064^{Aa}
+2% NaCl	4.172 ± 0.032^{Ab}	2.498 ± 0.242^{Bb}	3.721 ± 0.063^{Cb}	4.079 ± 0.061^{Ab}
+4% NaCl	5.711 ± 0.102^{Ac}	5.107 ± 0.274^{Bc}	5.244 ± 0.261^{Bc}	5.382 ± 0.018^{Ac}
+0.1% K sorbate	3.693 ± 0.063^{Aa}	3.105 ± 0.031^{Bd}	3.063 ± 0.192^{Bade}	3.349 ± 0.296^{Aba}
+0.2% K sorbate	4.354 ± 0.104^{Ad}	3.873 ± 0.197^{Be}	3.632 ± 0.236^{Bbd}	3.367 ± 0.385^{Ba}
+0.05% cysteine	3.884 ± 0.035^{Ae}	2.767 ± 0.038^{Bb}	3.344 ± 0.092^{Bde}	3.669 ± 0.125^{Ba}
+0.05% cysteine+2% NaCl	5.847 ± 0.308^{Ac}	3.934 ± 0.077^{Be}	3.699 ± 0.052^{Cb}	4.251 ± 0.153^{Dbd}
+0.05% cysteine+4% NaCl	8.123 ± 0.355^{Af}	5.182 ± 0.159^{Bc}	5.128 ± 0.338^{Bc}	5.580 ± 0.368^{Bc}

Table 4. (continuation)

+0.05% cysteine +0.1% K-sorbate	4.704±0.066^{Ag}	3.086±0.222^{Bd}	3.249±0.119^{Be}	4.678±0.296^{Ad}
+0.05% cysteine +0.2% K-sorbate	5.635±0.246^{Ac}	3.135±0.097^{Be}	3.895±0.128^{Bb}	5.421±0.060^{Ac}
	ΔpH_{48}			
MRS	1.95	1.88	1.82	1.75
+2% NaCl	1.92	1.73	1.72	1.66
+4% NaCl	1.63	1.64	1.66	1.61
+0.1% K-sorbate	1.77	1.82	1.79	1.71
+0.2% K-sorbate	1.69	1.72	1.71	1.67
+0.05% cysteine	1.79	1.80	1.77	1.68
+0.05% cysteine+2% NaCl	1.67	1.64	1.64	1.58
+0.05% cysteine+4% NaCl	1.51	1.51	1.47	1.43
+0.05% cysteine +0.1% K-sorbate	1.72	1.74	1.73	1.65
+0.05% cysteine +0.2% K-sorbate	1.62	1.65	1.64	1.61
	μ_{pH} (h^{-1})			
MRS	0.227	0.265	0.284	0.298
+2% NaCl	0.220	0.256	0.272	0.288
+4% NaCl	0.167	0.244	0.260	0.266
+0.1% K-sorbate	0.203	0.228	0.240	0.269
+0.2% K-sorbate	0.193	0.197	0.225	0.239
+0.05% cysteine	0.228	0.265	0.283	0.297
+0.05% cysteine+2% NaCl	0.223	0.245	0.257	0.269
+0.05% cysteine+4% NaCl	0.153	0.240	0.260	0.252
+0.05% cysteine +0.1% K-sorbate	0.195	0.221	0.227	0.256
+0.05% cysteine +0.2% K-sorbate	0.127	0.187	0.210	0.223
	λpH (h)			
MRS	5.817	4.678	5.329	5.337
+2% NaCl	7.608	5.340	5.574	6.010
+4% NaCl	8.114	6.693	7.239	7.608
+0.1% K-sorbate	6.572	5.770	5.574	5.970
+0.2% K-sorbate	7.479	5.831	5.761	7.201
+0.05% cysteine	5.444	3.672	4.773	4.820
+0.05% cysteine+2% NaCl	6.338	4.131	5.079	5.423
+0.05% cysteine+4% NaCl	7.815	6.577	6.931	7.375
+0.05% cysteine +0.1% K-sorbate	6.165	4.820	4.707	5.159
+0.05% cysteine +0.2% K-sorbate	6.193	5.663	5.167	6.612

Table 5. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus curvatus* 15.35 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.188 ± 0.005^{Aa}	0.210 ± 0.010^{Ba}	0.178 ± 0.003^{Ca}	0.132 ± 0.006^{Da}
+2% NaCl	0.145 ± 0.002^{Ab}	0.159 ± 0.001^{Bb}	0.144 ± 0.005^{Ab}	0.081 ± 0.007^{Cb}
+4% NaCl	0.088 ± 0.006^{Ac}	0.107 ± 0.007^{Bc}	0.113 ± 0.021^{Acgh}	0.064 ± 0.012^{Cc}
+0.1% K sorbate	0.087 ± 0.005^{Ac}	0.118 ± 0.013^{Bc}	0.087 ± 0.017^{Abcd}	0.045 ± 0.010^{Cc}
+0.2% K sorbate	0.075 ± 0.004^{Ad}	0.073 ± 0.005^{Ad}	0.077 ± 0.002^{Ad}	0.027 ± 0.005^{Bd}
+0.05% cysteine	0.251 ± 0.005^{Ae}	0.256 ± 0.006^{Ae}	0.236 ± 0.001^{Be}	0.234 ± 0.010^{Be}
+0.05% cysteine+2% NaCl	0.203 ± 0.005^{Af}	0.217 ± 0.011^{Aa}	0.163 ± 0.008^{Bf}	0.132 ± 0.007^{Ca}
+0.05% cysteine+4% NaCl	0.080 ± 0.003^{Acd}	0.116 ± 0.012^{Bc}	0.116 ± 0.004^{Bg}	0.109 ± 0.016^{Bf}
+0.05% cysteine +0.1% K-sorbate	0.153 ± 0.006^{Ab}	0.215 ± 0.005^{Ba}	0.152 ± 0.008^{Abf}	0.089 ± 0.006^{Cbf}
+0.05% cysteine +0.2% K-sorbate	0.136 ± 0.005^{Ag}	0.173 ± 0.004^{Bf}	0.133 ± 0.004^{Ah}	0.052 ± 0.004^{Cc}
	λ (h)			
MRS	3.099 ± 0.106^{Aa}	2.558 ± 0.041^{Ba}	2.920 ± 0.016^{Aa}	4.923 ± 0.263^{Ca}
+2% NaCl	4.245 ± 0.084^{Ab}	2.632 ± 0.106^{Ba}	2.961 ± 0.074^{Ca}	5.260 ± 0.047^{Db}
+4% NaCl	5.480 ± 0.159^{Ac}	3.973 ± 0.196^{Bb}	4.861 ± 0.004^{Cb}	6.219 ± 0.213^{Dc}
+0.1% K sorbate	4.814 ± 0.029^{Ad}	3.020 ± 0.062^{Bc}	4.549 ± 0.187^{Cb}	5.777 ± 0.080^{Dd}
+0.2% K sorbate	5.914 ± 0.054^{Ae}	3.729 ± 0.045^{Bd}	4.847 ± 0.338^{Cb}	6.906 ± 0.029^{De}
+0.05% cysteine	2.800 ± 0.042^{Af}	2.129 ± 0.089^{Be}	2.625 ± 0.051^{Cc}	4.589 ± 0.056^{Df}
+0.05% cysteine+2% NaCl	5.223 ± 0.070^{Ag}	3.717 ± 0.051^{Bd}	3.940 ± 0.101^{cd}	4.936 ± 0.077^{Da}
+0.05% cysteine+4% NaCl	5.816 ± 0.165^{Ae}	5.225 ± 0.147^{Bf}	5.887 ± 0.439^{Ae}	5.620 ± 0.279^{Abg}
+0.05% cysteine +0.1% K-sorbate	4.262 ± 0.185^{Ab}	3.040 ± 0.032^{Bg}	3.663 ± 0.023^{Cf}	4.906 ± 0.060^{Da}
+0.05% cysteine +0.2% K-sorbate	5.035 ± 0.274^{Ad}	3.487 ± 0.016^{Bh}	3.956 ± 0.026^{Cd}	5.762 ± 0.084^{Dg}
	ΔpH_{48}			
MRS	1.45	1.52	1.54	1.96
+2% NaCl	1.44	1.46	1.50	1.79
+4% NaCl	1.31	1.42	1.46	1.52
+0.1% K sorbate	1.22	1.27	1.38	1.62
+0.2% K sorbate	0.98	1.22	1.31	1.52
+0.05% cysteine	1.44	1.46	1.50	1.89
+0.05% cysteine+2% NaCl	1.40	1.42	1.46	1.52
+0.05% cysteine+4% NaCl	1.28	1.33	1.35	1.52
+0.05% cysteine +0.1% K-sorbate	0.99	1.10	1.21	1.36
+0.05% cysteine +0.2% K-sorbate	0.64	0.76	0.80	1.28

Table 5. (continuation)

	μ_{pH} (h^{-1})			
MRS	0.192	0.217	0.221	0.287
+2% NaCl	0.070	0.081	0.087	0.204
+4% NaCl	0.044	0.069	0.067	0.139
+0.1% K sorbate	0.160	0.192	0.208	0.239
+0.2% K sorbate	0.099	0.153	0.195	0.215
+0.05% cysteine	0.137	0.181	0.204	0.245
+0.05% cysteine+2% NaCl	0.070	0.081	0.188	0.215
+0.05% cysteine+4% NaCl	0.041	0.067	0.069	0.139
+0.05% cysteine +0.1% K-sorbate	0.079	0.120	0.184	0.193
+0.05% cysteine +0.2% K-sorbate	0.069	0.123	0.128	0.146
	λpH (h)			
MRS	5.876	5.825	7.492	12.936
+2% NaCl	7.181	6.701	8.296	25.067
+4% NaCl	11.481	10.616	11.917	28.098
+0.1% K sorbate	7.110	6.954	7.751	28.312
+0.2% K sorbate	7.363	7.110	9.080	28.566
+0.05% cysteine	7.026	6.043	8.140	12.936
+0.05% cysteine+2% NaCl	7.181	6.701	8.296	25.010
+0.05% cysteine+4% NaCl	11.481	10.616	11.197	28.098
+0.05% cysteine +0.1% K-sorbate	7.461	7.445	8.525	29.084
+0.05% cysteine +0.2% K-sorbate	8.883	7.724	9.386	45.271

Table 6. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus curvatus* 22.24 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	**Growth temperature (°C)**			
	30	**35**	**37**	**40**
	μ (ΔO. D. h^{-1})			
MRS	0.120 ± 0.002^{Aa}	0.197 ± 0.018^{Ba}	0.175 ± 0.008^{Ba}	0.157 ± 0.008^{Ca}
+2% NaCl	0.110 ± 0.005^{Ab}	0.196 ± 0.001^{Bb}	0.165 ± 0.006^{Ca}	0.142 ± 0.009^{Da}
+4% NaCl	0.055 ± 0.001^{Ac}	0.107 ± 0.006^{Bc}	0.091 ± 0.006^{Cb}	0.082 ± 0.009^{Cb}
+0.1% K sorbate	0.033 ± 0.005^{Ad}	0.100 ± 0.009^{Bc}	0.071 ± 0.001^{Cc}	0.046 ± 0.002^{Dc}
+0.2% K sorbate	0.015 ± 0.003^{Ae}	0.088 ± 0.002^{Bd}	0.049 ± 0.004^{Cd}	0.036 ± 0.003^{Dd}
+0.05% cysteine	0.136 ± 0.003^{Af}	0.216 ± 0.004^{Ba}	0.214 ± 0.004^{Be}	0.175 ± 0.012^{Ce}
+0.05% cysteine+2% NaCl	0.092 ± 0.003^{Ag}	0.143 ± 0.004^{Be}	0.128 ± 0.007^{Cf}	0.117 ± 0.014^{Dfg}
+0.05% cysteine+4% NaCl	0.064 ± 0.006^{Ah}	0.106 ± 0.007^{Bc}	0.121 ± 0.011^{BCg}	0.132 ± 0.008^{Caf}

Table 6. (continuation)

+0.05% cysteine +0.1% K-sorbate	0.100±0.005^{Abg}	0.170±0.016^{Ba}	0.133±0.012^{Cfg}	0.100±0.003^{Ag}
+0.05% cysteine +0.2% K-sorbate	0.091±0.006^{Ag}	0.135±0.003^{Bf}	0.117±0.004^{Cfg}	0.093±0.003^{Ah}
	λ (h)			
MRS	4.828±0.006^{Aa}	2.999±0.060^{Ba}	4.215±0.110^{Ca}	4.000±0.169^{Ca}
+2% NaCl	6.168±0.146^{Ab}	3.933±0.071^{Bb}	4.589±0.106^{Cb}	4.628±0.082^{Cb}
+4% NaCl	7.065±0.076^{Ac}	4.592±0.294^{Bc}	5.887±0.161^{Cc}	6.354±0.174^{Cc}
+0.1% K-sorbate	6.160±0.043^{Ab}	3.934±0.220^{Bb}	5.377±0.340^{Cd}	5.493±0.178^{Dd}
+0.2% K-sorbate	10.734±0.588^{Ad}	4.236±0.190^{Bc}	6.400±0.209^{Ce}	6.637±0.167^{Cc}
+0.05% cysteine	4.777±0.422^{Aa}	2.611±0.106^{Bd}	3.068±0.103^{Cf}	3.654±0.144^{De}
+0.05% cysteine+2% NaCl	5.760±0.119^{Ae}	2.995±0.111^{Ba}	4.201±0.422^{Cabg}	4.589±0.103^{Cb}
+0.05% cysteine+4% NaCl	7.102±0.167^{Ac}	3.904±0.303^{Bf}	4.990±0.133^{Cd}	5.614±0.136^{Df}
+0.05% cysteine +0.1% K-sorbate	4.916±0.104^{Aa}	2.744±0.195^{Ba}	3.808±0.055^{Cg}	4.587±0.103^{Db}
+0.05% cysteine +0.2% K-sorbate	5.941±0.089^{Ae}	3.180±0.071^{Be}	4.773±0.039^{Ch}	5.313±0.167^{Df}
	ΔpH_{48}			
MRS	1.79	1.77	1.70	1.66
+2% NaCl	1.79	1.67	1.66	1.36
+4% NaCl	1.63	1.59	1.58	1.34
+0.1% K-sorbate	1.66	1.36	1.25	1.15
+0.2% K-sorbate	0.14	0.14	0.08	0.04
+0.05% cysteine	1.73	1.61	1.51	1.19
+0.05% cysteine+2% NaCl	1.63	1.58	1.49	1.09
+0.05% cysteine+4% NaCl	1.62	1.53	1.50	0.94
+0.05% cysteine +0.1% K-sorbate	1.72	1.56	1.49	1.18
+0.05% cysteine +0.2% K-sorbate	1.52	1.32	1.20	1.10
	μ_{pH} (h^{-1})			
MRS	0.084	0.137	0.192	0.207
+2% NaCl	0.136	0.157	0.195	0.214
+4% NaCl	0.032	0.061	0.065	0.077
+0.1% K-sorbate	0.046	0.085	0.139	0.190
+0.2% K-sorbate	0.039	0.065	0.091	0.141
+0.05% cysteine	0.053	0.132	0.175	0.179
+0.05% cysteine+2% NaCl	0.136	0.153	0.179	0.190
+0.05% cysteine+4% NaCl	0.042	0.063	0.091	0.176
+0.05% cysteine +0.1% K-sorbate	0.052	0.126	0.158	0.179
+0.05% cysteine +0.2% K-sorbate	0.047	0.075	0.143	0.145
	λpH (h)			
MRS	7.325	4.419	6.012	6.449
+2% NaCl	7.507	4.845	6.069	6.622

Table 6. (continuation)

+4% NaCl	9.625	6.033	6.651	7.595
+0.1% K sorbate	23.282	4.796	6.260	6.521
+0.2% K sorbate	28.208	6.568	7.103	6.625
+0.05% cysteine	8.216	4.948	6.033	6.765
+0.05% cysteine+2% NaCl	10.212	5.827	7.391	6.883
+0.05% cysteine+4% NaCl	10.240	6.143	7.677	10.259
+0.05% cysteine +0.1% K-sorbate	26.491	6.634	7.573	7.616
+0.05% cysteine +0.2% K-sorbate	28.986	7.249	7.863	7.911

Table 7. Growth parameters (μ and λ) and acidification parameters (ΔpH_{48}, μ_{pH} and λ_{pH}) for *Lactobacillus plantarum* 009 grown in modified MRS broth, at different temperatures. Growth parameters values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.01$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.01$). Acidification parameters values are averages of two samples.

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.025 ± 0.002^{Aa}	0.140 ± 0.005^{Ba}	0.144 ± 0.011^{Ba}	0.109 ± 0.014^{Cae}
+2% NaCl	0.033 ± 0.004^{Ab}	0.153 ± 0.006^{Bb}	0.188 ± 0.017^{Cbd}	0.114 ± 0.008^{Da}
+4% NaCl	0.025 ± 0.001^{Aa}	0.104 ± 0.013^{Bc}	0.113 ± 0.008^{Bc}	0.077 ± 0.011^{Cb}
+0.1% K sorbate	0.029 ± 0.002^{Ab}	0.073 ± 0.006^{Bd}	0.144 ± 0.006^{Ca}	0.047 ± 0.004^{Dc}
+0.2% K sorbate	0.018 ± 0.003^{Ac}	0.044 ± 0.006^{Be}	0.122 ± 0.005^{Cc}	0.029 ± 0.005^{Dd}
+0.05% cysteine	0.083 ± 0.002^{Ad}	0.159 ± 0.005^{Bb}	0.181 ± 0.004^{Cd}	0.114 ± 0.011^{Dae}
+0.05% cysteine+2% NaCl	0.099 ± 0.003^{Ae}	0.158 ± 0.011^{Bb}	0.205 ± 0.008^{Cb}	0.136 ± 0.013^{Be}
+0.05% cysteine+4% NaCl	0.071 ± 0.005^{Af}	0.116 ± 0.005^{Bc}	0.114 ± 0.012^{Bc}	0.109 ± 0.008^{Ba}
+0.05% cysteine +0.1% K-sorbate	0.089 ± 0.004^{Ad}	0.095 ± 0.007^{Ac}	0.174 ± 0.005^{Bbd}	0.090 ± 0.005^{Af}
+0.05% cysteine +0.2% K-sorbate	0.030 ± 0.005^{Aab}	0.074 ± 0.010^{Bd}	0.140 ± 0.005^{Ca}	0.064 ± 0.003^{Bg}
	λ (h)			
MRS	11.796 ± 2.472^{Aa}	3.801 ± 0.135^{Bae}	3.438 ± 0.161^{Ca}	4.598 ± 0.045^{Da}
+2% NaCl	7.539 ± 0.157^{Ab}	3.622 ± 0.059^{Baf}	2.702 ± 0.207^{Cb}	3.968 ± 0.141^{Db}
+4% NaCl	19.489 ± 0.230^{Ac}	5.459 ± 0.173^{BCc}	5.738 ± 0.156^{Bc}	6.055 ± 0.439^{Cc}
+0.1% K sorbate	15.196 ± 0.495^{Ad}	5.444 ± 0.214^{Bc}	4.385 ± 0.197^{Cd}	4.722 ± 0.216^{Ca}
+0.2% K sorbate	15.796 ± 0.605^{Ad}	5.916 ± 0.260^{Bd}	4.666 ± 0.060^{Ce}	5.738 ± 0.173^{Bc}
+0.05% cysteine	6.556 ± 0.033^{Ae}	3.729 ± 0.023^{Be}	2.569 ± 0.098^{Cb}	4.388 ± 0.205^{Dae}
+0.05% cysteine+2% NaCl	5.128 ± 0.486^{Af}	3.599 ± 0.052^{Bf}	2.728 ± 0.174^{Cb}	3.309 ± 0.086^{Dd}
+0.05% cysteine+4% NaCl	7.994 ± 0.126^{ag}	5.129 ± 0.243^{Bc}	4.426 ± 0.123^{Cd}	5.940 ± 0.176^{Dc}

Table 7. (continuation)

+0.05% cysteine +0.1% K-sorbate	6.796±0.130^{Ah}	5.611±0.003^{Bc}	4.598±0.029^{Ce}	5.015±0.051^{De}
+0.05% cysteine +0.2% K-sorbate	6.802±0.130^{Ah}	5.679±0.158^{Bcd}	4.707±0.046^{Ce}	5.142±0.047^{Df}
	ΔpH_{48}			
MRS	1.90	1.89	1.79	1.74
+2% NaCl	1.81	1.75	1.62	1.53
+4% NaCl	1.62	1.66	1.63	1.30
+0.1% K-sorbate	1.76	1.74	1.75	1.72
+0.2% K-sorbate	1.73	1.73	1.72	1.65
+0.05% cysteine	1.92	1.85	1.81	1.80
+0.05% cysteine+2% NaCl	1.85	1.74	1.70	1.65
+0.05% cysteine+4% NaCl	1.78	1.63	1.57	1.50
+0.05% cysteine +0.1% K-sorbate	1.76	1.74	1.70	1.65
+0.05% cysteine +0.2% K-sorbate	1.73	1.61	1.61	1.55
	μ_{pH} (h^{-1})			
MRS	0.122	0.137	0.136	0.134
+2% NaCl	0.111	0.122	0.132	0.125
+4% NaCl	0.081	0.109	0.125	0.109
+0.1% K-sorbate	0.098	0.121	0.124	0.104
+0.2% K-sorbate	0.092	0.111	0.110	0.097
+0.05% cysteine	0.158	0.166	0.172	0.153
+0.05% cysteine+2% NaCl	0.158	0.171	0.175	0.167
+0.05% cysteine+4% NaCl	0.102	0.143	0.153	0.149
+0.05% cysteine +0.1% K-sorbate	0.142	0.143	0.146	0.142
+0.05% cysteine +0.2% K-sorbate	0.094	0.136	0.149	0.126
	λpH (h)			
MRS	2.888	0.964	0.936	1.419
+2% NaCl	4.669	1.954	1.398	1.623
+4% NaCl	7.194	4.751	3.310	5.194
+0.1% K-sorbate	4.024	0.993	0.946	2.999
+0.2% K-sorbate	5.861	1.855	0.957	3.401
+0.05% cysteine	1.820	0.896	0.738	1.253
+0.05% cysteine+2% NaCl	3.637	1.901	0.606	1.311
+0.05% cysteine+4% NaCl	6.858	3.783	0.993	4.225
+0.05% cysteine +0.1% K-sorbate	4.100	1.383	0.986	3.345
+0.05% cysteine +0.2% K-sorbate	6.507	2.597	1.225	5.379

Temperature

Figures 1 – 7 show the effect of temperature on growth and acidification parameters calculated for the different strains. Strains *Lb. sakei* 15.18, *Lb. curvatus* 15.35 and 22.24 grew best at 35°C (higher μ, lower λ). At 40°C, these strains displayed the lowest μ and longest λ values. Strains *Lb. sakei* 15.05 and *Lb. plantarum* 009 grew best at 37°C. The μ values were lowest and the λ values highest at 30°C in most cases. In the case of 15.05, there was no significant ($p<0.01$) difference between the μ values obtained at 30 and at 40°C, in MRS and in K-sorbate containing broths. The most favourable temperature for the growth of *Lb. sakei* 15.36 was also 35°C. The temperature at which μ was minimal and λ longest was, for this strain, 30°C. *Lb. sakei* 15.39 grew equally well at 35 and 37°C. Growth at these two temperatures was significantly ($p<0.01$) better than at 30 or 40°C. No significant ($p<0.01$) difference was observed for this strain between μ and λ values obtained at 30 and at 40°C.

The temperature-associated variation in ΔpH_{48} values differed with the species. All *Lb. sakei* strains showed a decrease in ΔpH_{48} values as temperature increased within the studied range, while both *Lb. curvatus* strains showed decreasing μ_{pH} values. The μ_{pH} values obtained for *Lb. plantarum* 009 were highest at 37°C, lowest at 30°C.

