

PCR-RFLP TYPIFICATION OF MICROBES USED IN THE PRODUCTION OF A FERMENTED FISH PRODUCT

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entirety or in part, been submitted at any university for a degree.

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ABSTRACT

The preservation of various fresh fish products is achieved by either smoking, salting, canning, freezing or fermenting a highly perishable raw product. Since many of these facilities are not readily available, the use of fermentation as a means of preserving the product has been extensively practiced. However, the fermentation of fish is a time consuming practise and only by accelerating the process would it be possible to ensure the production of a more cost effective and readily available safe end-product.

The quality of the fermented fish product is partially determined by the fermentation conditions and the metabolic activity of the microbes present. The rapid identification of the microbes present during the fermentation would enable the selection of possible starters to ensure an accelerated production of high quality fermented fish products. This study was thus undertaken to develop identification fingerprints for bacteria isolated from fermented fish products. A 1300 bp fragment of the 16S rRNA genes of each of the bacteria previously isolated was successfully amplified using the PCR technique. The isolates included strains of the genera *Bacillus*, *Staphylococcus*, *Sphingomonas*, *Kocuria*, *Brevibacillus*, *Cryseomonas*, *Vibrio*, *Stenotrophomonas* and *Agrobacterium*. The data obtained can, therefore, be used in the identification of these microbes isolated from other similar fermented fish products. The fingerprints could also be used to assist in determining the dominant microbial populations responsible for the characteristic qualitative changes occurring in the fish product during fermentation.

The microbial composition of a fermenting fish product partially determines the quality of the end-product, therefore, the use of selected bacterial starters could result in the accelerated production of a microbial safe fermented fish product. A further objective of this study was to accelerate the production of a fermented fish product by inoculating macerated trout with either selected lactic acid bacteria (LAB) or with selected bacteria with high proteolytic activity over a 30 day fermentation period. The LAB included a combination of *Lactobacillus plantarum*, *Lactococcus diacetylactis* and *Pediococcus cerevisiae* strains, whereas the bacteria with high proteolytic activity included strains of *Kocuria varians*, *Bacillus subtilis*, two strains of *B. amyloliquefaciens* and a combination of these

bacterial species. The quality of the fermented product was determined using changes in product pH, titratable acidity (%TA) and free amino nitrogen (FAN) formation as efficiency parameters.

The data obtained during the fermentation of the macerated trout showed that the selected starters did not have a significant effect on the pH decrease in the product over a 30 day fermentation period. The LAB strains did not have a significant effect on the %TA of the fermenting fish product, yet the presence of these bacteria appeared to limit the FAN production in the product. The bacteria with high proteolytic activity resulted in slightly enhanced %TA values and a higher FAN content in the fermented product. It was also determined that the LAB and *Kocuria varians*, in contrast to the *Bacillus* spp. inoculums, did not survive the fermentation conditions well, possibly due to the low pH environment. The presence of the starter bacteria in the fermenting fish mixture at the end of the fermentation was also successfully determined with the use of the PCR-RFLP technique.

The fermented fish product, obtained at the end of the fermentation period, had a good aroma and compared favourably to similar commercially available fermented fish products. The use of different microbial starters could in future enable the production of a diverse range of high quality products, which could be produced and marketed locally.

UITTREKSEL

Die preservering van 'n verskeidenheid vars vis produkte word bereik deur die hoogs bederfbare produk te rook, te sout, te blik, te vries of te fermenteer. Aangesien baie van hierdie fasiliteite nie gereedelik beskikbaar is nie, is die gebruik van fermentasie as 'n preserverings metode al ekstensief beoefen. Die fermentasie van vis is egter 'n tydsame proses en slegs deur die versnelling van die proses sal dit moontlik wees om die produksie van 'n meer koste effektiewe en gereedlike beskikbare veilige eindproduk te verseker.