These results show that fast ensiling (which is essential for product quality and safety) can only be obtained at relatively high fermentation temperatures. In spite of being widely used in the laboratory as the growth temperature for all LAB, 30°C may still be too low to ensure a quick enough decrease in pH, at least for the group of strains studied.

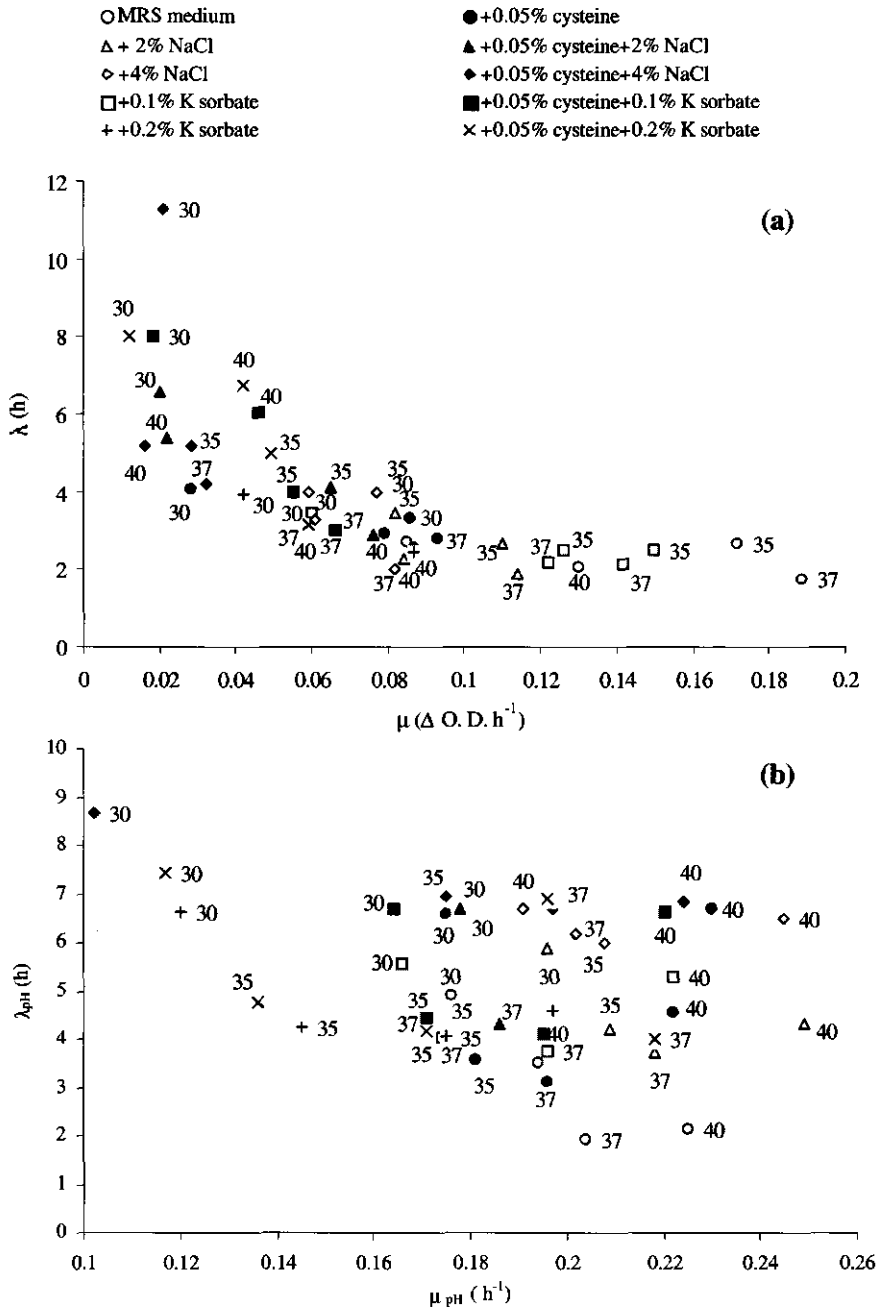

Fig. 1. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. sakei* 15.05. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

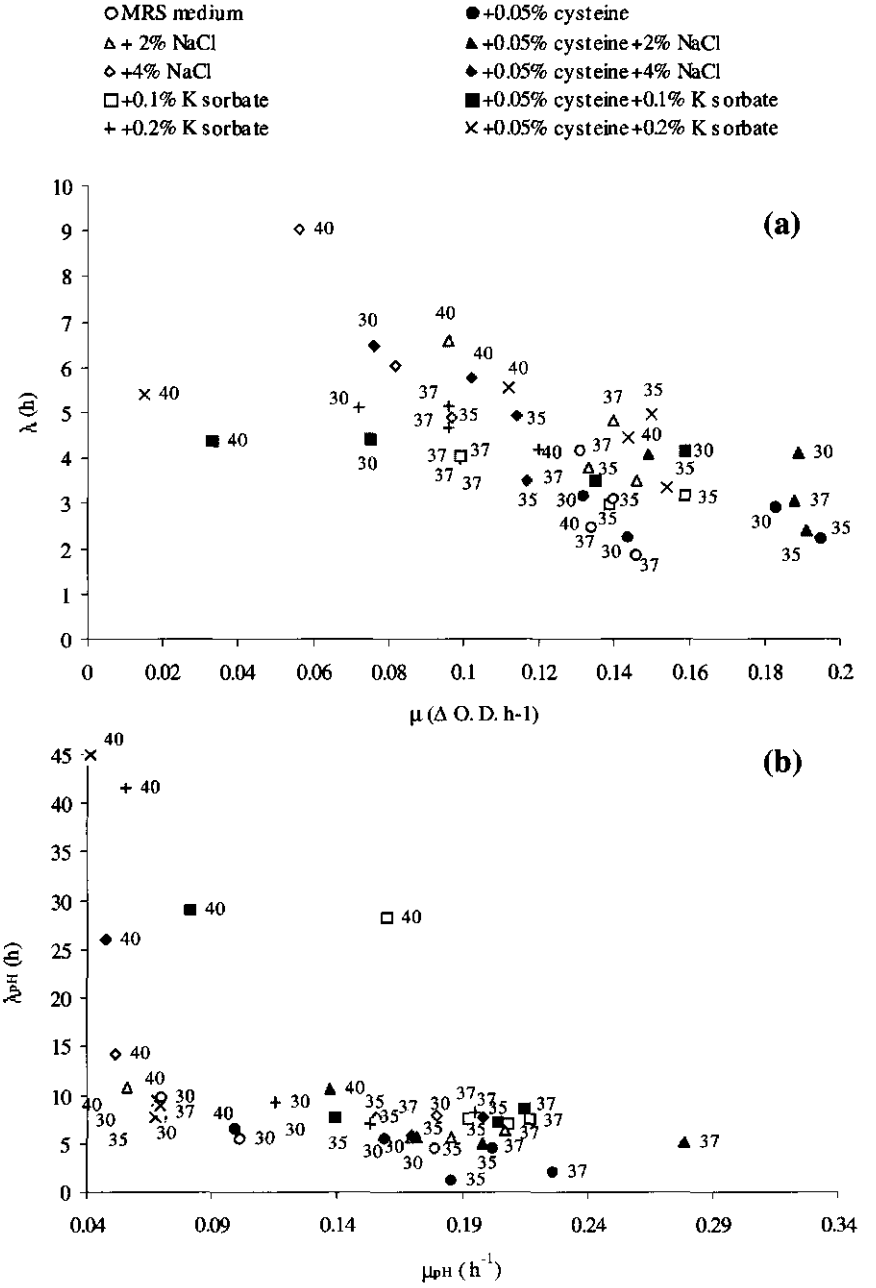

Fig. 2. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. sakei* 15.18. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

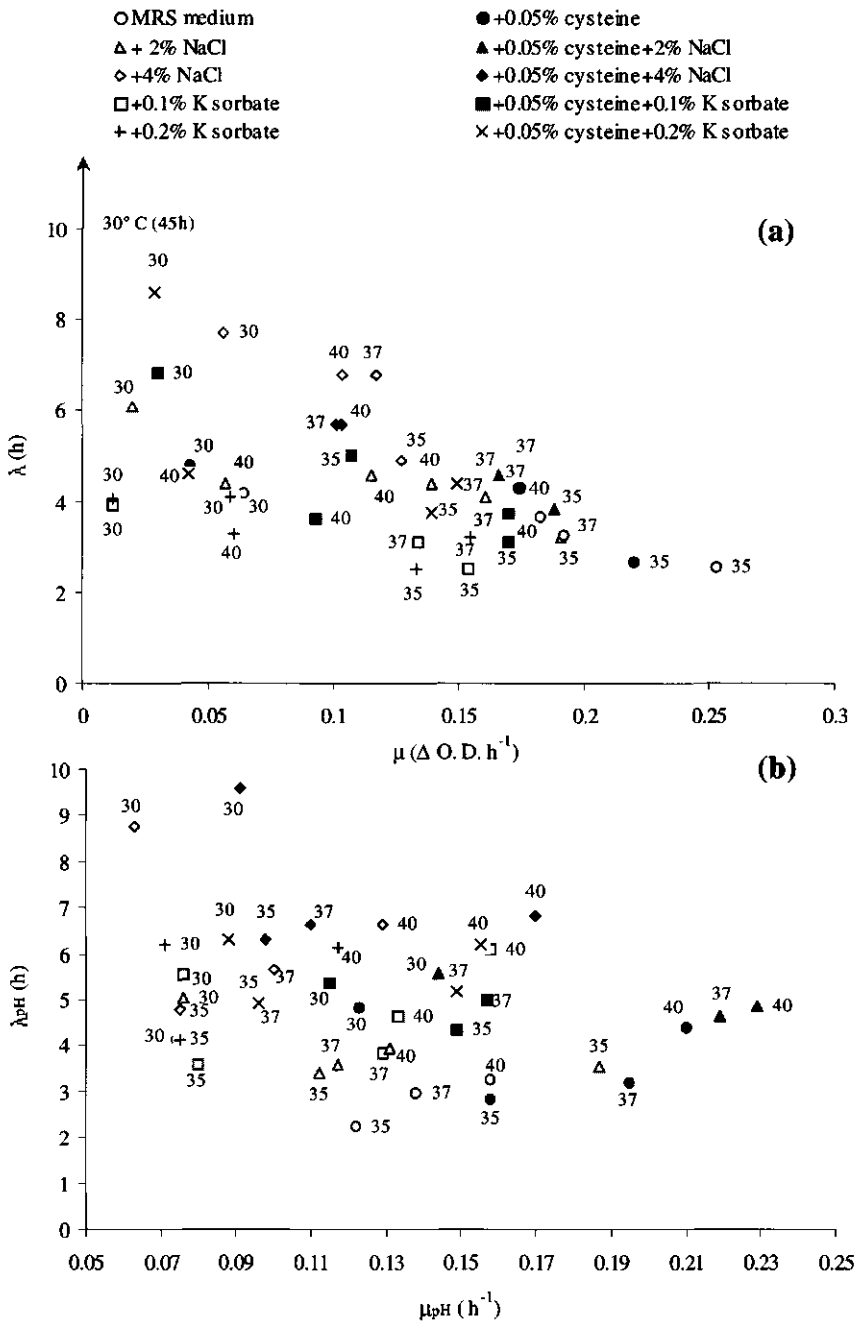

Fig. 3. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. sakei* 15.36. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

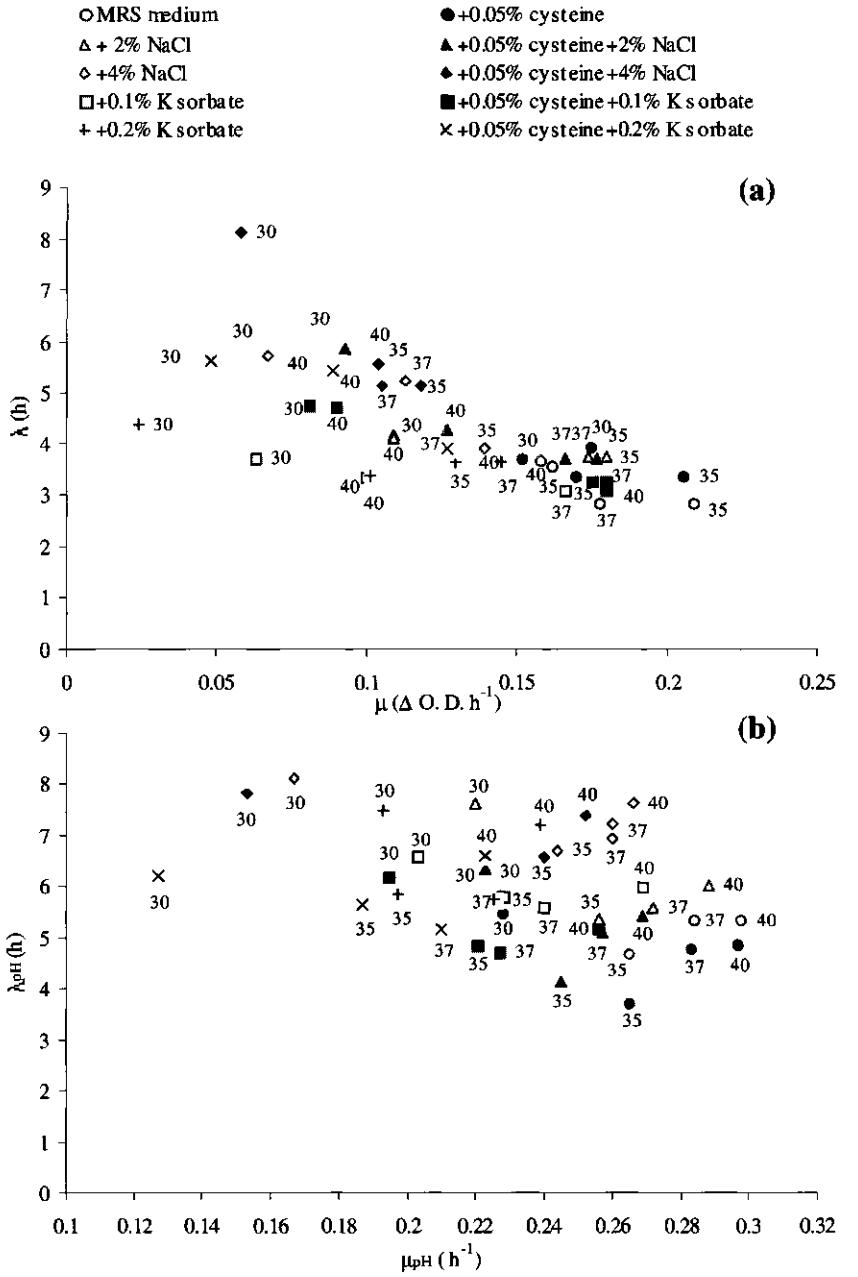

Fig. 4. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. sakei* 15.39. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

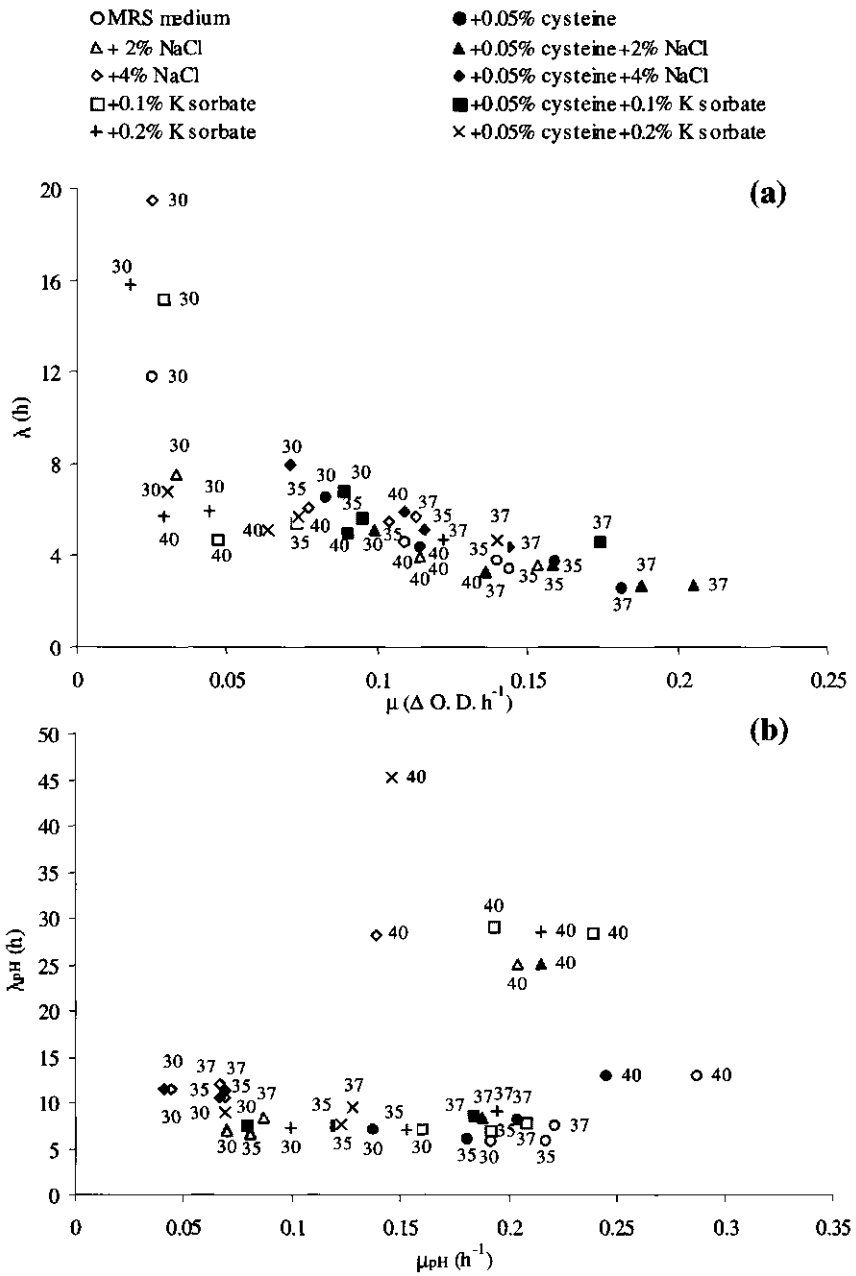

Fig. 5. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. curvatus* 15.35. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

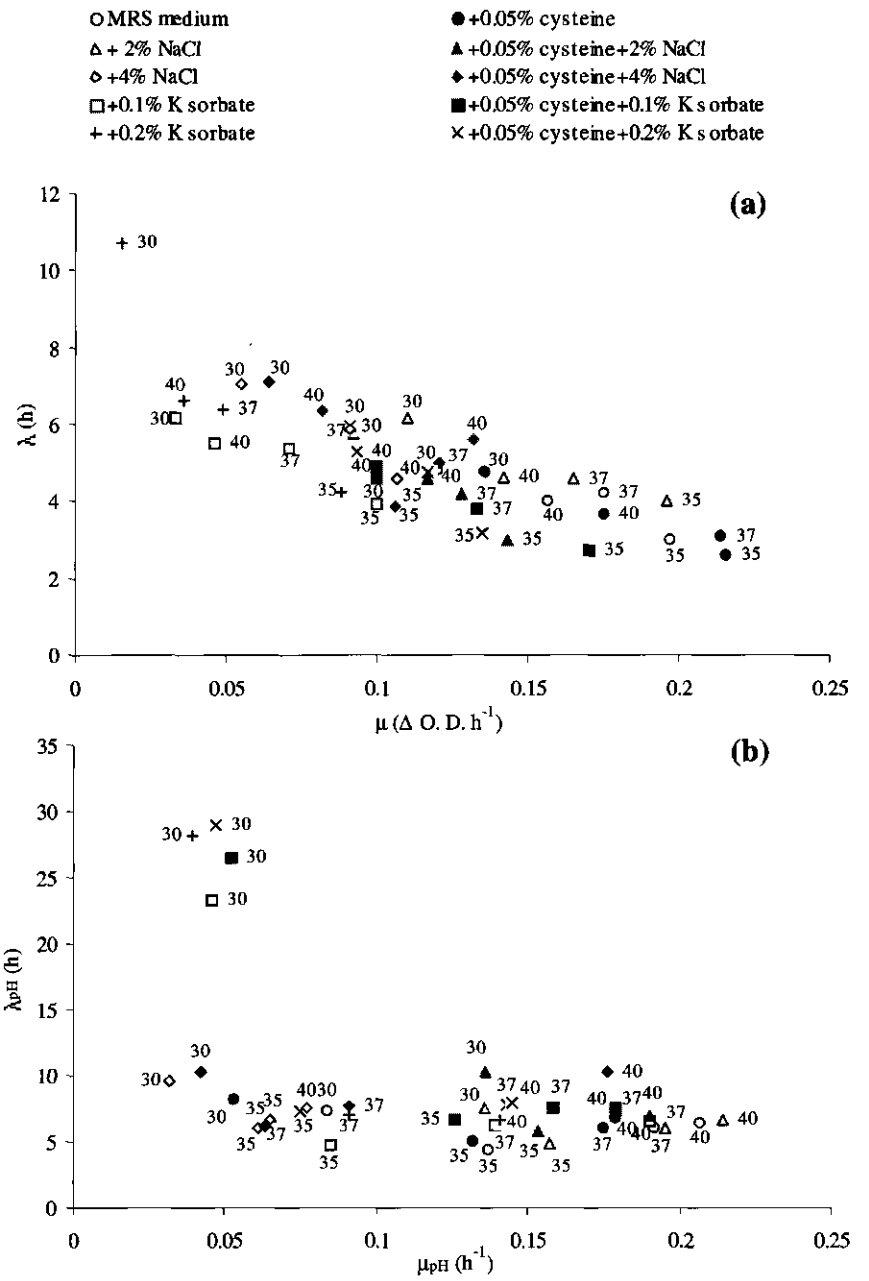

Fig. 6. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. curvatus* 22.24. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

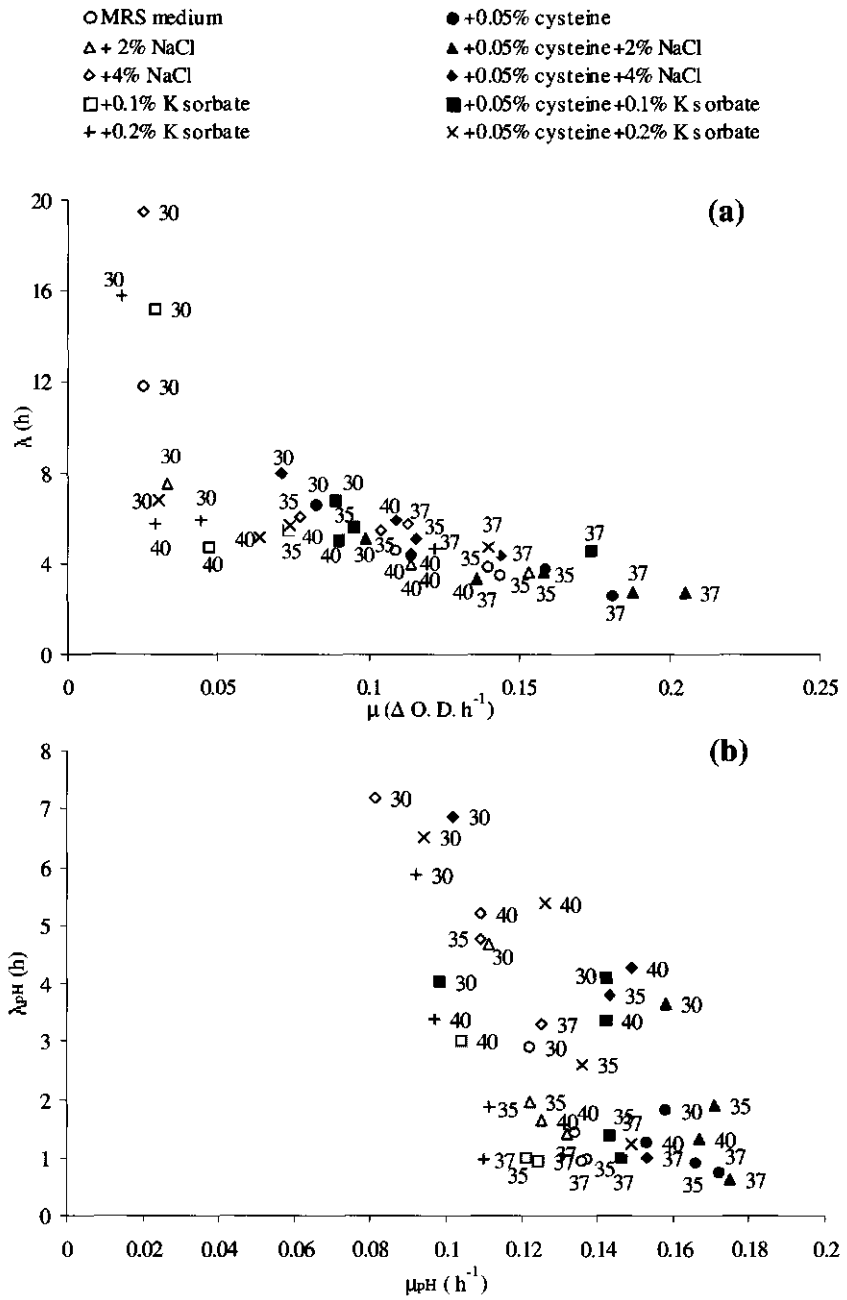

Fig. 7. Effect of temperature on μ, μ_{pH}, λ and λ_{pH} values obtained in different media formulations inoculated with *Lb. plantarum* 009. The data labels indicate the incubation temperatures. (a) growth parameters (b) acidification parameters.

Sodium chloride

The effect of sodium chloride on the growth and acidification parameters calculated for each strain is shown in Figures 8 and 9, respectively. NaCl addition to the basal MRS formula or to MRS + cysteine resulted in growth inhibition for all *Lb. sakei* and *Lb. curvatus* isolates, at most temperatures and cysteine concentrations. In a few cases, however, the difference between λ values obtained in MRS+2% NaCl and in MRS was not significant ($p<0.01$, *Lb. curvatus* 15.35 at 35 and 37°C in the absence of added cysteine). In addition, *Lb. sakei* 15.18 showed only a significant ($p<0.01$) increase in λ values in the presence of 2% NaCl. The μ values obtained for this isolate with 2% added NaCl did not differ significantly ($p<0.01$) from those obtained in MRS, except for a slight decrease at 40°C. In the case of *Lb. plantarum* 009, however, growth was enhanced in the presence of 2% NaCl, as could be seen from the significantly ($p<0.01$) higher μ values obtained. Lag times were also generally higher, except at 35 and 37°C, in the presence of added cysteine. The growth of both 15.18 and 009 was inhibited by adding 4% NaCl.

NaCl addition always brought about a decrease in ΔpH_{48} as well as an increase in λ_{pH}. Two isolates (*Lb. sakei* 15.39 and *Lb. curvatus* 15.35) also showed a decrease in μ_{pH} when growing in sodium chloride-containing media. In the case of *Lb. sakei* 15.05, an increase in μ_{pH} occurred in broths containing 2% and 4% NaCl. The growth rates of *Lb. sakei* 15.18 and *Lb. curvatus* 22.24 were highest for 2% NaCl-containing broths. *Lb. sakei* 15.36 and *Lb. plantarum* 009 showed a decrease in μ_{pH} when grown in media with 4% NaCl, but the effect of 2% NaCl on the μ_{pH} values of these isolates depended on the presence or absence of cysteine. In the absence of cysteine, μ_{pH} decreased, while an increase was observed when cysteine was present.

With some strains, 2% NaCl can be used as an additional hurdle for BFS production. Due to its very low λ_{pH} values, *Lb. plantarum* 009 is the most promising strain for ensilaging under these conditions.

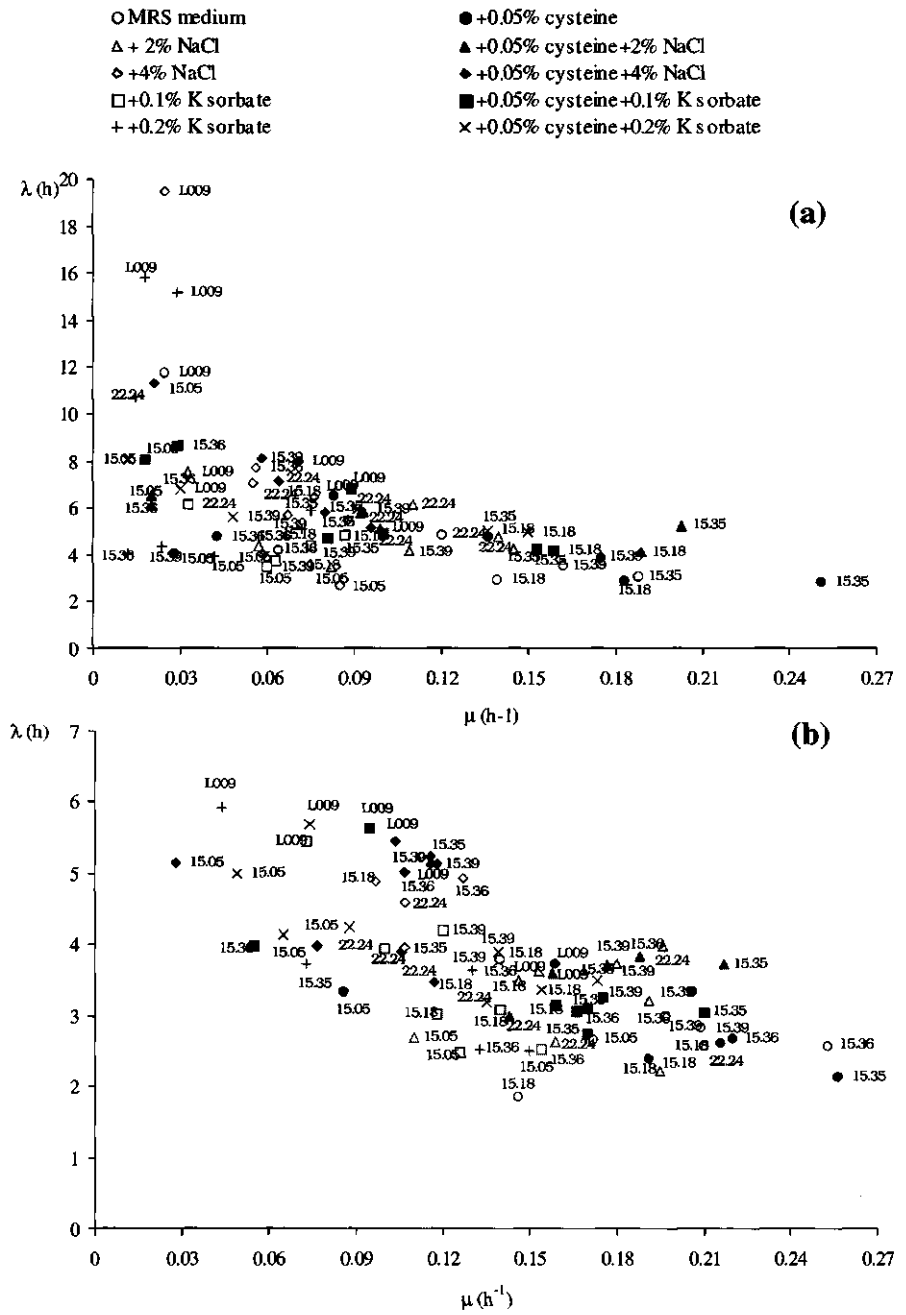

Fig. 8. Effect of seven LAB strains on μ and λ values obtained in different media formulations upon incubation at different temperatures. The values shown are the averages of four determinations. (a) 30°C, (b)35°C, (c) 37°C, (d) 40°C.

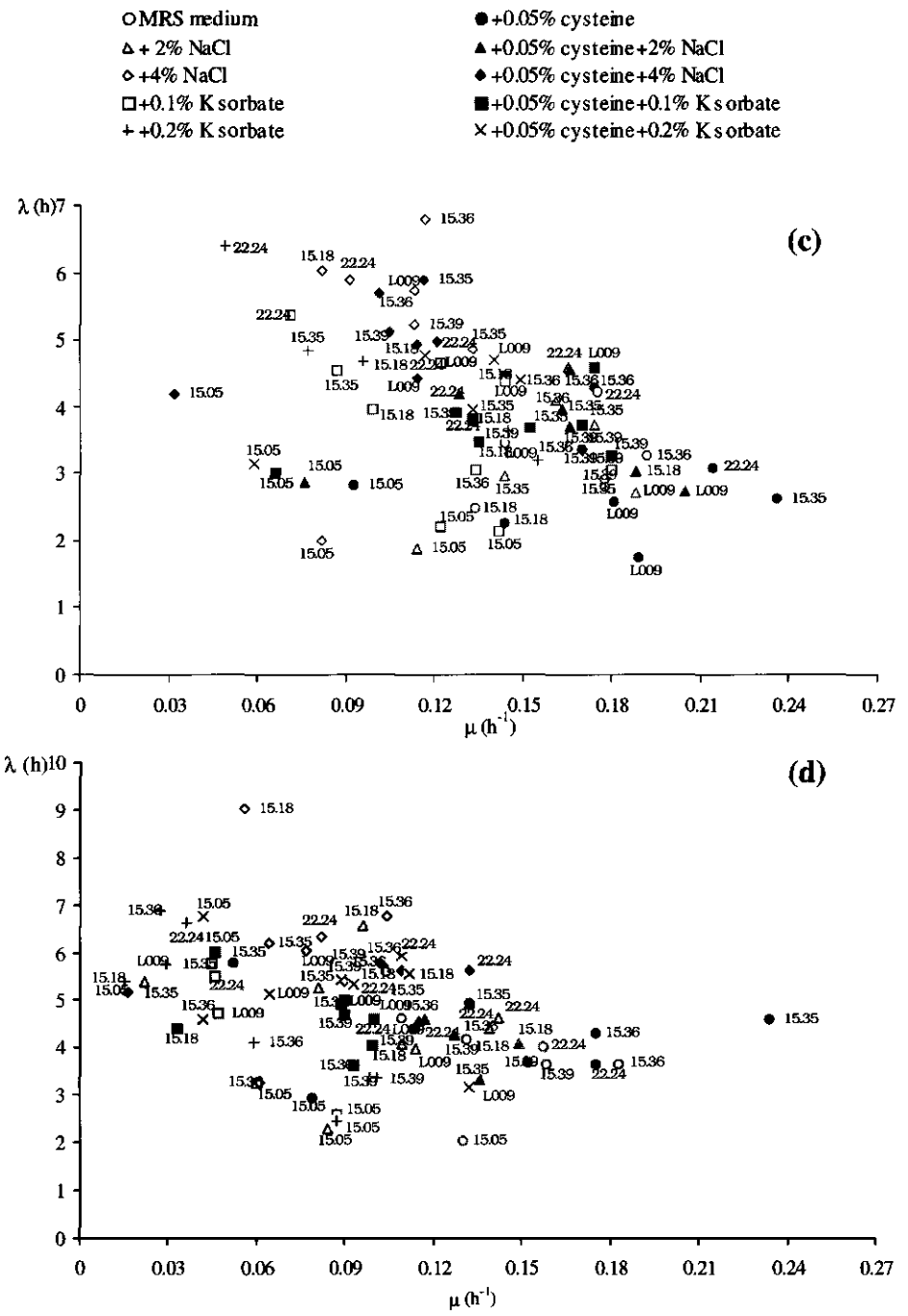

Fig. 8. (continuation).

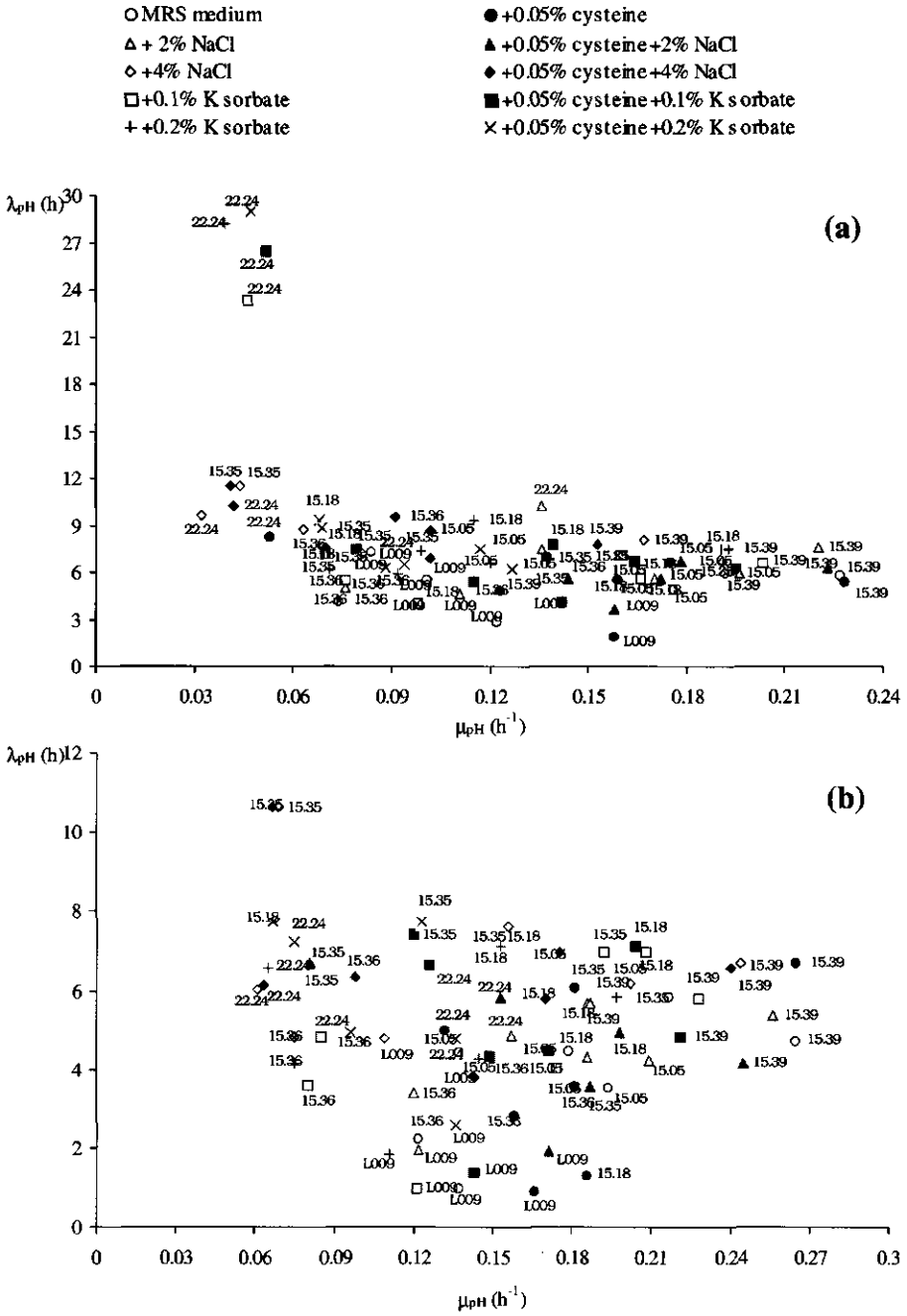

Fig. 9. Effect of seven LAB strains on pH decrease rates and lag times obtained in different media formulations upon incubation at 30°C. The values shown are the averages of four determinations. (a) 30°C, (b)35°C, (c) 37°C, (d) 40°C.

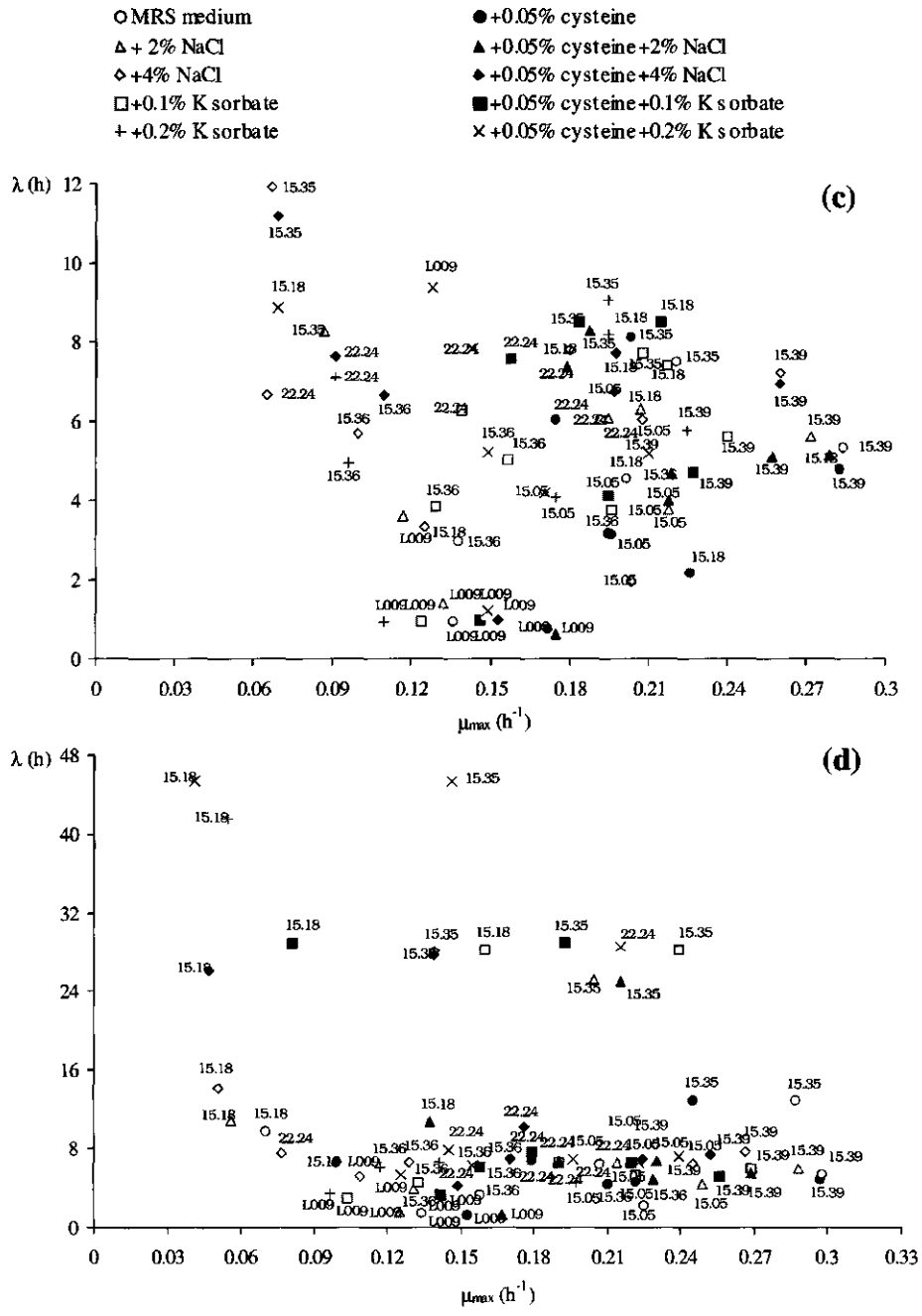

Fig. 9. (continuation)

Potassium sorbate

The effect of potassium sorbate on the growth and acidification parameters calculated for each strain is shown in Figures 8 and 9, respectively. Mostly, adding potassium sorbate to MRS medium or MRS medium with added cysteine resulted in growth inhibition (lower μ, longer λ). In a few cases, however, K-sorbate addition did not result in significant ($p<0.01$) differences. The μ values determined for *Lb.sakei* strain 15.36 in media containing K-sorbate did not differ significantly ($p<0.01$) from those obtained in the absence of K-sorbate at 30 and 35°C, in the absence of cysteine. In the case of *Lb.sakei* strain 15.39, λ values generally increased with K-sorbate addition, but μ values did not increase significantly ($p<0.01$) at 37°C, although they did at all other temperatures. The λ values of 22.24 were generally longer in media containing K-sorbate, with the exception of media containing 0.1% K-sorbate, incubated at 30 and 35°C. *Lb. plantarum* 009 did not show significantly ($p<0.01$) lower μ values when 0.1% K-sorbate was added to the media and incubation was done at 30 and 37°C, in the absence of cysteine. Adding K-sorbate led to lower ΔpH_{48} and extended lag phases in all studied strains under all tested growth conditions. For *Lb. sakei* 15.05, 15.36 and 15.39, as well as for *Lb. curvatus* 15.35 and 22.24 and *Lb. plantarum* 009, a decrease (slight in the case of 22.24) was observed also in μ_{pH} values. *Lb. sakei* 15.18 showed increased μ_{pH} values when 0.1% K-sorbate was added, but the addition of 0.2% K-sorbate led to lower rates.

Potassium sorbate considerably inhibited growth and acidification by most strains at the concentrations tested. For this reason, its use in BFS formulations fermented with these strains may lead to insufficient or delayed pH decrease and should be avoided. It could, however, be used as an antimycotic spray on the exposed surfaces of the silage, which have been described as more prone to mould and yeast growth (Fagbenro and Jauncey, 1993a and b).

Low Eh

The effect of cysteine addition on the growth and acidification parameters calculated for each strain is shown in Figures 8 and 9, respectively. Cysteine addition inhibited both the growth and acidification of *Lb. sakei* strains15.05 and 15.36. Strain 15.05 had longer λ and λ_{pH}, lower μ and ΔpH_{48}. μ_{pH} was also lower in MRS and MRS + NaCl, but did not vary significantly ($p<0.01$) in MRS + K-sorbate. Longer λ and λ_{pH} values were obtained for strain 15.36. ΔpH_{48} and μ_{pH} were lower, but μ was lower only in the absence of K-sorbate. In MRS+ K-sorbate, higher μ values were obtained. Growth and acidification by *Lb. curvatus* 15.35 were also inhibited, but only when NaCl was also added to the medium. This was reflected in longer lag times for growth and acidification, lower acidification rates and smaller ΔpH_{48} values. The growth rate was, however, higher in all cysteine-containing samples. In MRS and MRS+ K-sorbate, growth was enhanced, with higher μ values associated to shorter lag phases, but acidification was still

inhibited. In the case of isolate *Lb. sakei* 15.39, the main effect of cysteine on growth and acidification was observed on the length of both lag times. Adding cysteine increased the lag time for growth, but decreased the lag time for pH decrease. Cysteine stimulated the growth of isolates *Lb. sakei* 15.18 and in general also that of *Lb. plantarum* 009. The latter, however, did not show any significant ($p<0.01$) difference in μ and λ values in a few cases (at 35°C in the presence of NaCl; at 37°C with 4% NaCl; at 40°C in MRS). Cysteine effect on the pH decrease by these isolates depended on the presence or absence of K-sorbate. In the presence of K-sorbate, acidification was inhibited, in its absence it was stimulated.

The Eh conditions in cysteine-containing broths simulate the low Eh attained during the ensilage of fish. Starter strains should be able to grow and decrease the pH under these conditions. Strains that have impaired growth and pH decrease, such as *Lb. sakei* 15.05 and 15.36 are less likely to be of use for BFS production than strains that display better growth and acidification properties in cysteine-containing media, such as *Lb. plantarum* 009 and *Lb. sakei* 15.18. Mackerel or blue-jack mackerel have been previously ensiled with some of these strains (this thesis, chapters 3 and 5). While 15.05 has failed to acidify mackerel:water:sucrose:NaCl pastes (43:43:12:2) at 30°C (chapter 5), ensiling blue-jack mackerel:sucrose:water:NaCl pastes (76:12:10:2) with *Lb. plantarum* 009 at 37°C resulted in a fast pH decrease, in low final pH values and in good quality BFS (chapter 3), which is in agreement with the findings of the present work.