Die kwaliteit van die gefermenteerde vis produk word gedeeltelik bepaal deur die fermentasie kondisies en die metaboliese aktiwiteit van die mikrobies teenwoordig. Die vinnige identifikasie van die mikrobies teenwoordig gedurende die fermentasie sal die seleksie van moontlike suursels om die versnelde produksie van hoë kwaliteit gefermenteerde vis produkte moontlik maak. Hierdie studie is dus onderneem om identifikasie vingerafdrukke vir bakterieë wat geïsoleer is van gefermenteerde vis produkte moontlik te maak. 'n 1300 bp fragment van die 16S rRNA gene van elkeen van die bakterieë wat voorheen geïsoleer is, is suksesvol geamplifiseer deur die PCR tegniek. Die isolate sluit in stamme van die genera *Bacillus*, *Staphylococcus*, *Sphingomonas*, *Kocuria*, *Brevibacillus*, *Cryseomonas*, *Vibrio*, *Stenotrophomonas* en *Agrobacterium*. Die data kan dus gebruik word in die identifikasie van hierdie mikrobies as dit geïsoleer word van ander gefermenteerde vis produkte. Die vingerafdrukke kan ook gebruik word om die dominante mikrobiese populasies wat verantwoordelik is vir die kenmerklike kwalitatiewe veranderinge wat plaasvind in die vis produk gedurende die fermentasie, te identifiseer.

Die mikrobiese samestelling van 'n fermenterende vis produk bepaal gedeeltelik die kwaliteit van die eindproduk, daarom kan die gebruik van geselekteerde bakteriese suursels die versnelde produksie van 'n mikrobies veilige gefermenteerde vis produk teweeg bring. 'n Verdere doel van hierdie studie was om die produksie van 'n gefermenteerde vis produk te versnel deur fyngemaakte forel met of geselekteerde melksuurbakterieë of met geselekteerde bakterieë met hoë proteolitiese aktiwiteit te inokuleer oor 'n 30 dag fermentasie periode. Die melksuurbakterieë het ingesluit 'n kombinasie van *Lactobacillus plantarum*, *Lactococcus diacetylactis* en *Pediococcus cerevisiae*, terwyl die

bakterieë met hoë proteolitiese aktiwiteit stamme van *Kocuria varians*, *Bacillus subtilis*, twee stamme van *Bacillus amyloliquefaciens* en 'n kombinasie van hierdie bakteriese stamme ingesluit het. Die kwaliteit van die gefermenteerde produk is bepaal deur die veranderinge in die pH, titreerbare suur (%TS) en vrye amino stikstof (VAS) vorming van die produk as effektiwiteits parameters te gebruik.

Die data wat verkry is gedurende die fermentasie van die fyngemaakte forel het gedui daarop dat die geselekteerde suursels nie 'n merkwaardige effek op die afname in pH in die produk oor 'n 30 dag fermentasie periode het nie. Die melksuurbakterieë het nie 'n merkwaardige effek op die %TS van die gefermenteerde vis produk gehad nie, terwyl dit geblyk het dat die teenwoordigheid van hierdie bakterieë die produksie van VAS in die produk belemmer het. Die bakterieë met hoë proteolitiese aktiwiteit het 'n effense verhoogde %TS en 'n hoër VAS inhoud in die gefermenteerde produk veroorsaak. Dit is ook bepaal dat die melksuurbakterieë en *Kocuria varians*, in teenstelling met die *Bacillus* spp. inokulums, nie die fermentasie kondisies goed oorleef het nie, moontlik as gevolg van die lae pH omgewing. Die teenwoordigheid van die suursel bakterieë in die fermenterende vis mengsel aan die einde van die fermentasie is ook suksesvol bepaal met die PCR-RFLP tegniek.

Die gefermenteerde vis produk, verkry aan die einde van die fermentasie periode, het 'n goeie aroma gehad en het goed vergelyk met soortgelyke kommersieel beskikbare gefermenteerde vis produkte. Die gebruik van verskillende mikrobiiese suursels kan in die toekoms die produksie van 'n diverse reeks hoë kwaliteit produkte wat plaaslik geproduseer en bemark kan word moontlik maak.

dedicated to my parents

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CONTENTS

Chapter		Page
	Abstract	iii
	Uittreksel	v
	Acknowledgements	viii
1	Introduction	1
2	Literature review	4
3	PCR-RFLP analysis of bacteria isolated from fermented fish (Nuoc nam type) products	40
4	Accelerated fish fermentation by the inoculation with selected starter bacteria	55
5	General discussion and conclusions	89

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Fermentation is one of the oldest forms of food preservation and has long been an essential part of the human diet. Fermented foods include various products with an enhanced aroma, flavour and nutritional value (Beddows, 1998). Fermentation is an efficient, low energy process that improves the shelf-life of food products without the need for refrigeration, which is often unavailable in developing countries or remote areas (Battcock & Azam-Ali, 1998).