Strain

Figures 8 and 9 show the growth and acidification parameters of the LAB strains. At all temperatures tested, the best growing strain was *Lb. curvatus* 15.35, in MRS medium with added cysteine. At 35°C and 37°C, the growth parameters of a few other strains were similar to those of 15.35, namely *Lb. sakei* 15.36 in MRS medium, *Lb. curvatus* 22.24 in MRS medium with added cysteine, and *Lb. plantarum* 009 in MRS medium with added cysteine and 2% NaCl. However, at 30°C and 40°C, *Lb. curvatus* 15.35 (in MRS medium with added cysteine) grew considerably better than all other strains.

The effect of the strain on pH decrease was more complex than on growth, since the strains that showed the lowest λ values differ from those with the highest μ. *Lb. plantarum* 009 had, at all temperatures, the lowest λ values, whereas *Lb. sakei* 15.39 had the highest μ values for acidification.

Ensiling has been attempted with all these strains (this thesis, chapters 3, 4 and 5). Ensiling was successful only with *Lb. plantarum* 009 and *Lb. curvatus* 15.35. In spite of its high pH decrease rate, *Lb. sakei* 15.39 performed poorly in silages. This might have been due to its long lag time and/or its poor growth at the temperature used (30°C). *Lb. plantarum* 009 was the most successful strain – this can be due to its very short acidification lag times and good growth at the temperature tested. *Lb. curvatus* 15.35 stabilised the silages probably due to its

excellent growth and/or antimicrobial properties, even though it did not perform particularly well in terms of acidification.

CONCLUSIONS

Significant ($p<0.001$) interaction has been found among strain, temperature, cysteine and chemical preservatives tested. By consequence, fermentation conditions must be optimised for each specific strain. For the group of strains studied, temperatures of 35– 37°C should be used in order to ensure a fast stabilisation of the silages. Some strains, e. g. *Lb. sakei* 15.05 and 15.36, showed inhibited growth or acidification in cysteine containing media, indicating that they might perform poorly in silages, where Eh values decrease very quickly. NaCl (2%, w/w) can be used with some strains, such as *Lb. plantarum* 009, but 4% NaCl caused considerable inhibition of the growth and acidification by most strains. Both studied K-sorbate concentrations inhibited growth and acidification by all strains. Its use is, however, still possible as an antimycotic spray. *Lb. plantarum* 009 and *Lb. curvatus* 15.35 seem particularly suitable for usage as BFS starters, respectively due to having the shortest pH decrease lag times and the best growth properties. These results agree with the previous data from ensilage tests (chapters 3 and 5). Thus, a fast growth rate and short lag times for growth and pH decrease seem to be the most important selection criteria for starter strains intended for fish silage preparation.

Chapter 8

Effect of temperature, redox potential, pH and additives on growth of selected biogenic amine producing bacteria in bacteriological culture media

ABSTRACT

The growth of five biogenic amine producing bacteria of concern for fish silage production (*Lb. sakei* 15.19, *W. hellenica* 15.32, *E. faecium* 22.12, *Lb. büchneri* 2A and *M. morganii* 6675) was studied in different model systems. The culture media used were ten different modifications of the MRS medium for the lactic acid bacteria (LAB) and BHI for *Morganella* (basal medium, +2 or 4% NaCl, +0.1 or 0.2% potassium sorbate, +0.05% cysteine, +0.05% cysteine+2 or 4% NaCl and +0.05% cysteine+0.1 or 0.2% K-sorbate). Four incubation temperatures, 30, 35, 37 and 40°C, were used for each strain and medium formulation. The pH values of the various media were adjusted to 6.0. The effect of pH values 4.0, 4.5 and 5.0 on growth of *M. morganii* was studied under the described experimental conditions as well. Growth was measured as the increase in absorbance of the medium. The obtained values were used to calculate growth parameters (μ and λ).

All tested conditions (low pH, reduced redox potential (Eh), NaCl or K-sorbate addition) inhibited the growth of *M. morganii*. The growth of the biogenic amine producing LAB under study was somewhat inhibited by NaCl or K-sorbate addition, but not by low Eh.

INTRODUCTION

In properly preserved fish silage, pH values below 4.5 are attained within 2 – 3 days (Lássen, 1995b). Simultaneously, a sharp decrease in redox potential (Eh) takes place within the ensiled fish material (Lássen, 1995b). Under these conditions, fish protein undergoes a certain degree of proteolysis, yielding short peptides and free amino acids (Haard *et. al.*, 1985). Certain microorganisms possess decarboxylase enzymes and are capable of converting the final products of proteolysis into biogenic amines such as histamine, tyramine, cadaverine or

putrescine (Baranowski, 1985, Halász *et. al.*, 1994). Amino acid decarboxylation is favoured under acidic, reducing conditions such as those prevailing in fish silage (Beutling, 1992). Accumulation of biogenic amines could thus occur, rendering the product unsafe for animal consumption (Krizek, 1991).

Among the bacterial genera found to produce biogenic amines in fish, *Morganella morganii* is reported to produce high amounts of these potential toxins (Ieniştea, 1971,Wei *et. al.*, 1990, Kim *et. al.*, 2000). Lactic acid bacteria isolated from fermenting mackerel pastes have also been demonstrated to produce one or more biogenic amines (Dapkevicius *et. al.*, 2000). Their proliferation in the silage may be difficult to control, since they belong to the same bacterial group as the starter microorganisms and have, thus, similar growth requisites.

Fermentation temperature is a key factor to ensure the fast fermentation required to stabilise biological fish silage (Fagbenro and Jauncey, 1993b). It also plays a very important role in determining the type of microorganisms that will predominate in the silage, by affecting their growth parameters.

Sodium chloride and potassium sorbate are sometimes used as additives for ensiling fish. Both have been found to help control biogenic amine accumulation in some fermented foods such as miso (Chin and Koehler, 1986) and sausages (Shalaby and Abd El-Rahman, 1995) and in culture media (Chander *et. al.*, 1989, Gardini *et al.*, 2001). Lactic acid bacteria are tolerant to acid (Adams and Nicolaides, 1997), but *M. morganii* has been found to be relatively sensitive to acid (this thesis, chapter 4) and might be controlled by the acidic conditions within the silage.

The present work aims at studying the effect of the above mentioned conditions (temperature, redox potential, sodium chloride, potassium sorbate and pH) on the growth parameters of biogenic amine producing microorganisms of concern for the ensilage of fish.

MATERIALS AND METHODS

Bacterial cultures

Growth properties were assessed for five isolates of biogenic amine producing bacteria, *Lactobacillus sakei* 15.19, *Weissella hellenica* 15.32, *Enterococcus* sp. 22.12 (Dapkevicius *et. al.*, 2000), *Lactobacillus büchneri* 2A (Joosten and Northolt, 1987), and *Morganella morganii* 6675 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The first three strains were chosen among eight histamine-producing LAB isolated from naturally fermented mackerel sugar pastes, representing the main bacterial groups found. *L. büchneri* was chosen for comparison purposes and *M. morganii* was included in the tests due to its important role in histamine accumulation in scombroid fish (Kim *et al.*, 2000).

Growth and acidification testing

Growth and acidification experiments for lactic acid bacteria were performed in ten modifications of the MRS medium (Merck, 10611, Darmstadt, Germany) at 30, 35, 37 or 40°C, as follows:

- MRS medium
- MRS medium with 2% or 4% (w/w) NaCl added
- MRS with 0.1 or 0.2% (w/w) K-sorbate added
- MRS with 0.05% (w/w) cysteine added
- MRS with 0.05% (w/w) cysteine and 2 or 4% NaCl added
- MRS with 0.05% (w/w) cysteine and 0.1 or 0.2% K-sorbate added.

M. morganii was cultivated in Brain Heart Infusion (BHI, OXOID, CM225, Basingstoke, UK) modified in a similar way to the above mentioned MRS modifications. Furthermore, growth of this microorganism was also assessed at pH 5.0, 4.5 and 4.0.

When applicable, the pH of the media was adjusted to the desired values (6.0, 5.0, 4.5, and 4.0) by adding 4 N NaOH or HCl. 250-ml portions of the broths were stored for 16 – 18h at the relevant temperature and subsequently inoculated to a level of about 10^4 CFU ml^{-1} with a 16 – 18h old culture of each of the strains.

Growth was monitored by monitoring the increase in optical density (O. D.) at 620 nm in a Microtiter Reader (340 ATTC, SLT-Labinstruments, Austria). The wells in the microtiter reader plates were filled with 150µl of medium inoculated with the desired strain until O.D. 0.070±0.007 and covered with 50µl sterile mineral oil. Four differently positioned wells were filled for each strain and each medium. Incubation at the desired temperature (30, 35, 37 or 40°C) was done in the microtiter reader, using the thermostatic device of the apparatus. Measurements were done every 30 min. Samples were automatically agitated by the apparatus before each measurement.

Modelling

Modelling of the growth and pH decrease curves was carried out with the modified Gompertz equation, which was fitted to the data by nonlinear regression with a Marquardt algorithm, according to Zwietering *et al.* (1990). Bacterial counts were expressed as O. D. as a function of time (h). Values for lag time λ (h), maximum specific growth rate μ (ΔO. D. h^{-1}), maximum O. D. reached *A* and their 95% confidence intervals were calculated in this way.

Statistical analysis

The results obtained in all experiments were submitted to ANOVA (Gomez and Gomez, 1983). Separation of the means was performed by the Duncan test (Duncan, 1951).

RESULTS AND DISCUSSION

Tables 1 – 5 present the growth parameters calculated from the obtained experimental data for each of the strains. The maximum population levels (*A* values) are not shown and will not be discussed under the scope of the present work. High optical density values cannot be used because photometric determinations of bacterial cell densities show a poor correlation with actual numbers of living cells.

Temperature

The most favourable temperature for the growth of *Lb. sakei* 15.19 was 35°C. At this temperature, μ was significantly ($p<0.05$) higher and λ significantly ($p<0.05$) shorter. Growth was significantly ($p<0.05$) inhibited at 40°C, with significantly ($p<0.05$) lower growth rate and/or significantly ($p<0.05$) longer lag times. The same was observed for *W. hellenica* 15.32. Growth of *Lb. büchneri* 2A was also significantly ($p<0.05$) inhibited at 40°C. Its highest μ values and lowest λ values were at 35°C. In most cases, growth was significantly ($p<0.05$) better at 35°C than at all other temperatures. No significant ($p<0.05$) difference was observed, however, in the cysteine + 4% NaCl formulation.

Isolate 22.12 displayed significantly ($p<0.05$) better growth at 37°C than at all other temperatures tested. At this temperature, λ values were always significantly ($p<0.05$) shorter and μ values were higher. Concerning the μ values, the difference between values obtained at 35 and 37°C was significant ($p<0.05$) for MRS only, in the absence of cysteine, and for all formulations except the 4% NaCl, in the presence of cysteine. *M. morganii* also displayed the lowest growth rate and highest λ at 40°C. Differences between the values obtained at 40°C and at the other temperatures were significant ($p<0.05$). The highest growth rate occurred at 35°C. However, no significant ($p<0.05$) differences were found between μ values obtained at 30 and 35°C in MRS + 2% NaCl (both in the presence and in the absence of cysteine) and in MRS + 4% NaCl (just in the absence of cysteine). Differences between the μ values obtained at 35 and 37°C were not significant ($p<0.05$) for 2% NaCl (both in the presence and in the absence of cysteine) and for 0.1% K-sorbate (just in the absence of cysteine).

Temperatures in the range of 35 – 37°C have been found to be most appropriate for the ensilage of fish (this thesis, chapters 3 and 7). At these temperatures, the studied biogenic amine producing bacteria grow also well and may pose potential problems.

Table 1. Growth parameters (μ and λ) for *Lactobacillus sakei* 15.19 grown in modified MRS media, at different temperatures. Values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.05$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.05$).

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.145 ± 0.003^{Aa}	0.201 ± 0.016^{Ba}	0.154 ± 0.007^{Ba}	0.128 ± 0.006^{Ca}
+2% NaCl	0.193 ± 0.006^{Ab}	0.210 ± 0.004^{Ba}	0.176 ± 0.009^{Cb}	0.159 ± 0.012^{Cb}
+4% NaCl	0.089 ± 0.006^{Ad}	0.111 ± 0.013^{Ab}	0.103 ± 0.017^{Bc}	0.045 ± 0.004^{Bc}
+0.1% K sorbate	0.096 ± 0.007^{Acd}	0.151 ± 0.005^{Bc}	0.147 ± 0.007^{Aad}	0.101 ± 0.007^{Cf}
+0.2% K sorbate	0.087 ± 0.002^{Ad}	0.147 ± 0.012^{Bc}	0.139 ± 0.004^{Cd}	0.059 ± 0.003^{Dg}
+0.05% cysteine	0.251 ± 0.008^{ABe}	0.261 ± 0.007^{Ad}	0.242 ± 0.009^{Be}	0.210 ± 0.005^{Ce}
+0.05% cysteine+2% NaCl	0.216 ± 0.003^{Af}	0.209 ± 0.004^{ABa}	0.205 ± 0.004^{Bf}	0.154 ± 0.007^{Cb}
+0.05% cysteine+4% NaCl	0.105 ± 0.005^{Ac}	0.115 ± 0.005^{Bb}	0.111 ± 0.002^{Ac}	0.061 ± 0.020^{Ccg}
+0.05% cysteine +0.1% K-sorbate	0.182 ± 0.011^{Abg}	0.212 ± 0.004^{Ba}	0.160 ± 0.015^{Aa}	0.111 ± 0.010^{Aaf}
+0.05% cysteine +0.2% K-sorbate	0.166 ± 0.007^{Ag}	0.180 ± 0.006^{Be}	0.147 ± 0.007^{Ca}	0.082 ± 0.006^{Ad}
	λ (h)			
MRS	2.347 ± 0.109^{Ad}	1.651 ± 0.090^{Bd}	2.460 ± 0.160^{Ad}	2.517 ± 0.092^{Ac}
+2% NaCl	2.494 ± 0.088^{Ad}	1.719 ± 0.133^{Bd}	3.011 ± 0.096^{Ce}	3.394 ± 0.161^{Dd}
+4% NaCl	5.984 ± 0.124^{Ac}	4.153 ± 0.380^{Bc}	5.090 ± 0.442^{Bc}	6.271 ± 0.185^{Ab}
+0.1% K sorbate	2.992 ± 0.042^{Aa}	2.156 ± 0.145^{Ba}	2.750 ± 0.090^{Ca}	4.643 ± 0.031^{Ag}
+0.2% K sorbate	4.522 ± 0.036^{Ab}	3.011 ± 0.096^{Bf}	3.500 ± 0.115^{Cb}	4.978 ± 0.083^{Aa}
+0.05% cysteine	2.861 ± 0.055^{Ae}	2.358 ± 0.073^{Ba}	2.508 ± 0.126^{Bad}	4.251 ± 0.034^{De}
+0.05% cysteine+2% NaCl	5.402 ± 0.070^{Af}	3.738 ± 0.098^{Cb}	3.993 ± 0.052^{Bc}	5.072 ± 0.154^{Ca}
+0.05% cysteine+4% NaCl	6.232 ± 0.168^{Ac}	5.064 ± 0.812^{Be}	5.124 ± 0.378^{Cc}	6.411 ± 0.301^{Db}
+0.05% cysteine +0.1% K-sorbate	$4.079\pm.0.023^{Cf}$	2.783 ± 0.232^{Baf}	3.699 ± 0.092^{Cb}	5.104 ± 0.224^{Da}
+0.05% cysteine +0.2% K-sorbate	4.739 ± 0.205^{Abg}	3.156 ± 0.028^{Bb}	4.035 ± 0.100^{Cf}	6.471 ± 0.098^{Db}

Table 2. Growth parameters (μ and λ) for *Weissella hellenica* 15.32 grown in modified MRS media, at different temperatures. Values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.05$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.05$).

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.146±.001	0.189±0.012	0.156±0.002	0.068±0.004
+2% NaCl	0.169±0.002	0.203±0.014	0.181±0.011	0.123±0.007
+4% NaCl	0.082±0.005	0.107±0.006	0.099±0.004	0.041±0.010
+0.1% K sorbate	0.125±0.006	0.172±0.054	0.135±0.088	0.037±0.005
+0.2% K sorbate	0.084±0.004	0.161±0.003	0.108±0.003	0.018±0.002
+0.05% cysteine	0.237±0.003	0.254±0.003	0.244±0.005	0.235±0.017
+0.05% cysteine+2% NaCl	0.207±0.005	0.216±0.004	0.205±0.005	0.148±0.014
+0.05% cysteine+4% NaCl	0.085±0.004	0.114±0.03	0.104±0.009	0.047±0.011
+0.05% cysteine +0.1% K-sorbate	0.196±0.001	0.209±0.006	0.193±0.017	0.097±0.004
+0.05% cysteine +0.2% K-sorbate	0.172±0.005	0.175±0.008	0.155±0.009	0.047±0.002
	λ (h)			
MRS	2.495±0.136	1.896±0.092	2.566±0.340	4.524±0.088
+2% NaCl	4.245±0.135	2.632±0.091	2.961±0.179	4.932±0.234
+4% NaCl	5.480±0.184	3.973±0.247	4.861±0.471	5.816±0.196
+0.1% K sorbate	2.800±0.097	2.129±0.066	2.920±0.240	5.222±0.091
+0.2% K sorbate	3.138±0.293	2.490±0.086	3.174±0.149	5.260±0.225
+0.05% cysteine	3.099±0.046	2.558±0.042	2.625±0.153	4.940±0.043
+0.05% cysteine+2% NaCl	4.589±0.060	3.717±0.195	3.940±0.08	4.936±0.137
+0.05% cysteine+4% NaCl	5.887±0.303	5.225±0.183	5.620±0.356	6.219±0.192
+0.05% cysteine +0.1% K-sorbate	3.933±0.071	3.319±0.175	3.355±0.066	5.968±0.340
+0.05% cysteine +0.2% K-sorbate	4.000±0.103	3.368±0.175	3.699±0.066	6.610±0.293

Table 3. Growth parameters (μ and λ) for isolate 22.12 (*Enterococcus faecium* species) grown in modified MRS media, at different temperatures. Values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.05$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.05$).

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.137±0.002	0.153±0.041	0.180±0.004	0.077±0.001
+2% NaCl	0.103±0.003	0.138±0.007	0.150±0.006	0.044±0.008
+4% NaCl	0.075±0.003	0.103±0.008	0.113±0.015	0.029±0.008
+0.1% K sorbate	0.125±0.003	0.145±0.005	0.163±0.041	0.072±0.006
+0.2% K sorbate	0.077±0.006	0.121±0.005	0.129±0.008	0.036±0.008
+0.05% cysteine	0.154±0.006	0.168±0.003	0.192±0.008	0.100±0.003
+0.05% cysteine+2% NaCl	0.171±0.015	0.183±0.006	0.212±0.006	0.146±0.003
+0.05% cysteine+4% NaCl	0.106±0.012	0.111±0.012	0.124±0.016	0.081±0.006
+0.05% cysteine +0.1% K-sorbate	0.150±0.012	0.162±0.003	0.178±0.009	0.099±0.010
+0.05% cysteine +0.2% K-sorbate	0.124±0.011	0.129±0.004	0.169±0.008	0.059±0.002
	λ (h)			
MRS	3.060±0.231	2.822±0.059	1.829±0.112	3.554±0.153
+2% NaCl	3.805±0.119	4.355±0.124	2.671±0.085	6.630±0.237
+4% NaCl	5.310±0.107	5.794±0.064	3.985±0.133	7.022±0.300
+0.1% K sorbate	3.075±0.077	3.101±0.157	2.054±0.076	3.685±0.155
+0.2% K sorbate	3.147±0.069	3.453±0.187	3.004±0.096	5.362±0.123
+0.05% cysteine	3.489±0.022	3.006±0.055	2.111±0.473	4.822±0.075
+0.05% cysteine+2% NaCl	5.358±0.060	4.649±0.064	3.631±0.109	6.865±0.393
+0.05% cysteine+4% NaCl	5.870±0.200	6.166±0.200	5.321±0.187	11.568±0.782
+0.05% cysteine +0.1% K-sorbate	4.313±0.167	3.902±0.098	2.852±0.153	5.033±0.035
+0.05% cysteine +0.2% K-sorbate	4.460±0.153	4.125±0.140	3.106±0.148	5.697±0.271

Table 4. Growth parameters (μ and λ) for *Lactobacillus büchneri* 2A grown in modified MRS media, at different temperatures. Values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.05$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.05$).

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
MRS	0.140±0.005	0.206±0.005	0.176±0.005	0.115±0.003
+2% NaCl	0.125±0.010	0.154±0.008	0.145±0.016	0.110±0.003
+4% NaCl	0.085±0.009	0.131±0.005	0.101±0.007	0.077±0.009
+0.1% K sorbate	0.111±0.004	0.183±0.006	0.120±0.008	0.077±0.006
+0.2% K sorbate	0.072±0.007	0.110±0.008	0.093±0.008	0.060±0.004
+0.05% cysteine	0.195±0.005	0.245±0.017	0.207±0.021	0.145±0.011
+0.05% cysteine+2% NaCl	0.139±0.024	0.198±0.006	0.167±0.009	0.124±0.001
+0.05% cysteine+4% NaCl	0.108±0.012	0.113±0.009	0.109±0.009	0.079±0.011
+0.05% cysteine +0.1% K-sorbate	0.189±0.007	0.196±0.007	0.175±0.015	0.117±0.014
+0.05% cysteine +0.2% K-sorbate	0.149±0.010	0.157±0.004	0.129±0.008	0.075±0.011
	λ (h)			
MRS	3.304±0.055	2.705±0.206	2.735±0.148	4.125±0.148
+2% NaCl	5.018±0.051	3.139±0.217	5.363±0.123	6.437±0.055
+4% NaCl	6.005±0.129	5.424±0.381	5.459±0.173	7.936±0.185
+0.1% K sorbate	4.313±0.137	2.852±0.154	3.453±0.187	4.822±0.075
+0.2% K sorbate	4.460±0.140	3.106±0.153	4.125±0.148	5.033±0.035
+0.05% cysteine	3.060±0.231	2.478±0.021	2.684±0.134	3.902±.0098
+0.05% cysteine+2% NaCl	4.576±0.133	3.035±0.365	4.077±0.758	5.363±0.163
+0.05% cysteine+4% NaCl	5.752±0.302	4.929±0.139	4.978±0.284	7.263±0.345
+0.05% cysteine +0.1% K-sorbate	3.147±0.069	2.766±0.313	2.852±0.098	3.685±0.076
+0.05% cysteine +0.2% K-sorbate	3.508±0.067	3.101±0.155	3.404±0.068	3.554±0.153

Table 5. Growth parameters (μ and λ) for *Morganella morganii* 6675 DSMZ grown in modified BHI, at different temperatures. Values are averages ± standard deviations of four samples. Within the same row, the values that display identical capital letters do not differ significantly ($p<0.05$). Within the same column, values displaying the same small letters do not differ significantly ($p<0.05$).

Growth medium	Growth temperature (°C)			
	30	35	37	40
	μ (ΔO. D. h^{-1})			
BHI	0.064±0.010	0.081±0.005	0.070±0.001	0.052±0.003
+2% NaCl	0.039±0.004	0.041±0.002	0.041±0.001	0.020±0.001
+4% NaCl	0.031±0.008	0.037±0.001	0.029±0.003	NG
+0.1% K sorbate	0.103±0.002	0.118±0.002	0.113±0.013	0.023±0.002
+0.2% K sorbate	0.098±0.007	0.119±0.006	0.102±0.001	NG
+0.05% cysteine	0.053±0.004	0.061±0.002	0.053±0.004	0.034±0.001
+0.05% cysteine+2% NaCl	0.013±0.003	0.013±0.002	0.011±0.002	0.001±0.003
+0.05% cysteine+4% NaCl	NG	NG	NG	NG
+0.05% cysteine +0.1% K-sorbate	NG	NG	NG	NG
+0.05% cysteine +0.2% K-sorbate	NG	NG	NG	NG
	λ (h)			
BHI	2.554±0.048	1.676±0.048	2.624±0.201	3.647±0.078
+2% NaCl	3.697±0.257	9.985±0.156	10.325±0.252	NG
+4% NaCl	2.711±0.082	2.446±0.076	3.028±0.018	12.095±0.969
+0.1% K sorbate	3.689±0.070	3.548±3.548	3.731±3.731	7.596±0.079
+0.2% K sorbate	4.661±0.324	3.830±0.034	4.080±0.005	NG
+0.05% cysteine	4.163±0.131	3.520±0.057	3.786±0.038	4.302±0.080
+0.05% cysteine+2% NaCl	12.258±1.058	7.941±0.413	11.054±0.226	12.599±0.300
+0.05% cysteine+4% NaCl	NG	NG	NG	NG
+0.05% cysteine +0.1% K-sorbate	NG	NG	NG	NG
+0.05% cysteine +0.2% K-sorbate	NG	NG	NG	NG

NG = no growth.

Cysteine addition

Decreasing the Eh value of the growth medium by means of cysteine addition resulted in increased growth rate for *Lb. sakei* 15.19. The difference was, in most cases, significant ($p<0.05$). However, it also brought about a slight but significant ($p<0.05$) increase in λ values. The same was observed for *W. hellenica* strains 15.32 and 22.12. *Lb. büchneri* displayed higher μ values in the presence of cysteine, but the difference was not always significantly ($p<0.05$) higher. The λ values for this strain decreased in the presence of cysteine, but the difference was not always significant ($p<0.05$). At 35°C, no significant ($p<0.05$) differences were observed. At 40°C, all differences were significant ($p<0.05$). At 30°C, there were no significant ($p<0.05$) differences between MRS and MRS + cysteine, as well as between MRS + 4% NaCl and MRS + cysteine + 4% NaCl. At 37°C, all formulations except MRS and MRS + cysteine differed significantly ($p<0.05$). Cysteine addition significantly ($p<0.05$) inhibited the growth of *M. morganii* in BHI. Very little growth occurred in the presence of cysteine and 2% NaCl and no growth was observed in media containing cysteine and 4% NaCl or cysteine and potassium sorbate (at both concentrations studied).

Eh values of –200 mV (obtained by cysteine addition) led to inhibition of the growth by *M. morganii*, but not of any of the LAB strains under study, making the latter of special concern for the safety of fish silage.

Sodium chloride

Adding 4% NaCl to the growth medium always inhibited the growth of *Lb. sakei* 15.19. In media containing 2% NaCl, lag times were significantly ($p<0.05$) longer. The effect of adding 2% NaCl on the growth rate depends on the Eh of the medium. In the absence of cysteine, the growth rate increased, whereas in cysteine containing media a decrease is observed. The same was observed for 15.39. For 22.12, an increase in μ was observed in the presence of cysteine (except at 37°C; at this temperature, the difference was not significant $p<0.05$). Both 2 and 4% NaCl significantly ($p<0.05$) inhibited the growth of *Lb. büchneri*. NaCl addition inhibited the growth of *M. morganii*. Both a significant ($p<0.05$) increase in the lag time and a significant ($p<0.05$) decrease in the growth rate were observed. When combined with low Eh (added cysteine), no growth was observed. NaCl inclusion in biological fish silage (BFS) formulas may help controlling the growth of biogenic amine producing microorganisms. It can, however, also inhibit growth and acidification by some of the potential starter strains (this chapter 7). Choosing a starter that can grow and acidify well in the presence of NaCl might reduce this problem. NaCl inhibition of biogenic amine producing bacteria has already been reported in the literature (Gardini *et al.*, 2001). Other authors have reported inhibition of biogenic amine production, but not of bacterial growth. They have attributed the lower concentrations of biogenic amines obtained in the presence of

NaCl to inhibition of the microbial decarboxylase enzymes (Chin and Koehler, 1986).

Potassium sorbate

The growth of *Lb. sakei* 15.19 was inhibited by K-sorbate, as it can be seen from the longer lag times and lower growth rates obtained. In most cases, the difference between growth parameters obtained in sorbate containing broths and in MRS or MRS + cysteine was significant ($p<0.05$). The same was observed for *W. hellenica* 15.32. The growth of isolate 22.12 was significantly ($p<0.05$) inhibited by adding 0.2% K-sorbate to the basal formula of the MRS medium. Adding 0.1% led to significantly ($p<0.05$) lower μ only at 30°C, in the absence of cysteine. In all other cases, the differences were not significant ($p<0.05$). The duration of the lag phase was also increased, but differences were significant ($p<0.05$) only in cysteine containing broth formulations. Potassium sorbate inhibited the growth of *Lb. büchneri* 2A. The differences were not significant ($p<0.05$) at 30 and 37°C. The effect of K-sorbate on the growth rate of *M. morganii* depended on the Eh of the medium. If cysteine was absent, a significant ($p<0.05$) increase in μ was observed, with the exception of media containing 0.2% K-sorbate when incubated at 40°C. Under these conditions, no growth was observed. *M. morganii* did not grow when K-sorbate was added to cysteine containing BHI. When growth did occur, K-sorbate addition resulted in significantly ($p<0.05$) longer lag times. Potassium sorbate inhibited the growth of the biogenic amine producing microorganisms, but it had also been found to inhibit the growth of potential starter LAB in previous work (this thesis, chapter 7). Its use in BFS to control biogenic amine formation is, thus, not practical.

Effect of pH on the growth of *M. morganii*

The effect of pH on growth was tested only for *M. morganii*, since the acid-resistant character of LAB is well-established (Adams and Nicolaides, 1997). Figure 1 shows the effect of pH, cysteine and temperature on the growth parameters of *M. morganii*. When the pH of the medium was adjusted to 4.0, no growth was observed at any of the studied temperatures. At pH 4.5, growth occurred only at 30°C in the absence of cysteine. At all other temperatures, no growth occurred. A decrease in pH from 6.0 to 5.0 led to a strong, significant ($p<0.05$) decrease in μ values at all studied temperatures. Cysteine addition led to very marked, decreases in μ values and significantly ($p<0.05$) longer lag times at all tested temperatures and pH values. *M. morganii* showed to be considerably sensitive to decreasing pH values, especially when the Eh of the medium is low, and would, thus, be inhibited under the conditions prevailing in a successful BFS.

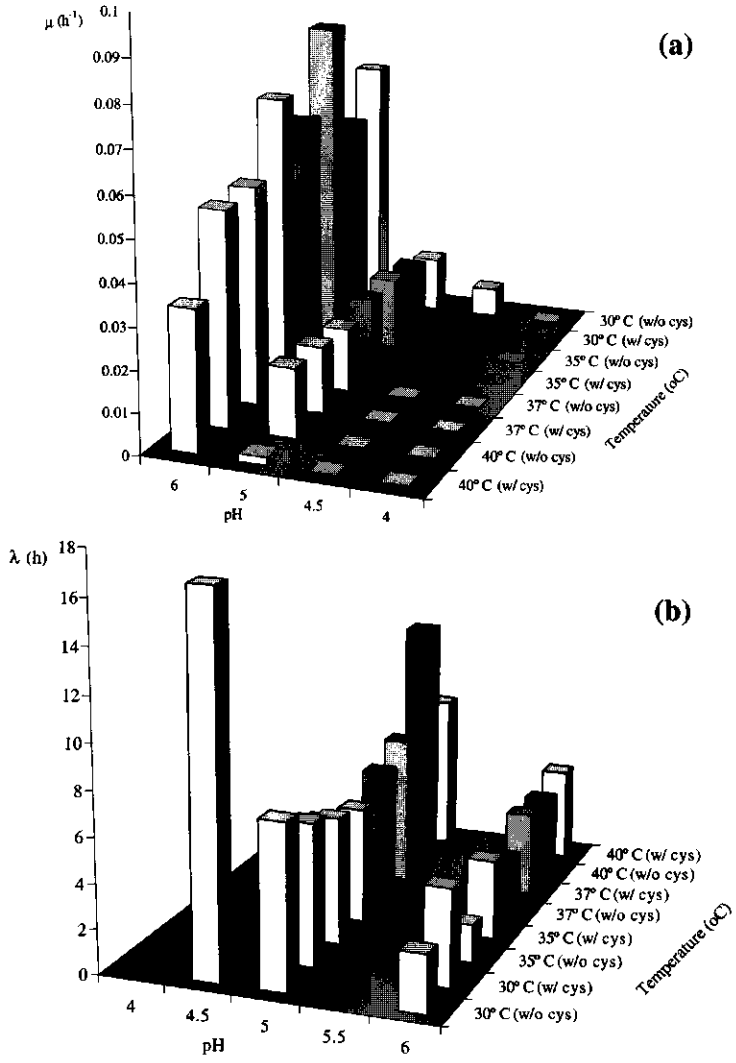

Fig. 1. Effect of temperature and pH on growth by *Morganella morganii* 6675 DSMZ. The values shown are averages of four samples. (a) Effect on growth rate. (b) Effect on lag time.w/ cys = with cysteine w/o cys = without cysteine

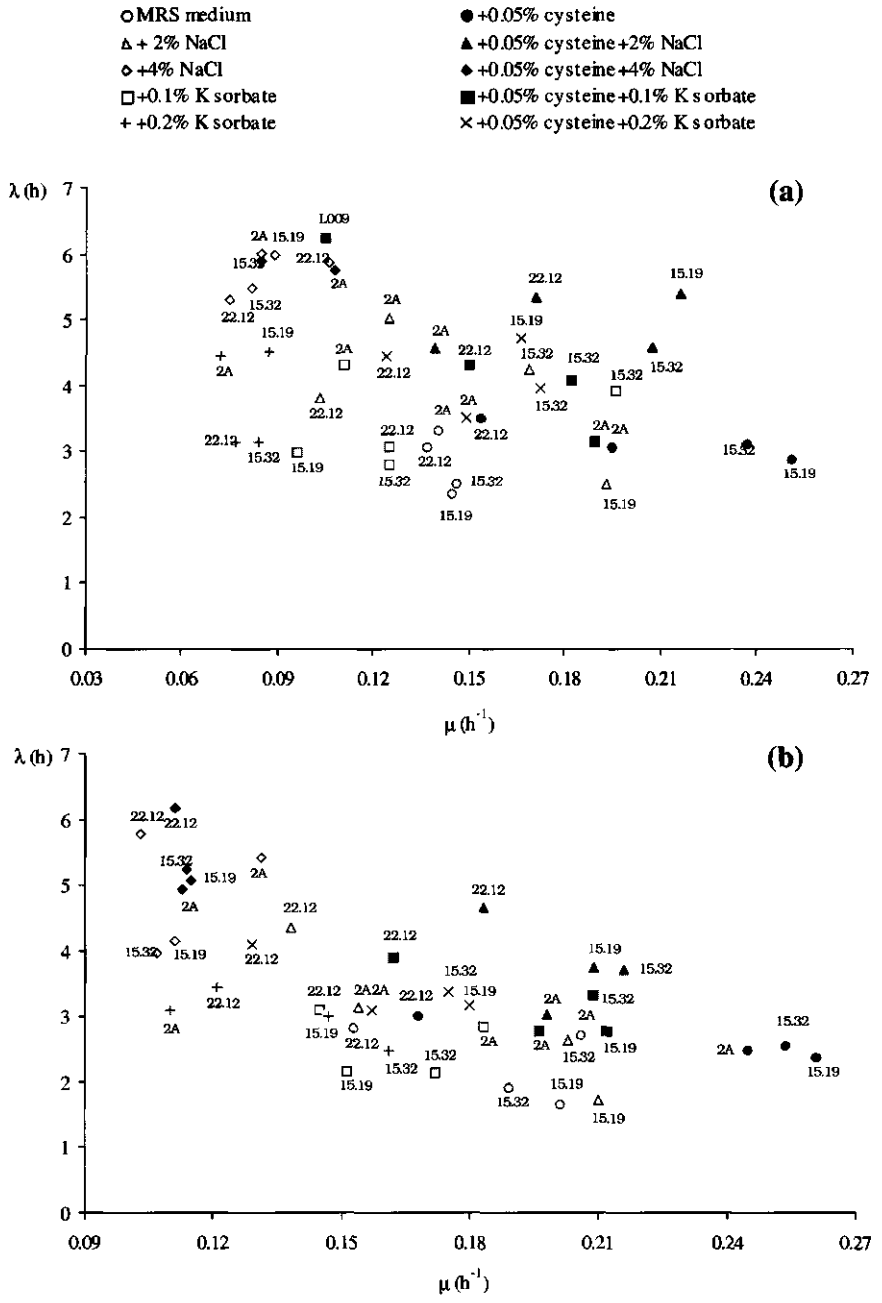

Fig. 2. Effect of ten medium formulations on μ and λ values of five strains of biogenic amine producing LAB upon incubation at different temperatures. The values shown are the averages of four determinations. (a) 30°C, (b) 35°C, (c) 37°C, (d) 40°C.

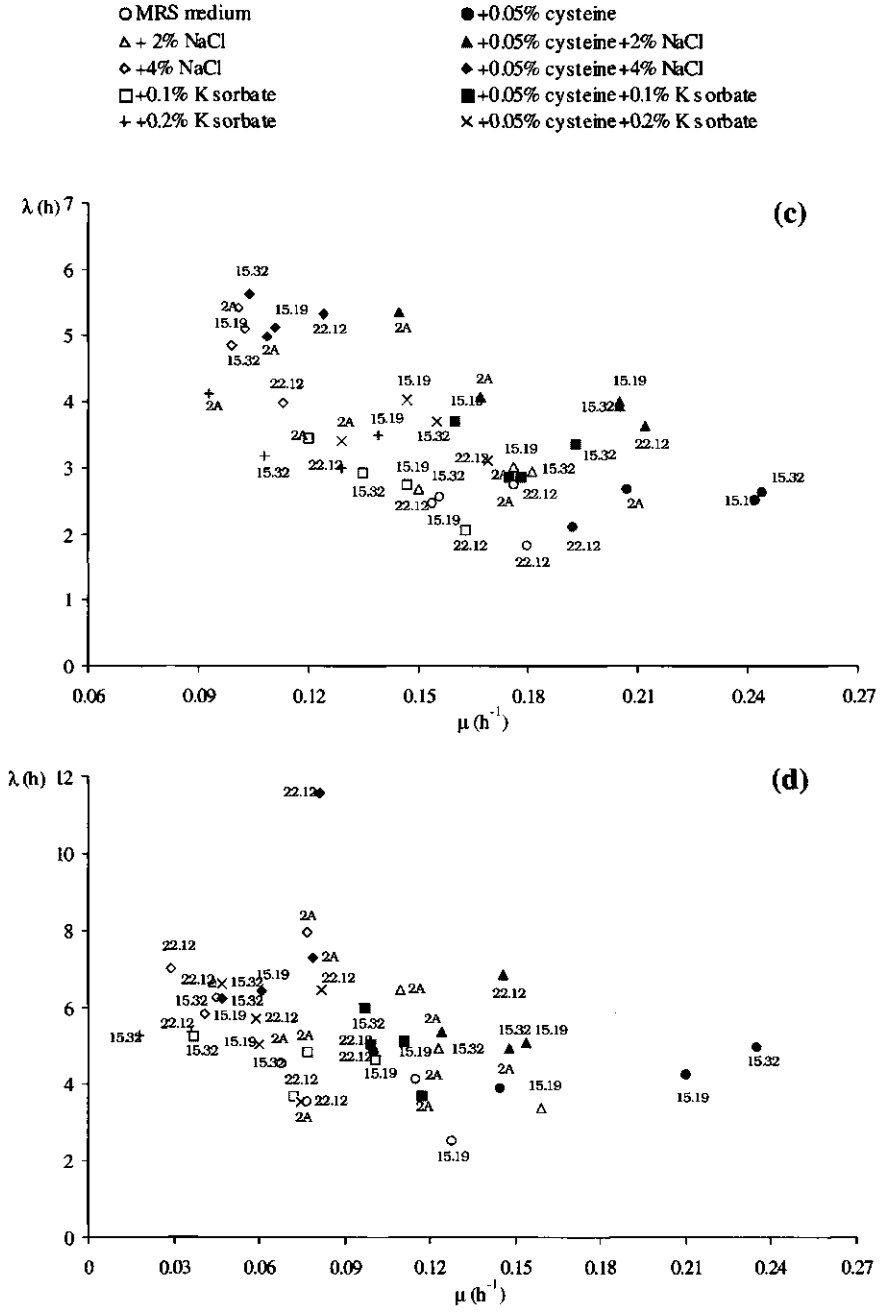

Fig. 2. (continuation)

Bacterial species

This discussion deals only with the results obtained for the LAB species. A comparison between the growth parameters obtained for *M. morganii* and the LAB is not included, since the growth media used for these two types of isolates differed. Figure 2 compares the growth properties of all LAB at the four temperatures tested. All strains but isolate 22.12 grew best in MRS medium with added cysteine. Isolate 22.12 had the highest μ values in MRS with added 2% NaCl, while its shortest lag times were obtained in MRS + cysteine. Reduced Eh favoured the growth of these strains, but in most cases, adding either NaCl or K-sorbate caused considerable growth inhibition. The highest μ values and shortest lag times corresponded, at all temperatures, to *Lb. sakei* 15.19 and *W. hellenica* 15.32.

CONCLUSIONS

Some authors have suggested that the main biological feature influencing biogenic amine formation is the extent of growth of microorganisms possessing decarboxylase activity (Yoshinaga and Frank, 1982, Gardini *et* al., 2001). Our data indicate that the growth of *M. morganii* will be efficiently inhibited under the ensilage conditions. The decrease in pH and Eh values and the presence of NaCl or K-sorbate (at reduced Eh) resulted in very little or no growth at all. The biogenic amine producing LAB, however, were able to grow well under conditions similar to those prevailing in the fish silage. Low Eh (-200 mV) did not inhibit their growth. Sodium chloride and potassium sorbate led, however, to some growth inhibition. Potassium sorbate was also found to inhibit potential starter LAB (this thesis, chapter 7), so its use as a selective agent against biogenic amine producing LAB is of no practical interest. Sodium chloride however, did not inhibit the growth of all potential starters (this thesis, chapter 7) and could, thus, be used as an additional hurdle, provided that an adequate halotolerant starter is used. Furthermore, two of the potential starters were shown to possess antimicrobial activity against the biogenic amine producing LAB (this thesis, chapter 4). The use of such starters for BFS production could provide an additional means of controlling growth of the undesirable LAB.

Chapter 9

General discussion

This thesis deals with the optimisation of safety and functionality aspects of the biological ensilage of fish. Knowledge in this area is rather scattered. Many important questions remain unclear. The aim of this work was to fill voids in the knowledge on the biological ensilage of fish.

Stabilisation of biological fish silage (BFS) by the starter LAB

Preservation of fish or fish residues by the biological ensilage process is based on the preservative effect of lactic acid bacteria (LAB). A quick pH decrease and a fast growth by the LAB starter strain are of utmost importance to ensure efficient competition with undesirable microorganisms. Fermentation temperature has been found to affect largely the growth and acidification of the substrate by potential starters, both in model systems (chapters 6 and 7) and in ensiled fish (chapter 3). In order to obtain a fast enough pH decrease, fermentation temperatures have to be close to the optimum temperature for the starter strains. Within the group of potential starter strains tested, the most appropriate temperatures for ensiling were found to be 35 – 37°C, which is in accordance with the findings of other authors (Ahmed and Mahendrakar, 1996a). However, at this temperature range, the growth of biogenic amine producing microorganisms is favoured too. Other means of controlling their growth and deleterious effect must be used to ensure high quality final products.

The starter strain also exerts a large influence on the outcome of the ensilage (Van Wyk and Heydenrych, 1985). Natural fermentation or back-slopping did not yield stable products in our case (chapter 4). Inoculation with an appropriate culture was regarded as necessary. We found that a strain with a short acidification lag time, such as *Lb. plantarum* 009, is most suitable (chapters 3 and 7). Ensiling with strains that show very rapid growth and that have good antibacterial properties, such as *Lb. curvatus* 15.35, may also yield stable silages although the pH decrease may be slower and higher final pH values may result.

Mixed-strain cultures are sometimes used by the food industry (Geisen and Holzapfel, 1996). The potential of mixed-strain cultures for BFS preparation has been investigated in chapter 6. Combinations of *Lb. curvatus* with *Lb. sakei* strains were tested. *Lb. curvatus* 15.35 showed bactericidal effect against all of the tested *Lb. sakei* strains, while *Lb. curvatus* 22.24 led to growth inhibition of all *Lb. sakei* strains tested, except *Lb. sakei* 15.05. Acidification was also not improved by the

use of culture combinations. Furthermore, culture combinations in which one of the strains had diamine oxidase activity did not result in histamine degradation (chapter 5). Mixed-strain starters based on the studied isolates were not regarded as suitable for the intended purpose.

Effect of potential additives and ensilage conditions on silage stability and functionality

For the sake of economy and simplicity of the process, BFS is usually made with minimal usage of additives. Sodium chloride, an antifungal agent (propionic acid or sorbate) and/or antioxidants have sometimes been used. Antioxidant usage in fish silage is not dealt with in this thesis. Sodium chloride has been investigated as a potential additive to fish silage. Concentrations of 2% NaCl (w/w) afforded some protection of the protein fraction in fish silage, while allowing the starter microorganisms to grow and produce acid rapidly enough to ensure that pH values below 4.5 were obtained within a reasonable delay (within 2 days). When 4% NaCl (w/w) were added however, starter growth and acid production were negatively affected and the corresponding silages showed signs of deterioration (chapter 3). NaCl has been found to delay starter growth and to decrease growth rates in most cases. In the case of *Lb. plantarum* 009, however, it was possible to use 2% NaCl (chapter 7). Furthermore, NaCl inhibited to some extent the growth of biogenic amine producing LAB (chapter 8).

Propionic acid did not show effective antifungal action (chapter 3). Potassium sorbate was found to possess also some inhibitory effect towards biogenic amine producing bacteria (chapter 8). However, it also inhibited significantly growth and acidification by all studied potential starters (chapter 7). It could therefore lead to eventual stability problems in BFS.

The effect of the reduced Eh on both potential starter strains and biogenic amine producing bacteria was studied in chapters 7 and 8. *M. morganii* showed scant growth in low Eh model systems. The effect of low Eh on potential starter or biogenic amine producing LAB is strain dependent. Strains that were inhibited in growth or acidification in model systems (*Lb. sakei* strains 15.05 and 15.36) may not be capable to lower Eh sufficiently. Thus, they may not be adequate as starters for the biological ensilage of fish.

Microbiological safety of fish silage

Biological fish silage has been regarded as a microbiologically safe product (Raa and Gildberg, 1982). The low final pH obtained, the antimicrobial action of undissociated molecules of lactic acid and other antimicrobial metabolites produced by LAB would ensure the inhibition or destruction of pathogenic organisms within the silage (James and Nair, 1977, Raa and Gildberg, 1982). The discovery of new, acid-resistant strains of pathogens such as *E. coli* O157:H7 may pose however, a new potential risk in this area. The LAB cultures tested in this

work as potential BFS starters inhibited growth of all studied pathogens (*L. monocytogenes* Scott A, *Salmonella*, and three strains of *E. coli* O157:H7) when used as inhibitor strains in the well-assay test. The antimicrobial action was attributed to the production of organic acids (this thesis, chapter 4). Although no challenge tests were performed, it may be presumed that these pathogens would be inhibited during the course of BFS production.