Fermented products, including fish sauces, pastes and vegetable blends (Steinkraus, 1995) account for the main protein source in the South East Asian diet and serve as condiments to rice dishes (Sanceda *et al.*, 1992; Al-Jedah & Ali, 2000). The traditional production of these fermented fish products is initiated by the dehydration of fish or other seafood mainly to prevent spoilage (Beddows, 1998). The fermentation of the fish tissue is then characterised by the release of proteolytic enzymes from the muscle tissues and the gastro intestinal tract of the fish, which convert insoluble fish protein to soluble amino acids and polypeptides (Stefansson, 1993). Enzymes secreted by the microbes present in the fish mixture also contribute to the degradation of the fish tissues and are essential to the flavour and aroma development of the fermented product (Steinkraus, 1995). However, the production of these fermented fish products are time consuming and can take from 6 – 18 months to complete. The acceleration of this process would, therefore, economically benefit the fermentation industry (Stefansson, 1993). The addition of commercial proteolytic enzymes and selected microbial starters to fermenting fish mixtures has been successfully used as a means of accelerating the proteolysis of the fish tissue (Al-Jedah & Ali, 2000; Lee *et al.*, 1989; Lubbe, 2000; Prochaska *et al.*, 1998; Venugopal, 1992).

Further improvement and regulation of the fermentation processes would be possible if the microbial content of the starters used during fermentation could be rapidly identified (Van der Vossen & Hofstra, 1996). Microbial identification based on morphological and physiological characteristics is time consuming and may not reflect the true composition of the fermenting mixture due to the inability to culture certain microbial strains on synthetic selective media (Borrell *et al.*,

1997; Ridley & Saunders, 1993). The development of molecular techniques has enabled the rapid and accurate identification of microbes present in food products, due to the sensitivity and specificity of these techniques (Johnson *et al.*, 1995; Suzzi *et al.*, 2000). These techniques are highly reliable and reproducible since identification is based on characteristics that do not vary under changing environmental conditions (Gautier *et al.*, 1996).

The objectives of this study were to identify the microbes isolated from fermented fish (Nuoc-Nam type) products, with the use of polymerase chain reaction (PCR) restriction fragment length polymorphisms (RFLPs). Furthermore, an attempt will be made to accelerate the production of a fermented fish product by the addition of lactic acid bacteria and bacteria with high proteolytic activity. This will be followed by the assessment of the quality of the product and the identification of the starters from the fermenting fish mixture using PCR-RFLPs.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

The populations of Thailand, Malaysia, Cambodia, the Philippines and Indonesia have a very low daily intake of total protein (Beddows, 1998). In these countries the most important sources of protein are fresh and processed fish products. The consumption of fresh fish products have in the past, however, been limited by the high price of fresh fish, the limited supply of fresh products and the poor keeping quality and inadequate marketing systems of these products (Van Veen, 1965).

Methods used during the processing of fish include drying, salting, smoking, steaming, pickling and fermentation, with fermentation being one of the oldest and most successful preservation techniques available (Avery, 1951; Prochaska *et al.*, 1998; Yankah *et al.*, 1993). Since the earliest times fermentation as a processing method was used by the Romans to produce two fish sauces, Garum and Alec (Steinkraus, 1995). Even today this method is still used to produce similar fermented products like Garos (Italy and Greece), Botargue (Italy and Greece), Shottsuru (Japan), Jeot-kal (Korea) (Lee *et al.*, 1977), Nampla (Thailand), Budu (Malaysia) (Beddows *et al.*, 1979), Bakasang (Malaysia) (Ijong & Ohta, 1996), Patis (Philippines) (Baens-Arcega, 1977) and Pissala (France) (Beddows, 1998). At present, the most frequently produced fermented fish sauce is Nuoc-Nam. This product is mainly produced in the Vietnamese province, Binh-Thuan and on the island of Phu Quoc (Steinkraus, 1995).

The world wide demand for fermented fish products exceeds the production of these products (Sanceda *et al.*, 1996). The single most limiting factor in the production is the long time periods required for the fermentation of a good quality fish sauce (Mabesa, 1987; Sanceda *et al.*, 1996), since the ageing process of the products can range from a few to 18 months, depending on the size of the fish that is used and the quality of the produced sauce (Steinkraus, 1995). This emphasises the need to develop an accelerated fermentation process that may lead to the production of high quality products in reduced production time (Beddows, 1998).