Potential toxicological risks associated with BFS

The environmental conditions prevailing within fish silage may also pose the potential risk of biogenic amine build-up (Haard *et al.*, 1985, Beutling, 1992). The presence of biogenic amines in fish silage may render it unfit for animal consumption (Krizek, 1991). Histamine has been a cause of concern in fish products for human and animal consumption. Histamine formation in fermented fish products occurs mainly as a result of microbial degradation of the amino acid histidine (Silla-Santos, 1996). Several potential BFS starter strains were tested for their antimicrobial activity against biogenic amine producing strains of *M. morganii*, *E. coli*, *Lb. büchneri* B300 and LAB isolated from naturally-fermented mackerel:sucrose pastes (*Lb. sakei* 15.19, *W. hellenica* 15.32, and *Enterococcus* sp. 22.12). Inhibition of *E. coli*, *M. morganii*, *Lb. büchneri*, *W. hellenica* and *Enterococcus* was common among the potential starter strains studied. However, biogenic amine producing *Lb. sakei* 15.19 was inhibited only by *Lb. plantarum* 009 and *Lb. curvatus* 15.35 (chapter 4).

Another potential risk associated with the preservation of fish residues for animal consumption is presented by the possible formation of toxic metabolites derived from the oxidation-prone, highly unsaturated fish lipids. The protective effect of lactic fermentation on food lipids has been documented (Bonestroo, 1992). Chapter 2 investigates the potential protective effect of lactic acid fermentation on fish lipids. Ensiling by the biological process led to considerable decreases in peroxide values when compared with the acid method. In spite of a report describing delay of lipid oxidation by formaldehyde in fish silage (Haard *et al.*, 1985) this compound led, in the present work, to an increased peroxide value in the oil extracted from acid silages.

Protein degradation and its control in BFS

When fish silage is to be used as a source of organic nitrogen for animal feeding, the extent of proteolysis is a main concern. Animals may use nitrogen sources in which protein has been degraded to a certain extent, but when too large a proportion of protein has been converted to non-protein nitrogen, finally even resulting in a breakdown of amino acids, adverse effects may occur both in monogastrics and ruminants (Ørskov, 1977, Espe *et al.*, 1992a). It is possible to arrest proteolysis in acid fish silage by adding formaldehyde (chapter 2). The use of this additive may, however, be objectionable (Haard *et al.*, 1985). When compared

to the acid ensilage method, BFS was also advantageous in this aspect, since it yielded considerably lower NPN values (chapter 3). Sodium chloride addition (2%, w/w) also led to lower NPN values, as well as to the formation of lower amounts of TVBN.

Physical changes within biological fish silage

The most obvious physical change that takes place during the ensilage of fish is the gradual liquefaction. Fish silage liquefies as a result of proteolysis and loss of water-binding capacity of the fish muscle (Hall *et al.*, 1985a, Jangaard, 1987). Although related, proteolysis and liquefaction are not completely overlapping (Haard *et al.*, 1985, Jangaard, 1987). Liquefaction is considered as completed when viscosity decreases below 5 $kg.m^{-1}s^{-1}$ (Haard *et al.*, 1985). Proteolysis may, however, proceed long after this viscosity level is reached (Haard *et al.*, 1985).

Fish silage is a non-homogenous product. Stratification upon storage or centrifugation has been described. A top layer of oil, followed by an intermediate liquid layer, and a bottom layer of sedimented solids have been observed (Mc Bride *et al.*, 1961, Espe *et al.*, 1992a). We found evidence that phenomena other than proteolysis may be involved in the formation of liquid in fish silage (chapter 3). When acid and biological fish silage materials were centrifuged at time = 0h, at 25 000 g, a liquid layer of different specific gravity was formed. In the presence of acids, the amount of liquid layer obtained upon centrifugation was very large when compared to that obtained from non-acidified biological silages. The addition of NaCl strongly decreased the formation of the liquid layer, leading to supernatant weights close to zero. The abrupt decrease in pH obtained by direct acid addition will decrease water-binding capacity of the major myofibrillar proteins (as described in Chapter 3) and NaCl addition changes the configuration of muscle proteins increasing their water-binding properties. The observed phenomena were previously described for meats from warm-blooded animals (Lawrie, 1991).

Conclusions

The biological ensilage has been found advantageous when compared to the acid process, since it yielded lower levels of peroxides in the silage oil and led to lower NPN values.

The most important factors in achieving stable BFS are a suitable fermentation temperature and an appropriate choice of starter. Temperatures of 35 – 37°C were regarded as most suitable from the point of view of starter growth and acidification. Strains with a short acidification lag time, such as *Lb. plantarum* 009, or with very fast growth and good antibacterial properties, such as *Lb. curvatus* 15.35, are most suitable. Combinations of strains did not yield benefits in terms of strain growth or acidification and were not regarded as suitable for the intended purpose.

BFS appeared to be microbiologically safe. Fermentation by LAB was shown to efficiently inhibit pathogens that had not yet been assessed in the context of fish silage, such as *L. monocytogenes* and *E. coli* O157:H7 as well as some spoilage microorganisms. Biogenic amine producing bacteria could pose safety risks. There were, however, potential starter strains that were able to inhibit growth by these bacteria. Histamine degradation by starter strains was also tested as an innovative means of ensuring low levels of this amine in the fermented product under study. Several strains were found to degrade histamine as single strain cultures and could find application in fish silage and other fish products in which histamine build-up poses a risk.

Sodium chloride (2% w/w) could be of value as an additive for BFS production. It is cheap and easily available. Furthermore, it inhibits growth by the biogenic amine producing bacteria studied, but starter strains can be found that are not affected by NaCl addition. NaCl additionally decreased NPN and TVBN values in BFS.

Propionic acid did not efficiently inhibit fungal growth in BFS. Inclusion of K-sorbate in the mixture was shown to inhibit growth and/or acidification by starter LAB, and could therefore bring about stability problems when used in BFS preparation.

References

Abdoul-Raouf, U. M., Beuchat, L. R. and Ammar, M. S. 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. *Appl. Environ. Microbiol.* **8**: 2364-2368.

Adams, M. R., Cooke, R. D. and Twiddy, D. R. 1987. Fermentation parameters involved in the production of lactic acid preserved fish-glucose substrates. *Int. J. Food Sci. and Technol.* **22**: 105-114.

Adams, M. R. and Nicolaides, L. 1997. Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control* **8** (5/6) 227-239.

Ahmed, J. and Mahendrakar, N. S. 1996a. Chemical and microbial changes in fish viscera during fermentation ensiling at different temperatures. *Bioresource Technol.* **59**: 45-46.

Ahmed, J. and Mahendrakar, N. S. 1996b. Growth and meat quality of broiler chicks fed with fermented fish viscera silage. *Int. J. Anim. Sci.* **11**: 1-5.

Ahmed, J. and Mahendrakar, N. S. 1996c. Autolysis and rancidity development in tropical freshwater fish viscera during fermentation. *Bioresource Technol.* **58**: 247-251.

Ahmed, J. and Mahendrakar, N. S. 1996d. Acceleration of fish viscera silage by prefermented starter culture. *Irish J. Agric. Food Res.* **35**: 171-177.

Ahmed, J., Ramesh, B. S., and Mahendrakar, N. S. 1996. Changes in microbial population during fermentation of tropical freshwater fish viscera. *J. Appl. Bacteriol.* **80**: 153-156.

Ali, M. Z., Gheyasuddin, S., Zaher, M., Hossain, M. A. and Islam, M. N. 1994. Evaluation of fish silage prepared from underutilized marine fishes as protein sources in the diet of major carp (*Cirrhinus mrigala*). *J. Aquac. Trop.*, **9**: 247-254.

Almås, K. A. 1990. *In*: Advances in Fisheries Technology and Biotechnology. Ed. Voight, M. N. and Botta, J. R., Technomic Publishing Co., Inc., Lancaster, Basel. p. 361-372.

Ang, J. F. and Hultin, O. H. 1989. Denaturation of cod myosin during freezing after modification with formaldehyde. *J. Food Sci.*, **4**: 814-818.

Anglesea, J. D. and Jackson, A. J. 1985. Thiaminase activity in fish silage and moist fish feed. *Anim. Feed Sci. and Technol.* **13**: 39-46.

Anon. 1988. Pescado. Determinação do teor de azoto básico volátil total (A. B. V. T.). Método de Conway. *Norma Portuguesa NP 2930.* Instituto Português de Qualidade. Lisbon.

AOAC. 1984. Official methods of Analysis of the Association of Official Analytical Chemistry. 14th Edition. Washington, D.C.

AOAC. 1990. Official Methods of Analysis, 15th ed. Association of Official Analytical Chemists, Washington, DC.

AOCS. 1993. cd 8-53. Official Methods and Recommended Practices of the American Oil Chemists'Society, 2p., Washington, D. C.

Aryanta, R. W., Fleet, G. H. and Buckle, K. A. 1991. The occurrence and growth of microorganisms during the fermentation of fish sausage. *Int. J. Food Microbiol.* **13**: 143-156.

Åsgård, T. and Austreng, E. 1981. Fish silage for salmonids: a cheap way of utilizing waste as feed. *Feedstuffs* **53**: 22-24.

Åsgård, T. and Austreg, E. 1985. Dogfish offal, ensiled or frozen, as feed for salmonids. *Aquacult.* **49**: 289-305.

Austreng, E. and Åsgård, T. 1986. Fish silage and its use. "Trend and Problems in Aquaculture". Proc. 2nd Int. Conf. Aquafarming, Verona, Italy. p. 26-229.

Backhoff, H. P. 1976. Some chemical changes in fish silage. *J. Food Technol.* **11**: 353-363.

Baranowski, J. 1985. Assay for histidine decarboxylase activity. *In*: Histamine in marine products: production by bacteria, measurement and prediction of formation. Ed. Pan, B. S., James, D.

Batista, I., Mendes, R. and Nunes, M. C. 1987. Ensilados ácidos e biológicos: preparação e estudo das alterações químicas ocorridas durante a armazenagem. Seminário sobre Aquacultura, Porto, 9-11 Abril 87.

Batterham, E. S., Gorman, T. B. S. and Chvojka, R. 1983. Nutritional value and mercury content of fish silage for growing pigs. *Anim. Feed Sci. Technol.* **9**: 169-179.

Beal, C., Louvet, P. and Courrieu, G. 1989. Influence of controlled pH and temperature on the growth and acidification of pure cultures of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398. *Appl. Microbiol. Biotechnol.*, **32**: 148-154.

Ben Embarek, P. K. 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. *Int. J. Food Microbiol.* **23**: 17-34.

Benjamin, M. and Datta, A. R. 1995. Acid tolerance of enterohaemorrhagic *E. coli*. *Appl. Environm. Microbiol.* **4**: 1669-1672.

Beutling, D. 1992. Prüfung von Starterorganismen auf Ihre Befähigung zur Bildung von Histamine und Tyramine. *Mh. Vet.-Med.* **47**: 587-591.

Bibek, R. 1992. Foods and microorganisms of concern. *In*: Food biopreservatives of microbial origin. Ed. Bibek, R. and Daeschel, M. CRC Press, Mexico. p. 25-27.

Bibek R. and Daeschel M. 1992. *In*: Food biopreservatives of microbial origin. Ed. Bibek, R and Daeschel M. CRC Press, Mexico. Preface.

Bigueras-Benitez, C. M. and Nacorda, H. M. E. 1992. Evaluation of fish silage as a protein source for poultry. *In*: Papers presented at the 8th Session of the IPFC Working Party on Fish Technology and Marketing, Yogjakarta, Indonesia, 24-27 September 1991. FAO Indo-Pacific Fisheries Committee, Bangkok, Thailand. . FAO FIIU/R470, Suppl. FAO, Rome, Italy, pp. 148-154.

Bligh, E.G. and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**: 911-917.

Bonestroo, M. H. 1992. Development of fermented sauce-based salads. Assessment of safety and stability. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Børrensen, T. 1992. *In*: Seafood Science and Technology. Ed. By Bligh, E. G. Fishing News Books, p. 278-288.

Brown, N. and Sumner, J. 1985. Fish silage. FAO Fisheries Report No. 317-Suppl., p. 404-412.

Buckenhüskes, H. J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiol. Rev.* **12**: 253-272.

Cassens, R. G., Greaser, M. L., Ito, T. and Lee, M. 1979. Reactions of nitrite in meat. *Food Technol.* **33**(7): 46-57.

Chander, H., Batish, V. K., Babu, S. and Singh, R. S. 1989. Factors affecting amine production by a selected strain of *Lactobacillus bulgaricus*. *J. Food Sc.* **54**: 940-942.

Chikindas, M. L., García Garcerá, M. J., Driessen, A. J. M., Ledeboer, A. M., Nissen-Meyer, J., Nes, I., Abee, T., Konings, W. N. and Venema, G. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl. Environm. Microbiol.* **59**: 3577-3584.

Chin, K. D. H. and Koehler, P. E. 1986. Effect of salt concentration and incubation temperature on formation of histamine, phenethylamine, tryptamine and tyramine during miso fermentation. *J. Food Prot.* **49**: 423-427.

Chirase, N. K., Moha, K. and Males, J. R. 1985. Fish silage for improving the nutritional value of wheat straw. *J. Anim. Sci.* **61**: 661-669.

Clausen, T.N., Damgaard, B.M., Borsting, C.F. and Hejlesen, C. 1999. Fatty and defatted herring scrap fed to mink (*Mustela vison*) in the reproduction and nursing periods. *Canadian J. Anim. Sci.* **79**: 457-465.

Collins, C. H., Lyne, P. M. and Grange, J. M. 1989. Collins and Lyne's Microbiological Methods, 6th Ed. Butterworth & Co., U. K.

Conner, D. E. and Kotrola, J. S. 1995. Growth and survival of *E. coli* O157:H7 under acidic conditions. *Appl. Environm. Microbiol.* **61**(1): 382-385.

Cooke, R. D., Twiddy, D. R. and Reilly, P. J. A. 1987. Lactic acid fermentation as a low-cost means of food preservation in tropical countries. *FEMS Microbiol. Rev.* **46**: 369-379.

Costilow, R. N. W., Ferguson, W. E. and Ray, S. 1955. Sorbic acid as a selective agent in cucumber fermentations. *Appl. Microbiol.* **3**: 341-345.

Daeschel, M. 1992. Procedures for the detection of antimicrobial activity of microorganisms. *In*: Food biopreservatives of microbial origin. Ed. Bibek, R. and Daeschel, M. CRC Press, Mexico. p. 57-81.

Daeschel, M. and Penner M. H. 1992. Hydrogen peroxide, lactoperoxidase system and reuterin. *In*: Food biopreservatives of microbial origin. Ed. Bibek, R. and Daeschel, M. CRC Press, Mexico. p. 155-177.

Dapkevicius, M. L. N. E., Batista, I., Nout, M. J. R., Rombouts, F. M. and Houben, J. H. 1998. Lipid and protein changes during the ensilage of blue whiting *Micromesistius poutassou* Risso) by acid and biological methods. *Food Chem.* **63**: 97-102.

Dapkevicius, M. L. N. E., Nout, M. J. R., Rombouts, F. M., Houben, J. H. and Wymenga, W. 2000. Biogenic amine formation and degradation by potential fish silage starter microorganisms. *Int. J. Food Microbiol.* **57**: 107-114.

Dave, R. I. and Shah, N. P. 1998. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *J. Dairy Sci.* **81**: 2804-2816.

Davidson, P. M. 1997. Chemical preservatives and natural antimicrobial compounds. *In:* Doyle, M. P, Beuchat, L. R., and Montville, T. J. (eds.) Food Microbiology: Fundamentals and Frontiers. American Society for Microbiology, U. S. A., p. 520-556.

Delort-Laval, J. 1985. Chemical treatments of oil meals. Abstract of Papers. Work Conference on Emerging Technologies in Fats and Oils Industry. (Cannes: AOCS). Ed. A. R. Baldwin, pp. 218-220.

Djadjasewaka and Djadjadiredja, R. 1980. Fish silage as a feed for freshwater fish. *In*: Fish silage production and its use. Papers presented at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 September, FAO Fisheries Report No. 230, Ed. Disney, J. G. and James, D., FAO, Rome, Italy. p. 74-76.

Dong, F. M., Fairgrieve, W. T., Skonberg, D. I. and Rasco, B. A. 1993. Preparation and nutrient analysis of lactic acid bacterial ensiled salmon viscera. *Aquacult.* **109**: 351-366.

Dossmann, M. U., Vogel, R. F. and Hammes, W. P. 1996. Mathematical description of the growth of *Lactobacillus sake* and *Lactobacillus pentosus* under conditions prevailing in fermented sausages. *Appl. Microbiol. Biotechnol.* **46**: 334-339.

Drebot, M. 1987. Rapport sur l'ensilage du poisson. *In*: Atelier sur l'ensilage du poisson. Ministère des Pêches et Océans, Canada, p. 79-87.

Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometr.* **11**: 1-42.

El-Hili, H. A. 1989. Effets d'une alimentation à base d'ensilage chez la daurade. *Bull. Inst. Natl. Science Technol. Océanogr. Pêche Salammbo.* **16**: 55-63.

Espe, M. and Haaland, H. 1992. The protein value of fish silage prepared from capelin stored under different conditions before ensiling. Effect of storing the silage for one year. *Fisk. Dir. Skr. Ser Enoering* **5**: 37-44.

Espe, M., Haaland, H. and Njaa, L. R. 1992a. Substitution of fish silage protein and a free amino acid mixture for silage protein and a free amino acid mixture for fish meal protein in a chicken diet. *J. Sci. Food Agric.* **58**: 315-319.

Espe, M., Haaland, H., Njaa, L. R. and Raa, J. 1992b. Growth of young rats on diets based on fish silage with different degrees of hydrolysis. *Food Chem.*, **44**: 195-200.

Espe, M., Raa, J. and Njaa, L. R. 1989. Nutritional value of stored fish silage as a protein source for young rats. *J. Sci. Food Agric.* **49**: 259-270.

Fagbenro, O. A. and Bello-Olusoji, A. 1997. Preparation, nutrient composition and digestibility of fermented shrimp head silage. *Food Chem.* **60**: 489-493.

Fagbenro, O. and Jauncey, K. 1993a. Chemical and nutritional quality of stored fermented fish (tilapia) silage. *Bioresource Technol.* **46**: 207-211.

Fagbenro, O. and Jauncey, K. 1993b. Chemical and nutritional quality of raw, cooked and salted fish silages. *Food Chem.* **48**: 331-335.

Fagbenro, O. and Jauncey, K. 1994a. Chemical and nutritional quality of fermented fish silage containing potato extracts, ginger or formalin extracts. *Food Chem.* **50**: 383-388.

Fagbenro, O. and Jauncey, K. 1994b. Growth and protein utilisation by juvenile catfish (*Clarias gariepinus*) fed moist diets containing autolysed protein from stored lactic-acid-fermented fish-silage. *Bioresource Technol.* **48**: 43-48.

Fagbenro, O. A. and Jauncey, K. 1998. Physical and nutritional properties of moist fermented fish silage pellets as a protein supplement for tilapia (*Oreochromis niloticus*). *Anim. Feed Sci. Technol* **71**: 11-18.

Faid, M., Karani, H., Elmarrakchi, A. and Achkari-Begdouri, A. 1994. A biotechnological process for the valorization of fish waste. *Bioresource Technol.* **59**: 237-241.

Faid, M., Zouiten, A., Elmarrakchi, A. and Achkari-Begdouri, A. 1997. Biotransformation of fish waste into a stable feed ingredient. *Food Chem.* **60**: 13-18.

Fontenot, J. P., Ayangbile, G. A., Samuels, W. A., Abanzige, M. D. A. and Allen, V. G. 1992. Ensiled seafood processing wastes and crop residues as feeds for ruminants. Pres. at Aquaculture '92, Orlando, FL (USA) 21-25 May 1992. *In*: Aquaculture '92: Growing toward the 21st century. World Aquaculture Society, USA. P. 96.

Freeman, H. C. and Hoogland, P. L. 1956. Processing of cod and haddock viscera:1. Laboratory experiments. *J. Fisheries Research Board Canada* **13**: 869-877.

Garbutt, J. 1997. Essentials of Food Microbiology. Arnold, London, UK. Chapter 6. Factors affecting the growth of micro-organisms, p. 84.

Gardini, F., Martuscellli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E. and Suzzi, G. 2001. Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of Enterococcus faecalis. *Int. J. Food Microbiol.*,. **64**: 105-117.

Gardner, H. W. 1978. Lipid hydroperoxides and their degradation. . *In:* Encyclopedia of Food Science. Ed. Peterson, M. S. and Johnson, A. H. AVI Publishers, Westport, Connecticut, U. S. A., p. 467.

Garvie, E. L. 1984. Genus *Pediococcus* Claussen. *In*: Bergey's Manual of Systematic Bacteriology, Vol. 2. Ed. Sneath P. H. A., Mair N. S., Sharpe, M. E. and Holt, J. G. Williams and Wilkins, Baltimore, p. 1208-1234.

Geisen, R. and Holzapfel, W. 1996. Genetically modified starter and protective cultures. *Int. J. Food Microbiol.* **30**: 315-324.

Gendron, A., Bryl, P. and Nicol, F. 1987. Possibilités offertes par l'ensilage de poisson au Québec. In: Anon., 1987. Rapport, Attelier sur l'ensilage du poisson. Pointe-de-l'Église, Nouvelle-Écosse, 16-17 juin, 1987. Min. Pêches Océans, Canada, séries Éducation Gén. n°7, p. 94-98.

Gibson, A. M. and Roberts, T. A. 1986. The effect of pH, water activity, sodium nitrite and storage temperature on the growth of *Clostridium perfringens* and faecal streptococci in laboratory media. *Int. J. Food Microbiol.* **3**: 195-210.

Gildberg, A. and Raa, J. 1977. Properties of a propionic acid/formic acid preserved silage of cod viscera. *J. Sci. Food Agric.* **2**: 467-653.

Giurca, R., Levin, R. E. 1992. Optimization of the lactic acid fermentation of hydrolyzed cod gurry with molasses. *J. Food Biochem.* **16**: 83-97.

Gomez, K. and Gomez, A. 1983. Statistical Procedures for Agricultural Research, 2nd ed. John Wiley & Sons, New Yourk, USA.

Gourama, H. and Bullerman, L. B. 1995. Antimycotic and antiaflatoxinogenic effect of lactic acid bacteria: a review. *J. Food Prot.* **58**: 1275-1280.

Gouygou, J. P., Sinquin, C. and Durand, P. 1987. High pressure liquid chromatography of histamine in fish. *J. Food Sci.* **4**: 925-927.

Gram, L. and Huss, H. H. 1996. Microbiological spoilage of fish and fish products. *Int. J. Food Microbiol.* **33**: 121-137.

Green, S. 1989. Fish silage is an easily produced protein source. *Pigs* **5**: 30-31.

Green, S., Wiseman, J. and Cole, D. J. A. 1988. Examination of stability, and its effect on the nutritive value, of fish silage in diets for growing pigs. *Anim. Feed Sci. Technol.* **21**: 43-56.

Guevara, Y. J., Bello, R. A. and Montilla, J. J. 1991. Evaluación del ensilado de pescado elaborado por via microbiologica como suplemento proteinico en dietas para pollos de engorde. *Arch. Latinoam. Nutrición* **41**: 246-256.

Haaland, H. and Njaa, L. R. 1989. Total volatile nitrogen – a quality criterion for fish silage? *Aquacult.* **79**: 31-36.

Haaland, H., Espe, M. Njaa, L. F. and Myklestad, H. 1990. Chemical composition and variation in some parameters during storage of 8 formic acid silages prepared from capelin. *Fisk. Dir. Skr., Ser. Enoering,* **3**: 59-74.