B. TRADITIONAL PRODUCTION

Fermented fish sauces and pastes are usually produced from different immature, invaluable or small fish species, including *Decapterus*, *Engraulis*, *Dorosoma*, *Clupeodus*, *Stolephorus* and shrimps (Steinkraus, 1995). The first step in the traditional production of these products is the dehydration of the fish tissue at a high salt concentration that lowers the moisture content to a level where spoilage cannot occur. A balance in the salt concentration must, however, be reached since a too high salt concentration would inhibit the growth of starter microbes, as well as the activity of proteolytic enzymes, which are partly responsible for the changes observed in the fermenting product. A too low salt concentration, on the other hand, would result in reduced cellular osmosis and unwanted microbial growth (Steinkraus, 1995). The initial salt concentration is, therefore, of great importance in determining the keeping quality of the product (Beddows, 1998).

The fish tissue also undergoes various physiological and biochemical changes during the production of fermented fish sauce (Enes-Dapkevicius *et al.*, 2000). Initially (0 – 25 d) the supernatant volume and the soluble nitrogen in the product increases. Then, during the period between 80 and 120 days, the fish tissue breaks down and the cellular proteins are hydrolysed, which is followed by the digestion of soluble proteins during the 140 – 200 day period. This changes the distribution of the nitrogenous protein compounds by transforming the soluble nitrogenous matter into amino acids and ammonia (Steinkraus, 1995).

The degradation of the fish proteins during the fermentation is in part induced by the action of endogenous enzymes (proteases) present in the fish tissue, which makes the production of fish sauce partly an autolytic process (Stefansson, 1993). The proteases are released by the cellular matter to attack the internal cell membranes and tissues of the fish with the formation of a cellular liquid (Beddows, 1998). The proteases present in the gastro-intestinal tract of the fish are especially active during proteolysis in the early months of the fermentation (Beddows, 1998; Steinkraus, 1995). The enzymes, however, vary in specificity and the production of different amino acids from identical insoluble proteins can have a profound impact on the acceptability of the product, since the concentration of individual amino acids and peptides effects the taste of the product (Steinkraus, 1995).

The proteolytic activity of the enzymes present in the fish tissue produces a liquid that can be extracted after the fermentation as a fish sauce or it can be decanted at regular intervals over a shorter production time to render a fish paste (Mizutani *et al.*, 1992). The fish paste can then be macerated and mixed with a variety of additives including fennel seeds, pepper, cinnamon, ginger, coriander, cumin and thyme, depending on the country of origin and the nature of the paste (Al-Jedah & Ali, 2000). If the fermentation period is very short and the process only softens the fish tissue, salted fish products are produced (Beddows, 1998).

The proteases present in the fermenting fish mixture can also be supplemented by the extracellular proteases produced by the microbial populations present in the product. These enzymes are active at high salt concentrations (Steinkraus, 1995) and their activity can be influenced by the fish species, the specific fish body parts used and the fishing season (Al-Jedah & Ali, 2000; Martinez, 2000).

C. ACCELERATED PRODUCTION

Various studies have been done on the acceleration of the fermentation of fish tissue, since this would reduce the production costs and possibly also enhance the quality of the product (Stefansson, 1993; Steinkraus, 1995). The addition of proteolytic enzymes derived from pineapple juice and the mycelial fungus, *Aspergillus oryzae*, has already been successfully used to accelerate the fermentation process (Dougan *et al.*, 1975; Steinkraus, 1995). Certain proteases, including Koji enzymes produced by *A. oryzae*, produce pleasant aromatic compounds such as trimethylamine and trimethylamine oxide, when present in the fermenting mixture. These compounds contribute greatly to the characteristic aroma of the end-product (Dougan *et al.*, 1975). In a study done by Ravipim-Chaveesuk *et al.* (1994) a product with a chemical and microbiological composition similar to Nampla was produced by the direct addition of proteases. They also reported that although the aroma and flavour of this enzymatic produced product corresponded to that of the fermented product, the colour of the sauce was darker. Enzymes derived from other plant, animal or microbial sources such as papain, ficin, bromelain, trypsin and chymotrypsin could also possibly be used to accelerate fish proteolysis or to improve the aroma of the product (Al-Jedah &

Ali, 2000). These attempts have, however, been reasonably unsuccessful, due to the inactivation of the proteolytic enzymes by the high salt concentration of the product (Avery, 1951).

Another attempt to accelerate the fermentation involved the addition of histamine to the fermenting mixture, which enabled the acceleration of the fish liquefaction rate without an increase in the histamine content of the product. In addition, the fermented end-product also contained elevated levels of other amino acids due to the higher degree of fish protein hydrolysis (Sanceda *et al.*, 1996). Beddows (1998) reported that although these hydrolyzed products are produced with a distribution and concentration of nitrogenous compounds similar to that of microbial fermented fish sauce, the end-product often lacks aromatic quality.

The addition of selected microbes with the ability to produce extracellular proteases to macerated fish was performed by Lubbe (2000) in an attempt to accelerate the fermentation. The selected microbes included *Lactococcus diacetylactis*, *Lactobacillus acidophilus*, *L. plantarum*, *Pediococcus cerevisiae*, *Kocuria varians*, *Cryseomonas luteola*, *Stenotrophomonas maltophilia*, *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *B. mycoides*, *B. amyloliquefaciens*, *B. lentus* and *B. licheniformis*. These microbes were found to successfully accelerate the fermentation and enhanced the quality of the end-product. In another study, different lactic acid bacteria (LAB) were tested for the ability to degrade biogenic amines in fermenting fish slurry by producing diamine oxidases. These selected microbes were able to successfully reduce the histamine content in fish silage during fermentation, thereby improving the health benefits of the fermented products (Enes-Dapkevicius *et al.*, 2000).

D. QUALITY

Fermented fish products can generally be described as a salty, clear brown liquid with a distinctive aroma (Sanceda *et al.*, 1986; Steinkraus, 1995). These products consist mainly of hydrolysed protein, minerals and calcium (Van Veen, 1965) and contains nine of the essential amino acids, which contributes to the nutritional value of the product (Steinkraus, 1995).

The characteristic aroma and flavour of fermented fish products are extremely important for consumer acceptability (Beddows, 1998). The aroma of

these products normally consists of three different flavours, ammonial, cheesy and meaty aromas. The ammonial flavour is mainly due to the presence of ammonia, trimethylamine and other amines, whereas low-molecular weight volatile fatty acids (VFA), ethanoic and *n*-butanoic acids, probably produced by LAB (Al-Jedah & Ali, 2000), are responsible for the cheesy flavour. The compounds responsible for the meaty aroma have not yet been identified (Sanceda *et al.*, 1986).

Even though the aroma of the fermented fish product is an important characteristic of the quality of the product, no standardization requirements of the final product exist (Yankah *et al.*, 1993). For example, the Food Composition Table for East Asia sets values per 100 g of high quality Nuoc-Nam at 268 kcal, 46 g moisture, 17.5 g protein, 12 g fat, 20.8 g carbohydrate, 3.4 g ash, 0.09 mg thiamine and 0.86 mg riboflavin. The composition of low quality sauce per 100 g is at 117 kcal, 75.7 g moisture, 6.8 g protein, 5.4 g fat, 9.5 g carbohydrate, 2.6 g ash, 0.03 mg thiamine and 0.27 mg riboflavin (Steinkraus, 1995).

Various factors contribute to the inconsistency of sauce quality, including different industrial processing procedures, the rate of deterioration of the fish tissues, the addition of spices (Gram, 1991; Van Veen, 1965; Yankah *et al.*, 1993) and the bacterial composition of the product (Avery, 1951). It has also been reported that, although the initial microbial population decreases rapidly during the fermentation process, certain pathogenic bacteria, including members of the genera *Bacillus* (Lubbe, 2000) and *Clostridium* may be present in the fermenting fish mixture (Beddows, 1998).

E. MICROBIAL COMPOSITION

The microbial content greatly influences the quality of fermented fish products during the fermentation (Steinkraus, 1995). The different microbial species present are known to produce volatile compounds such as methyl ketones, organic acids and carbonyl compounds, which are believed to be responsible for the characteristic aroma and flavour (Saisithi *et al.*, 1966). The most important microbes isolated from fermented fish products include *Bacillus* spp. (Crisan & Sands, 1975; Lubbe, 2000), LAB (Østergaard *et al.*, 1998), *Staphylococcus* spp. (Lubbe, 2000; Tanasupawat *et al.*, 1992), *Micrococcus* spp. (Sanchez & Klitsaneephaiboon, 1983), *Corynebacterium* spp. (Saisithi *et al.*, 1966),

Enterobacter spp. (Ijong & Ohta, 1996), *Moraxella* spp. (Ijong & Ohta, 1996) and *Pseudomonas* spp. (Sands & Crisan, 1974).