Haard, N. F., Kariel, N., Herzberg, G., Feltham, L. A. W. and Winter, K. 1985. Stabilisation of protein and oil in fish silage for use as a ruminant feed supplement. *J. Sci. Food Agric.* **36**: 229-241.

Halász, A., Baráth, A., Simon-Sarkadi, L. and Holzapfel, W. 1994. *Trends Food Sci. Technol.* **5**: 42-49.

Hall, G. M., Keeble, D., Ledward, D. A. and Lawrie, R. A. 1985a. Silage from tropical fish 1. Proteolysis. *J. Food Technol.* **20**: 561-572.

Hall, G. M., Ledward, D. A. and Lawrie, R. A. 1985b. Silage from tropical fish 2. Undigested fraction. *J. Food Technol.* **20**: 573-580.

Hamden, I. Y., Deane, D. D. and Kunsman, J. E. 1971. Effect of potassium sorbate on yogurt cultures. *J. Milk Food Technol.* **34**: 307.

Hammes, W. P. 1990. Bacterial starter cultures in food production. *Food Biotechnol.* **4**: 383-397.

Hammoumi, A., Faid, M., El yachioui, M. and Amarouch, H. 1998. Characterization of fermented fish waste used in feeding trials with broilers. *Process Biochem.*, **33**: 423-427.

Hardy, R. W., Shearer, K. D., Stone, F. E. and Wieg, D. H. 1983. Fish silage in aquaculture diets. *J. World Maricult. Soc.* **14**: 695-703.

Hardy, R. W., Shearer, K. D. and Spinelli, J. 1984. The nutritional properties of co-dried fish silage in rainbow trout (*Salmo gairdneri*) dry diets. *Aquacult.* **38**: 35-44.

Hassan, T. E. and Heath, J. L. 1986. Biological fermentation of fish waste for potential use in animal and poultry feeds. *Agric. Wastes* **12**: 1-15.

Hassan, T. E. and Heath, J. L. 1987. Chemical and nutritive characteristics of fish silage produced by biological fermentation. *Biol. Wastes* **20**: 18-21.

Houtsma, P. C., de Wit, J. C. and Rombouts, F. M. 1993. Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. *Int. J. Food Microbiol.* **20**: 247-257.

Hsieh, R. J. and Kinsella, J. E. 1989. Oxidation of polyunsaturated fatty acids: mechanisms, products, and inhibition with emphasis on fish. *Adv. Food Nutr. Res.* **33**: 233-339.

Ienişţea, C. 1971. Bacterial production and destruction of histamine in foods, and food poisoning caused by histamine. *Nahrung* **15**: 109-113.

Ingram, M. and Kitchell, A. G. 1967. Salt as a preservative for foods. *J. Food Technol.* **2**:1.

Jack, R. W., Tagg, J. R. and Bibek, R. 1995. Bacteriocins of Gram-positive bacteria. *Microbiol. Rev.* **59**: 171-200.

Jackson, A. J., Kerr, A. K. and Bullock, A. M. 1984a. Fish silage as a dietary ingredient for salmon. II. Preliminary growth findings and nutritional pathology. *Aquacult.* **40**: 283-291.

Jackson, A. J., Kerr, A. K. and Cowey, C. B. 1984b. Fish silage as a dietary ingredient for salmon. I. Nutritional and storage characteristics. *Aquacult.* **38**: 211-220.

Jackson, J. O. 1996. The Grim Sweepers. Giant high-tech vessels roam the world's waters, scooping up their once bottomless bounty. *Time* **148**: 58-61.

Jackson, T. C., Acuff, G. R. and Dickson, J. S. 1997. Meat, Poultry and Seafood. *In*: Food Microbiology. Fundamentals and Frontiers. Ed. Doyle, M. P., Beuchat, L. R., and Montville, T. J., ASM Press, Washington D. C., USA, pp.83-100.

James, M. A. and Nair, M. R. 1977. Comparative study of fish ensilage prepared by microbial fermentation and formic acid ensilage. Proc. Conf. on Handling, Processing and Marketing of Tropical Fish. Tropical Products Institute, London, UK, p. 273-275.

Jangaard, P. M. 1987. Le poisson ensilé: historique et développements récents. *In*: Anon., 1987. Rapport, Attelier sur l'ensilage du poisson. Pointe-de-l'Église, Nouvelle-Écosse, 16-17 juin, 1987. Min. Pêches Océans, Canada, séries Éducation Gén. n°7, p. 8-37.

Jay, J. M. 2000. Modern Food Microbiology. 6th Ed. Aspen Publ., Inc., Gaithersburg, Maryland, USA.

Jeppesen, V. F. and Huss, H. H. 1993. Characteristics and antagonistic activity of lactic acid bacteria isolated from chilled fish products. *Int. J. Food Microbiol.* **18**: 305-320.

Johnsen, F. and Skrede, A. 1981. Evaluation of fish viscera silage as a feed resource. Chemical characteristics. *Acta Agric. Scand.* **31**: 22-28.

Johnson, R. J., Brown, N., Eason, P. and Sumner, J. 1985. The nutritional quality of two types of fish silage for broiler chickens. *J. Sci. Food Agric.* **36**: 1051-1056.

Joosten, H.M. L. J. and Northolt, M. D. 1987. Conditions allowing the formation of biogenic amines in cheese; decarboxylative properties of some non-starter bacteria. *Neth. Milk Dairy J.* **41**: 259-280.

Jyhshiun, L. Lee, I.-S., Frey, J. Slonczewski J. L. and Foster, J. W. 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. *J. Bacteriol.* **117**: 4097-4101.

Kandler, O. and Weiss, N. 1984. Genus *Lactobacillus* Beijerinck 1901, 212. *In* Bergey's Mannual of Systematic Bacteriology (Vol. 1). Ed. Holt, J. G. Publ.) Williams and Wilkins, Baltimore, USA. Pp. 1209-1234.

Kherrati, B., Faid, M., Elyachioui, M. and Wahmane, A. 1998. Process for recycling slaughterhouses wastes and by-products by fermentation. *Bioresource Technol.* **63**: 75-79.

Kim, S.-H., Ben-Gingirey, B., Barros-Velázquez, J. Price, J. and Haejumg, A. 2000. Histamine and Biogenic Amine Production by *Morganella morganii* Isolated from temperature-abused albacora. *J. Food Prot.* **63**(2): 244-251.

Kjos, N. P., Skrede, A. and Øverland, M. 1999. Effects of dietary fish silage and fish fat on growth performance and sensory quality of growing-finishing pigs. *Canadian J. Anim. Sci.* **79**: 139-147.

Kompiang, I. P., Yushadi, S. and Cresswell, D. C. 1980. Microbial fish silage: chemical composition, fermentation characteristics and nutritional value. *In*: Fish silage production and its use. Ed. Disney, J. G. and James, D. FAO Fisheries Report No. 230, Food and Agriculture Organisation of the United Nations, Rome, Italy. p. 38-43.

Kreuzer, R. 1954. Untersuchungen zur Durchfürung der Fischgärungssilage. *Arch. für Fish.* **5**: 47.

Kritzinger, N. M., Wessels, J. P H., Potgieter, B. J. and Atkinson, A. 1979. Fish silage as a protein source in the diets of baconer pigs. Abstract of Progress Report No. 197, FIRI Annual Report, p. 69.

Krizek, M. 1991. The determination of biogenic amines in silage. *Arch. Anim. Nutr., Berlin* **1**: 97-104.

Kumagai, H., Matsui, H., Koichi, O. and Yamada, H. 1969. Properties of crystalline tyramine oxidase from *Sarcina lutea*. *Biochim. Biophys. Acta* **17**: 1-8.

L'Aventure, J. 1987. Essais sur l'ensilage du poisson. In: Atelier sur l'ensilage du poisson. Ministère des Pêches et Océans, Canada.

Lahsen, A. 1991. Histamine food poisoning: and update. *Fish Technol. News.* **11**: 3-5.

Lall, S. 1987. Le poisson ensilé: aliment pour le saumon et la truite. *In*: Atelier sur l'ensilage du poisson. Min. des Pêches et Océans, Canada, p. 90-91.

Lamprecht, E., Post, E., van Wyk, H. J. and Riley, F. R. 1982. A preliminary report on the preparation of naturally-fermented fish silage. 36th Annual Report of the Director – FIRI, p. 49-50.

Lapie, L. P. and Bigueras-Benitez, C. M. 1992. Feeding studies on tilapia (*Oreochromis* sp.) using fish silage. *In*: Papers presented at the 8th Session of the IPFC Working Party on Fish Technology and Marketing, Yogjakarta, Indonesia, 24-27 September 1991. FAO Indo-Pacific Fisheries Committee, Bangkok, Thailand. Publ, by: FAO, Rome, Italy, pp. 165-177. FAO FIIU/R470, Suppl.

Larson, R. A. 1988. The antioxidants of higher plants. *Phytochem.* **27**: 969-978.

Lássen, T. M. 1995a. Biological quality of fermented fish offal and chicken by-products. *Agric. Sci. Finland* **4**: 27-33.

Lássen, T. M. 1995b. Evaluation of conditions for fermentation of fish offal. *Agric.Sci. Finland* **4**:11-17.

Lássen, T. M. 1995c. Lactic acid fermentation of fish offal and chicken by-product with different starter cultures. *Agric. Sci. Finland* **4**:19-26.

Lawrie, R. A. 1991. Water-holding capacity and juiciness. *In* Meat Science. 5th Ed. Pergamom Press, Oxford, England, p.191-197.

Leuschner, R. G., Heidel, M. and Hammes, W. P. 1998. Histamine and tyramine degradation by food fermenting microorganisms. *Int. J. Food Microbiol.* **39**: 1-10.

Levin, R. E. and Witkowski, R. 1991. Characteristics and identity of obligately aerobic spoilage yeasts from fish silage. *J. Appl. Bacteriol.* **71**: 354-359.

Lieber, E. R. and Taylor, S. L. 1978. Thin-layer chromatographic screening methods for histamine formation in tuna fish. *J. Chrom.* **153:** 143-152.

Lindgren, S. and Pleje, M. 1983. Silage fermentation of fish or fish waste products with lactic acid bacteria. *J. Sci. Food Agric.* **34**: 1057-1067.

Lindgren, S. and Refai, O. 1984. Amylolytic lactic acid bacteria in fish silage. *J. Appl. Bacteriol.* **57**: 221-228.

Lin, T. M. and Park, J. W. 1998. Solubility of salmon myosin as affected by conformational changes at various ionic strengths and pH. *J. Food Sci.* **63**: 215-218.

Lin, M. Y. and Yen, C. L. 1999. Reactive oxygen species and lipid peroxidation product-scavenging ability of yogurt organisms. *J. Dairy Sci.* **82**: 1629-1634.

Lisac, H. 1961. The liquid ensilage of mediterranean sardines for animal feeding. *Proc. Gen. Fish. Council Mediterranean*, **6**: 111-119.

Lo, K. U., Liao, P. H., Bullock, C. and Jones, Y. 1993. Silage production from salmon mortalities. *Aquacult. Eng.* **12**: 37-45.

Lovern, J. A. 1965. Trash fish – is there money in it? *World Fishing*, June **65**: 87-89.

Lupín, H. M. 1983. Ensilado biológico de pescado. Una propuesta para la utilización de residuos de la pesca continental en América Latina. Paper pres. at Seminario sobre manipuleo, processamiento, mercadeo y distribución de los productos de la pesca continental en América Latina. COPESCAL, México, 12p., il.

Luten, J. B., Bouquet, W., Seuren, L. A. J., Burggraaf, M. M., Riekwel -Booy, G., Durand, P., Etienne, M., Gouygou, J. P., Landrein, A., Ritichie, A., Leclerq, M. and Guinet, R. 1991. Biogenic amines in fishery products: standardization methods within EC. In H. H. Huss *et al.* (eds.), Quality assurance in the fish industry. Amsterdam, Elsevier Science publishers.

Machin, D. H., Panigrahi, S., Bainton, J. and Morris, T. R. 1990. Performance of broiler chicken fed on low and high oil fish silages in relation to changes taking place in lipid and protein components. *Anim. Feed Sci. Technol* **28**: 199-223.

Mackie, I. M., Hardy, R. and Hobbs, G. 1971. Fermented fish products. FAO Fisheries Reports, No. 100, Food and Agriculture Organisation of the United Nations, Rome, Italy. 54 p.

Maijala, R., Ereola, S., Lievonen, S., Hill, P. and Hirvi, T. 1995. Formation of biogenic amines during ripening of dry sausages as affected by starter culture and thawing time of raw materials. *J. Food Sci.* **60**: 1187-1190.

Mc Bride, J. R., Idler, D. R. and Mc Leod, R. A. 1961. The liquefaction of British Columbia herring by ensilage, proteolytic enzymes and acid hydrolysis. *J. Fish. Res. Bd. Can.* **18**: 93-111.

Mc Donald, 1981. The Biochemistry of Silage. Wiley and Sons, Chichester, UK.

Meister, A. 1965. Biochemistry of the Amino Acids. Vol. 1. 2nd Ed. Academic Press, New York, U. S. A.

Man, J. M. 1999. Enzymes. . *In*: Principles of Food Chemistry. Ed. Man, J. M. Publ. Aspen Publ., Inc., Gaithersburg, Maryland, USA. p. 389-428.

Montaner, M. I., Parin, M. A. and Zugarramurdi, A. 1995. Technical-economic comparison of chemical and biological fish silage. *Alimentaria* **33**: 43-51.

Morzel, M., Fransen, N. G. and Arendt, E. 1997. Defined starter cultures used for fermentation of salmon fillets. *J. Food Sci.* **62**: 1214-1217.

Myer, R. O., Johnson, D. D., Otwell, W. S., Walker, W. R. and Combs, G. E. 1988. Potential utilization of scallop viscera silage for solid waste management and as a feedstuff for swine. *Nutrition Reports Int.* **37**: 499-514.

Myer, R. O., Johnson, D. D., Otwell, W. S., Walker, W. R. and Combs, G. E. 1990. Evaluation of scallop viscera silage as a high-protein feedstuff of growing-finishing swine. *Anim. Feed Sci. and Technol.* **13**: 43-53.

Nche, P. F. 1995. Innovations in the production of kenkey, a traditional fermented maize product of Ghana. Nutritional, physical and safety aspects. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Niinivaara, F. P., Pohja, M. S. and Kumolainen, S. E. 1964. Some aspects about using bacterial pure cultures in the manufacture of fermented sausages. *Food Technol.* **18**: 25.

Nilsson, R. and Rydin, C. 1963. Fermentation as a means of preserving organic materials. *Acta Chem. Scand.* **17**: 174-179.

Nwokola, E. and Sim, J. 1990. Comparative evaluation of fermented fish waste, fermented whole herring and fish meal. *Poultry Sci.* **69**: 270-275.

Ologhobo, A. D., Balogun, A. M. and Bolarinwa, B. B. 1988. The replacement value of fish silage for fish meal in practical broiler rations. *Biol. Wastes* **35**: 117-125.

Ørskov, E. R. 1977. Nutritional principles and evaluation of by-products, waste products and new feeds for ruminants. *Livestock Prod. Sci.*, **4**: 165-175.

Ostyakova, E. B. and Kosvina, L. A. 1975. Changes in fractional composition of lipids of whole small fish during preservation with formalin solutions. *Rybnoe Chojasjstvo*, **11**: 81-83.

Owens, J. D. and Mendoza, L. S. 1985. Enzymatically hydrolysed and bacterially fermented fishery products. *J. Food Technol.* **20**: 237-293.

Owusu-Ansah, Y. J. and Hultin, H. O. 1987. Effect of in situ formaldehyde production on solubility and cross-linking of proteins of minced red hake muscle during frozen storage. *J. Food Biochem.* **11**: 17-30.

Petersen, H. 1953. Acid preservation of fish and fish offal. *FAO Fish. Bull.* **6**: 18-26.

Piard, J. C. and Desmazeaud, M. 1993, Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end products. *In*: Antimicrobials in foods. Ed. Branen, A. L. and Davidson, P. M. Marcel Dekker Inc., U. S. A. p. 533-541.

Pond, W. G. and Manner, J. H. 1984. Fish silage and liquefied fish. Swine production and nutrition. AVI Publishing Co., Inc., Westport, Connecticut, USA, p. 397-400.

Potter, D. P., Tatterson, I. N. and Wignall, J. 1978. Fish by-products – fish meal and fish silage. *Process Biochem.* Jan., p. 22-25.

Prescott, C. E. and Brown, S. M. 1998. Five-year growth response of western red cedar, western hemlock, and amabilis fir to chemical and organic fertilizers. *Can. J. Forest Res.* **28**: 1328-1334.

Prescott, C. E., Weetman, F. G. and Barker, J. E. 1996. Causes and amelioration of nutrient deficiencies in cutovers of cedar-hemlock forests in coastal British Columbia. *Forestry Chron.* **72**: 293-302.

Raa, J. 1980. Biochemistry of microbial fish spoilage and preservation by lactic acid bacteria and added acid. *In*: Global Impacts of Applied Microbiology GIAM 6. Ed. Emejuawe, S. U. Ogunbi, U., Sanni, S. O., Academic Press, p. 3-16.

Raa, J. and Gildberg, A. 1976. Autolysis and proteolytic activity of cod viscera. *Int. J. Food Technol.* **11**: 619-628.

Raa, J. and Gildberg, A. 1982. Fish silage: a review. *CRC Crit. Rev. Food Sci. Nutr.* **16**: 383-419.

Raa, J., Gildberg, A. and Strøm, T. 1983. *In*: Ledward, D. A., Taylor, A. J. and Lawrie, R. A. (Eds.). Valorizing Waste for Feeds and Food. Butterworths, London, UK, p. 117-132.

Rangkuti, M., Batubara, L. and Agus, M. 1980. Fish silage for pig production. *In*: Fish silage production and its use. Papers presented at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 Sept. Ed. Disney, J. G. and James, D. FAO Fisheries Report No. 230, p. 70-73.

Rattagool, P., Swachamthamwongratana, S. and Wongchinda, N. 1980a. Fish silage in Thailand: further trials on broiler chicken. . *In*: Fish silage production and its use.

Papers presented at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 Sept. FAO Fisheries Report No. 230, Ed. Disney, J. G. and James, D., FAO, Rome, Italy. p.59-63.

Rattagool, P., Wongchinda, N. and Swachamthamwongratana, S. 1980b. Studies on the nutritive value of fish silage for broiler chickens. *In*:. Fish silage production and its use. Papers presented at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 Sept. FAO Fisheries Report No. 230. Ed. Disney, J. G. and James, D., FAO, Rome, Italy, p. 38-54.

Rattagool, P., Wongchinda, N. and Swachamthamwongratana, S. 1980c. Fish silage in Thailand: feeding trials on broiler chicken. *In*: Fish silage production and its use. Papers presented at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 Sept. FAO Fisheries Report No. 230. Ed. Disney, J. G. and James, D., FAO, Rome, Italy, p. 55-58.

Reece, P. 1981. Recovery of high-quality oil from mackerel and sprat by the ensilage process. *J. Sci. Food Agric.* **32**: 531-538.

Restaino, L., Komatsu, K. K. and Syracuse, M. J. 1981. Effects of acids on potassium sorbate inhibition of food-related microorganisms in culture media. *J. Food Sci.* **47**: 134-138, 148.

Roa, P. D. 1965. Ensilage of fish by microbial fermentation. *Fish. News Int.* **4**: 283-284, 286.

Rodriguez, T., Montilla, J. J. and Bello, R. A. 1990. Ensilado de pescado a partir de la fauna de acompaniamiento del camarón. 2. Prueba de comportamiento en pollos de engorde. *Arch. Latinoamericanos de Nutrición* **40**: 548-559.

Rogers, D. and Bevan, E. A. 1978. Group classification of killer yeasts based on cross-reactions between strains of different species and origin. *J. Gen. Microbiol.* **105**: 199-202.

Rollan, G. C., Coton, E. and Lonvaud-Funel, A. 1995. Histidine decarboxylase activity of *Leuconostoc oenos* 9204. *Food Microbiol.* **12:** 455-461.

Roscoe, H. G. and Kupfer, D. 1972. A new histaminase assay.*Anal. Biochem.* **47:** 418-425.

Rouvinen, K.I., Anderson, D. M. and Alward, S. R. 1996. Use of silver hake and herring and the corresponding silages in mink diets during the growing-furring period. *Can. J. Anim. Sci.*, **76**: 127-133.

Sanders, J. W, Leehouts, K. J., Haandrikman, A. J., Venema, G. and Kok, J. 1995. Stress response in *Lactococcus lactis*: cloning expression analysis, and mutation of the lactococcal superoxide dismutase gen. *J. Bacteriol.* **177**: 5254-5260.

Sainclivier, M. 1985. L'industrie alimentaire halieutique. Vol. 2. Des techniques ancestrales à leurs réalisations contemporaines. Salage, Séchage, Fumage, Marinage, Hydrolysats. Bulletin Scientifique et Technique de l'École Nationale Supérieure Agronomique et du Centre de Recherches de Rennes, France. Chap. VII. Les Hydrolysats, p. 308-366.

Schillinger, U. and Lücke, F. -K. 1989. Antibacterial activity of Lactobacillus sake isolated from meat. *Appl. Environm. Microbiol.* **55**: 1901-1906.

Schomburg, D. and Stephan, D. 1993. Enzyme Handbook. Springer Verlag, Berlin, Germany.

Schrøder, K., Clausen, E., Sandberg, A. M. and Raa, J. 1980. Psychrotrophic *Lactobacillus plantarum* from fish and its ability to produce antibiotic substances. *In*: Advances in Fish Science Technol., Proc. Torry Jubileum Conference. Torry Research Station, Aberdeen, UK, p. 480-483.

Shalaby, A. R. and Abd El-Rahman, H. A. 1995. Effect of potassium sorbate of biogenic amines during sausage fermentation. *Nahrung* **39**: 308-315.

Shore P. A. 1971. Fluorimetric assay of histamine. *Meth. Enzym.* **17**B: 842-845.

Shqueir, A. A., Kellems, R. O. and Church, D C. 1984. Effects of liquefied fish, cottonseed meal and feather meal on in vivo and in vitro rumen characteristics of sheep. *Can. J. Animal Sci.* **64**: 881-888.

Siebert, G. and Schmidt, A. 1965. Fish tissue enzymes and their role in deteriorative changes in fish. *In*: FAO Int. Symposium Fish Technology and Utilisation. Fishing News Books, Ltd., London, UK.

Silla-Santos, M. H. 1996. Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* **29**: 213-231.

Silla-Santos, M.H. (2001) Toxic nitrogen compounds produced during processing: biogenic amines, ethyl carbamides, nitrosamines. *In*: Adams , M.R. and Nout, M.J.R. (Eds.) Fermentation and Food Safety. Aspen Publishers, Inc. Gaithersburg, Md., USA, p. 119-140.

Silva, C. G., da Ponte, D. J. B. and Enes Dapkevicius, M. L. N. 1998. Storage temperature effect on histamine formation in big eye tuna and skipjack. *J. Food Sci.* **63**: 644-647.

Sinnel, H. J. 1980. Interacting factors affecting mixed populations. *In*: Microbial Ecology of Foods. Vol. I. Ed. Silliker, J. H. Academic Press, London, UK, p. 251-281.

Skrede, A. and Nes, I. F. 1988. Slaughterhouse by-products preserved by *Lactobacillus plantarum* fermentation as a feed for mink and foxes. *Anim. Feed Sci. Technol.* **20**: 287-298.

Smail, D. A., Huntley, P. J. and Munro, A. L. S. 1990. Fate of four fish pathogens after exposure to an ensiling mixture containing fish farm mortalities. Proceedings of Counc. Meeting for the Int. Council for the Exploration of the Sea, Copenhagen, Denmark, 7 p. ICES-CM-1990/F: 50.

Stanton, W. R. and Yeoh, Q. L. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. *In*: Proceedings of the Conference on Handling, Processing and Marketing of Tropical Fish. Tropical Products Institute, London, UK.

Stecchini, M. L., Del Torre, M. and Munari, M. 2001. Determination of peroxy radical-scavenging of lactic acid bacteria. *Int. J. Food Microbiol.* **64**: 183-188.

Stoffels, G., Nissen-Meyer, J., Gudmundsdottir, A., Sletten, K., Holo, H. and Nes, I. F. 1992. Purification and characterisation of a new bacteriocin isolated from a *Carnobacterium* sp. *Appl. Environm. Microbiol.* **58**: 1417-1422.

Straub, W., Kicherer, M., Schilcher, S. M. and Hammes, W. P. 1995. The formation of biogenic amines by fermentation microorganisms. *Lebensm. Untersuch. Forsch.* **201**: 79-82.

Strøm, A., Olafsen, J. A. and Larsen, H. 1979. Trimethyl amine oxide: a terminal electron acceptor in anaerobic respiration of bacteria. *J. Gen. Microbiol.* **112**: 315-320.

Strøm, T .and Eggum, B. O. 1981. Nutritional value of fish viscera silage. *J. Sci. Food Agr.* **32**: 115-120. {Agric. Is OK}

Suzuki, T. 1981. Fish and krill processing technology. Appl. Sci. Publ., London, UK.

Synnes, O. M. and Opstad, S. 1995. Effect of fish silage on the yield and chemical composition of Italian ryegrass (*Lollium multiflorum* Lam.). *Acta Agric. Scand., Section B: Soil and Plant Sci.* **45**: 68-72.

Szakács, R., Radvánsky, B. and Gyenes, J. 1988. Large-scale production of animal feed from meat industry by-products by lactic acid fermentation. Biotechnology and Food Industry. Proc. Int. Symposium, Budapest, Hungary. p. 609-615.

Tatterson, I. N. 1982. Fish silage – Preparation, properties and uses. *Anim. Feed Sci. Technol.* **7**: 153-159.

Tatterson, I. N. and Windsor, M. L. 1974. Fish silage. *J. Sci. Food Agr.* **25**: 369-379.{Agric is OK}

Taylor, S. L. 1986. Histamine food poisoning: toxicology and clinical aspects. *CRC Crit. Rev. Toxicol.* **17**:s 91-128.

Taylor, S. L. and Sumner, S. S. 1986. Determination of histamine, putrescine and cadaverine. *In*: Seafood Quality Determination. Proceedings of an International Symposium coord. by the University of Alaska, Anchorage, 10-14 Nov. Ed. Kramer, D. E. and Liston, J. Elsevier Sicence Publ., Amsterdam, The Netherlands.

Ten Brink, B., Damink, C., Joosten, H. M. L. J., Joosten, J. H. and Huis in't Veld, J. 1990. Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* **11**: 73-84.

Tibbetts, G W. and Seerley, R. W. 1988. Poultry viscera ensiled with *Lactobacillus acidophillus* for growing and finishing swine diets. *Nutr. Rep. Int.* **38**: 905-913.

Torrissen, O., Tideman, F. and Raa, J. 1981. Ensiling in acid – a method to stabilize astaxanthin in shrimp processing by-products and improve uptake of this pigment by rainbow trout (*Salmo gairdeneri*). *Aquacult.* **26**: 77-83.

Trow-Smith, R. 1974, Fish puree – a growth gravy for pigs. *Farmers Weekly*, October, 25.

Twiddy, D. R., Cross, S. J. and Cooke, D. R. 1987. Parameters involved in the production of lactic acid preserved fish-starchy substrate combinations. *Int. J. Food Sci. Technol.* **22**: 115-121.

Ulvestad, O. P. 1987. La production et l'utilisation du poisson ensilé pour nourrir les animaux. *In*: Atelier sur l'ensilage du poisson. Min. des Pêches et Océans, Canada.

Van Lunen, T. 1987. L'utilisation du poisson ensilé pour nourrir les animaux. *In*: Atelier sur l'ensilage du poisson. Min. des Pêches et Océans, Canada, p. 69 -75.

Van Wyk, H. J. and Heydenrych, C. M. S. 1985. The production of naturally fermented fish silage using various lactobacilli and different carbohydrate sources. *J. Sci. Food Agric.* **36**: 1093-1103.

Van Wyk, H. J., Heydenrych, C., Walters, S. Williams, C. and Mol, T. 1983a. Fish silage; preliminary experiments in reducing the cost of production of naturally fermented silage. 37th Annual Report of the Director, FIRI, p. 46.

Van Wyk, H. J., Heydenrych, C., Williams, C., Garry, D. and Milkovitch, S. 1983b. Naturally fermented fish silage II. 37th Annual Report of the Director, FIRI, p. 41-45.

Venugopal, V. 1995. Methods for processing and utilization of low cost fishes: a critical appraisal. *J. Food Sci. Technol.*, **32**: 1-12.

Verité, R., Poncet, C., Chabi, S. and Pion, R. 1977. Utilisation des tourteaux traités au formol par les vaches laitières. *Ann. Zootech.*, **26**: 167-181.

Vignolo, G. M., Holgado, A. P. R. and Oliver, G. 1988. Acid production and proteolytic activity of *Lactobacillus* strains isolated from dry sausages. *J. Food Prot.*, **51**: 481-484.

Viken, N. I. and Bjørge, F. 1987. Les usines de production d'ensilage. *In*: Anon., 1987. Rapport, Attelier sur l'ensilage du poisson. Pointe-de-l'Église, Nouvelle-Écosse, 16-17 juin, 1987. Min. Pêches Océans, Canada, séries Éducation Gén. n°7, p.38-59.

Vizcarra-Magana, L.A., Avila, E. and Sotelo, A. 1999. Silage preparation from tuna fish wastes and its nutritional evaluation in broilers. *J. Sci. Food Agric.*, **79**: 1915-1922.

Voigt, M. N. and Eitenmiller, R. R. 1978. Role of histidine and tyrosine decarboxylases and mono- and diamine oxidases in amine build-up in cheese. *J. Food Prot.* **41**: 182-186.

Wei, C. I., Chen, C.-M., Koburger, J. A., Otwell, W. S. and Marshall, M. R. 1990. Bacterial growth and histamine production on vacuum packaged tuna. *J. Food Sci.* **55**: 59-63.

Wessels, J. P. H., Rutokowski, H. P. M. and Mulder, M. C. 1978. The effect of enzymes on the tryptophan content of fish silage. 32nd Annual Report of the Director – FIRI, p. 49.

Wet, P. J., Wessels, J. P. H. and Post, B. 1982. Utilisation of fish silage as an important protein source in a milk replacer for lambs. 36th Annual Report of the Director – FIRI, p. 49 (Summary only).

Wheaton, F. W. and Lawson, T. B. 1985. Other preservation methods. In *Processing Aquatic Food Products*. John Wiley & Sons, New York, USA. p. 274-275.

Whipple, M. J. and Rohovec, J.S. 1994. The effect of heat and low pH on selected viral and bacterial fish pathogens. *Aquacult.*, **123**: 179-189.

White, M. B., Anderson, D. M. and Rouvinen, K. I. 1999. Apparent digestibility coefficients of raw ground, acid and fermented silver hake (*Merluccius bilinearis*) feedstuffs for mink and an evaluation of the storage stability of the silages. *Canadian J. Anim. Sci.*, **79**: 375-381.

Whittemore, C. T. and Taylor, A. G. 1976. Nutritive value to the growing pig of deoiled liquefied herring offal preserved with formic acid (fish silage). *J. Sci. Food Agric.* **27**: 239-243.

Wijtzes, T., Bruggeman, M.R., Nout, M.J.R. and Zwietering, M.H. (1997) A computerised system for the identification of lactic acid bacteria. *Int. J. Food Microbiol.* **38**: 65-70.

Windsor, M. and Barlow, S. 1981. Introduction to fishery by-products. Fishing News Books, Ltd., Farnham, Surrey, England.

Winter, K. A. and Feltham, L. 1983. Fish silage: the protein solution. Agriculture Canada Report.

Wirahadikusumah, S. 1968. Preventing *Clostridium botulinum* Type E poisoning and fat rancidity by silage fermentation. *Lantsbrukshögsk. Annaler, Sweden* **35**: 823-825.

Wood, J. F., Capper, B. S. and Nicolaides, L. 1985. Preparation and evaluation of diets containing fish silage, cooked fish preserved with formic acid and low-temperature-dried fish meal as protein sources for mirror carp (*Cyprinus carpio*). *Aquacult.* **44**: 27-40.

Woolford, M. K. 1972. The microbiological screening of potential additives. Grassland Research Institute, U. K., Annual Report 74-75.

Yeoh, Q. L. 1980. The status of research on fish silage in Malaysia. In: Disney, J. G. and James, D. (Eds.). Fish Silage Production and Its Use. Papers pres. at the IPFC Workshop on fish silage production and its use. Jakarta, Indonesia, 17-21 Sept. FAO Fisheries Report No. 230, p. 19-23.

Yoshinaga, D. H. and Frank, H. A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwomus pelamis*). *Appl. Environm. Microbiol.* **44**: 447-452.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M. and van't Riet, K. 1990. Modeling of the bacterial growth curve. *Appl. Environm. Microbiol.* **56**: 1875-1881.

Summary

This thesis deals with stability, safety, and functionality aspects of biological fish silage (BFS) obtained by lactic acid fermentation. BFS may provide an economically viable, environment friendly way of upgrading fish waste.

The biological silage has been found advantageous when compared to the so-called acid process, since it yielded lower levels of peroxides in the silage oil and led to lower non-protein nitrogen values.

The most important factors in achieving stable biological fish silages are an appropriate choice of starter, an adequate carbohydrate source as substrate and a suitable fermentation temperature. Temperatures of 35 – 37°C were regarded as most suitable from the point of view of starter growth and acidification. Strains with a short acidification lag time, such as *Lb. plantarum* 009, or with very fast growth and good antibacterial properties, such as *Lb. curvatus* 15.35, are most suitable. Combinations of strains in mixed cultures did not yield benefits in terms of strain growth or acidification and were not regarded as suitable for our purposes.

BFS appeared to be microbiologically safe. Fermentation by lactic acid bacteria was shown to efficiently inhibit pathogens such as *L. monocytogenes* and *E. coli* O157:H7 that to our knowledge had not yet been studied in the context of fish silage, as well as some spoilage microorganisms. Biogenic amine producing bacteria could pose safety risks. There were, however, potential starter strains that were able to inhibit growth of these bacteria. Histamine degradation by some of the most promising available LAB strains was also tested as an innovative means of ensuring low levels of this amine in the fermented product. Several strains were found to degrade histamine as single strain cultures and these could find application in fish silage and other fish products in which histamine accumulation might pose a certain risk.

Sodium chloride (2% w/w) could be of value as an additive for BFS production. It inhibits growth of biogenic amine producing bacteria, but starter strains can be found that are not affected by the addition. NaCl additionally decreased non-protein nitrogen and total volatile basic nitrogen values in BFS.

Propionic acid did not efficiently inhibit fungal growth in BFS. Inclusion of potassium sorbate in the medium was shown to inhibit growth and/or acidification by potential starter lactic acid bacteria and therefore could bring about stability problems when used in this way in BFS preparation.

Samenvatting

Dit proefschrift behandelt diverse aspecten van de stabiliteit, veiligheid en functionele eigenschappen van biologische vissilage (BVS) verkregen door melkzuurfermentatie. BVS biedt een economisch haalbare en milieuvriendelijke mogelijkheid ter verwerking van visafval.

Het biologisch ensileren vergelijkt gunstig met het zogeheten zuur-ensileren, aangezien er minder peroxiden in de oliefractie, en lagere niet-eiwit stikstofgehalten blijken te worden gevormd.

De belangrijkste vereisten voor het verkrijgen van een stabiele biologische vissilage zijn de keuze van de juiste startercultuur, een geschikte suikerbron als substraat en de juiste fermentatietemperatuur. Temperaturen van 35 - 37°C werden het geschiktst gevonden voor de groei en zuurvorming door de starters. Starterstammen zoals *Lb. plantarum* 009 met een korte aanlooptijd voor zuurvorming, of stammen zoals *Lb. curvatus* 15.35 met zeer snelle groei en goede antibacteriële eigenschappen, zijn het interessantst. Combinaties van stammen in gemengde startercultures leverden geen meerwaarde op met betrekking tot groei en/of zuurvorming en bleken daarom niet bruikbaar.

BVS blijkt microbiologisch veilig te zijn. De fermentatie door melkzuurbacteriën bleek een effectieve remming te geven van pathogenen zoals *L. monocytogenes* en *E. coli* O157:H7 waarvan het gedrag in BVS nog niet eerder was onderzocht, alsmede van een aantal bederf micro-organismen. Een veiligheidsrisico is de bacteriële vorming van biogene amines. Er werden echter stammen gevonden die in staat zijn om biogene-amine-vormende bacteriën te remmen. De afbraak van histamine door deze stammen werd ook onderzocht als een innovatieve mogelijkheid om de concentratie van dit amine in BVS zo laag mogelijk te houden. Een aantal stammen waren als reincultuur in staat histamine af te breken, en deze zouden kunnen worden toegepast in vissilage en andere visproducten waaraan het risico van hoge histaminegehalten verbonden kan zijn.

Natriumchloride (keukenzout 2% w/w) kan een waardevolle toevoeging aan BVS betekenen. Het remt de groei van biogene-amine-vormende bacteriën, maar niet van sommige starterstammen. Keukenzout toevoeging leidde ook tot lagere gehalten van niet-eiwit stikstof en totaal vluchtige basische stikstof in BVS.

Propionzuur was niet in staat groei van gisten en schimmels in BVS te remmen. Toevoeging van kaliumsorbaat in het medium remde de groei en/of zuurvorming door mogelijke starter- melkzuurbacteriën en zou dus kunnen leiden tot verminderde stabiliteit van BVS.

Resumo

Na presente tese, focam-se aspectos relacionados com a estabilidade, segurança, qualidade e funcionalidade da ensilagem biológica de pescado. Este processo poderá constituir uma alternativa viável do ponto de vista económico e aceitável do ponto de vista ambiental para a valorização de resíduos do pescado.

Verificou-se que o processo biológico de ensilagem era mais vantajoso do que o processo ácido, por resultar em menores níveis de peróxido nos óleos das silagens e em menores concentrações de azoto não proteico.

Os factores mais importantes para a estabilização das silagens biológicas são uma temperatura de fermentação adequada e uma escolha criteriosa da cultura de arranque. Temperaturas da ordem de 35 – 37°C foram consideradas como as mais adequadas do ponto de vista do crescimento e acidificação pela cultura de arranque. Estirpes com curtos períodos de latência para a acidificação, como *Lb. plantarum* 009, ou com crescimento muito rápido e boas propriedades antibacterianas, como o *Lb. curvatus* 15.35, são as mais apropriadas para culturas de arranque. Combinações de estirpes não produziram resultados positivos em termos de crescimento e acidificação das culturas e arranque e não foram, por esse motivo, consideradas úteis para os fins propostos.

As silagens biológicas aparentaram ser seguras do ponto de vista microbiológico. A fermentação pelas bactérias do ácido láctico demonstrou inibir eficazmente patogénicos que não tinham ainda sido estudados no âmbito da silagem de peixe, como a *L. monocytogenes* e a *E. coli* O157:H7, bem como microorganismos que tomam parte no processo de deterioração. Bactérias produtoras de histamina poderiam constituir um potencial risco. Contudo, encontraram-se culturas de arranque que inibiam o crescimento destes microrganismos. Testou-se também um método inovador para a limitar a acumulação de histamina, que consistiu na degradação desta amina biogénica por potenciais culturas de arranque. Várias das estirpes estudadas possuíam esta propriedade e poderão ser usadas para limitar os níveis de histamina nas silagens de peixe ou em outros produtos das pescas.

O cloreto de sódio (2%, p/p) pode ser útil como aditivo para a produção de silagens biológicas de pescado, por ser barato e fácil de obter. Verificou-se, contudo, que inibe o crescimento de bactérias produtoras de aminas biogénicas, havendo no entanto potenciais culturas de arranque que toleram estes níveis de NaCl. Para além disso, a adição de NaCl conduziu a uma diminuição dos níveis de azoto não proteico e de bases voláteis totais nas silagens biológicas. O ácido propiónico não demonstrou uma acção fungicida eficaz e o sorbato de potássio

conduziu a inibição do crescimento e acidificação pelas potenciais culturas de arranque, podendo causar problemas de instabilidade nas silagens.

Trumpas tezių turinys

Šiose tezėse yra nagrinėjami biologinio žuvies siloso (BŽS) stabilumo, kokybės ir saugumo aspektai. BŽS gamyba įgalina ekonomišku ir ekologišku būdu perdirbti žuvies atliekas.

Buvo nustatyta, kad biologinis silosas yra pranašesnis už silosą paruoštą pridedant rūgščių, nes pirmuoju būdu pagamintas produktas pasižymi žemesne peroksidų bei neproteininio azoto (angl., *non-protein nitrogen* arba NPN) koncentracija.

Tinkama fermentacijos temperatūra bei sėkmingas starterinių kultūrų parinkimas yra vieni iš svarbiausių veiksnių nulemiančių biologinio siloso stabilumą. Buvo nustatyta, kad 35 – 37°C temperatūra yra tinkamiausia starterio augimui ir rūgščių koncentracijos silose didėjimui. Kultūros, kurios pasižymi trumpu vėlinimosi laiku (*Lb. plantarum* 009) arba labai sparčiu augimu ir stipriomis antibakterinėmis savybėmis (*Lb. curvatus* 15.35), buvo pripažintos tinkamiausiomis. Kelių mikrobiologinių kutūrų deriniai nepagerino starterio augimo ir rūgščių koncentracijos augimo, ir todėl tolimesniuose tyrimuose nebuvo naudoti.

BŽS pripažintas mikrobiologiškai saugiu produktu. Gauti rezultatai parodė, kad fermentacijai naudojant pieno rūgšties bakterijas yra įmanoma efektyviai pristabdyti gedimą sukeliančių mikroorganizmų bei tokių patogenų kaip *L. monocytogenes* and *E. coli* O157:H7 augimą. Šis faktas žuvies siloso kontekste yra naujas. Potencialus rizikos faktorius yra biogeninius aminus gaminčios bakterijos. Tyrimų metu buvo izoliuotos starterinės kultūros pajėgios sulėtinti šių bakterijų augimą. Novatoriškas sprendimas buvo pritaikytas histamino kontrolei tiriamame produkte. Tuo tikslu buvo įvertintos potencialių starterių histamino degradavimo savybės. Buvo nustatyta, kad kai kurie iš potencialių starterių yra pajėgūs degraduoti histaminą, kas įgalina juos panaudoti žuvies siloso bei kitų žuvies produktų gamyboje, kur besikaupiantis histaminas kelia pavojų produkto saugumui.

Natrio chloridas (2%) gali būti vertingas priedas gaminant BŽS. Tai pigi ir lengvai prieinama medžiaga. Be to, natrio chloridas stabdo tirtų histaminą gaminančių bakterijų augimą. Tačiau reikia paminėti, kad kai kurioms startinėms kultūroms natrio chloridas nepadarė jokio poveikio. NaCl taip pat sumažino NPN bei pilno lakaus bazinio azoto (angl., *total volatile basic nitrogen* arba TVBN) dydžius BŽS pavyzdžiuose.

Propioninė rūgštis pridėta į BŽS neturėjo įtakos pelėsių augimui. Tačiau tyrimai pademonstravo slopinantį natrio sorbato poveikį potencialaus pieno rūgšties bakterijų starterio augimui bei rūgščių koncentracijos didėjimui. Dėl šios

priežasties, panaudojus natrio sorbatą BŽS gamyboje ima kristi produkto stabilumo rodikliai.

List of publications

Full papers

Enes Dapkevicius, M. L. N., I Batista, M. J. R. Nout, F. M. Rombouts and J. H. Houben, 1998. Lipid and protein changes during the ensilage of blue whiting (*Micromesistius poutassou* Risso) by acid and biological methods. *Food Chem.* **63**: 97-102.

Silva, C. C. G., D. J. B. Ponte, and M. L. N. Enes Dapkevicius 1998. Effect of storage temperature on histamine formation in tuna. *J. Food Sci.* **103**: 644-647.

Enes Dapkevicius, M. L. N., Nout, M. J. R., Rombouts, F. M., Houben, J. H. and Wymenga, W. 2000. Biogenic amine formation and degradation by potential fish silage starter microorganisms. *Int. J. Food Microbiol.* **57:** 107-104.

Other publications

Enes, M. L. N. 1992. Biological fish silage as an alternative to fishmeal production. Paper presented at the 22nd WEFTA Meeting, Instituto de Investigação das Pescas e do Mar Lisboa, Portugal.

Enes, M. L. N., M. J. R. Nout, F. M. Rombouts, J. H. Houben and W. Schaik, 1997. Upgrading fish waste by ensilage: keeping the biogenic amine content low. Poster, NVVL Annual Symposium, Ede, The Netherlands.

Enes, M. L. N., M. J. R. Nout and F. M. Rombouts, 1995. Choosing starters for the production of fermented animal foods. Poster, 5th Congress of Animal Science "New Trends in Animal Science", Terceira Island, Azores, Portugal. Published in *Revista Portuguesa de Zootecnia,* nº extra, Resumos, pg. 72.

Enes Dapkevicius, M. L. N., M. J. R. Nout, F. M. Rombouts, J. H. Houben, and W. Wymenga, 1996. Biogenic amine formation and degradation as criteria for the selection of safe fish silage starter microorganisms. Paper presented at the "Food Micro '96", Congress, Budapest, Hungary.

Enes Dapkevicius, M. L. N., M. J. R. Nout, F. M. Rombouts, and J. H. Houben, 1998. Potential of lactic acid bacteria for the upgrading of fish waste into animal feed components. 8th Congress of Animal Science "Management and Animal Welfare", Terceira Island, Azores, Portugal.

Enes Dapkevicius, M. L. N. E., B. Afonso, C. C. G. da Silva, and J. E. S. Matos, 2001. Antibacterial activity of azorean honeys against *Staphylococcus arueus* strains. 11th Congress of Animal Science "Animal Production in the Periphery of the European Union", Terceira Island, Azores, Portugal.

Curriculum vitae

Maria de Lurdes Nunes Enes Dapkevicius was born on the 3rd January 1962, in the island of S. Jorge (Azores, Portugal). After primary and secondary studies in Angra, Azores, she graduated in Animal Science at the University of the Azores in 1987. The theme of her final project for graduation was "Management and Economics of Milk Farms in Terceira Island (Azores, Portugal)". She started working as a training teaching assistant in Microbiology at the University of the Azores in the same year. In 1992, she passed the exams of Pedagogical Aptitude and Scientific Ability demanded by the Portuguese Law for assessing the status of teaching assistant. The theme of her research work for these exams was "Biological ensilage of fish". In 1993, she worked for 6 months at the Food Microbiology group of WAU under the scope of the ERASMUS program, for training in the identification and biogenic amine production by lactic acid bacteria. In 1994, she started her PhD research at WAU, funded by two EU projects. In 1998, she returned to the Azores, to resume her work as a teaching assistant at the University. She is presently teaching General Microbiology to the courses of Agricultural, Zootechnical and Environmental Engineering and Dairy Science and Technology to the Zootechnical Engineering course.

Acknowledgements

The present study was funded by EC grants ERB 4001GT 94/0050 and FAIRGT 95 6118 (FLAIR Programme).

The collaboration of Berta Borges, Wieke Wymenga, Ana Costa, Willem van Schaik and Gera Jie-A-Looi in some of the laboratory tests is acknowledged. Confirmatory tests for the identification of the most promising isolates were carried out by Dr. Norbert Weiss, at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.