Bacillus

The genus *Bacillus* includes mesophylic Gram-positive rods that have an optimum growth temperature of 28° - 35°C and grow at pH values ranging from 4.9 – 9.3. These bacteria are primarily soil inhabitants and have the ability to form endospores that are resistant to adverse conditions (Holt *et al.*, 1994). Bacilli are chemoheterotrophic, aerobic or facultatively anaerobic and the cells are either peritrichously flagellated or non-motile (Prescott *et al.*, 1996).

This genus encompasses a wide variety of species that have been isolated from many different foods, including meats (Batt, 2000), spices (Dahl, 2000), dairy products (Larsen & Jørgensen, 1997), dried fruit (Batt, 2000) and fried or boiled rice (Hsieh *et al.*, 1999; Sutherland, 1993). *Bacillus* species isolated from fermented fish sauce are mostly capable of producing measurable amounts of metabolites and volatile acids that contribute to the characteristic aroma of the fermented product (Saisithi *et al.*, 1966). The species from this genus are usually dominant during the fermentation, possibly due to their resistance to the high salt concentrations in the product (Batt, 2000; Lubbe, 2000).

Sanchez & Klitsaneephaiboon (1983) identified a range of *Bacillus* species that could be involved in the production of aromatic compounds, including *B. pumilus*, *B. coagulans* and *B. subtilis*. Other *Bacillus* species identified from fermented fish products include *B. licheniformis* (Beddows, 1998) and *B. sphaericus* (Crisan & Sands, 1975). Other *Bacillus* species identified in different grades of Nampla are given in Table 1 (Crisan & Sands, 1975).

Lactobacillus

Lactobacillus species are characterised as non-motile, non-endospore forming, Gram-positive rods that grow in complex media at a pH range varying between 4.5 and 6.4 (Prescott *et al.*, 1996). These microbes are obligatory heterofermentative and produce a mixture of lactic acid, ethanol, acetic acid and CO₂ (Ijong & Ohta, 1996).

Table 1. *Bacillus* species present in different grades of Nampla (Crisan & Sands, 1975).

Species	First grade			Second grade	
	1 month	7 months	final	1 day	1 month
<i>B. cereus</i> I	-	+	-	-	-
<i>B. cereus</i> II	-	-	-	-	+
<i>B. circulans</i>	-	-	-	+	-
<i>B. licheniformis</i> I	+	-	+	+	-
<i>B. licheniformis</i> II	-	+	-	-	-
<i>B. megaterium</i>	-	-	+	-	-
<i>B. pumilus</i>	-	-	-	-	+
<i>B. subtilis</i>	-	-	+	-	-

During the fermentation these microbes may inhibit the growth of various pathogens and undesirable microbes, ensuring the quality of the fermented product (Avhurhi & Owens, 1990; Teixeira, 2000). This preserving action is achieved by the production of anti-bacterial substances, such as anti-bacterial peptides (bacteriocins) and lactic acid (Glatman *et al.*, 2000). The bacteriocins act only on closely related Gram-positive species, while the lactic acid has a broader action due to the resulting lower pH of the product (Østergaard *et al.*, 1998).

Lactobacilli are often used as starters in the manufacturing of fermented food products due to its tolerance of low water activity and the ability to out compete other microbes (Ijong & Ohta, 1996). These microbes are involved in the production of fermented milks (Tynkkynen *et al.*, 1999), cheeses, yoghurts (Roy *et al.*, 2000), fermented sausages (Cocolin *et al.*, 2000), fermented vegetables and sourdough (Teixeira, 2000). *Lactobacillus* spp., specifically *L. plantarum*, have also been identified as the predominant microbial group isolated from Thai fermented fish sauce products (Østergaard *et al.*, 1998).

Pediococcus

Pediococci are Gram-positive, tetrad-forming, catalase-negative cocci that are non-motile and do not form endospores. They can ferment glucose to produce lactic acid without the production of gas (Weiss, 1992). The optimum growth temperature of members of this genus is usually between 30° and 35°C, while the optimum pH range is usually between pH 7.0 - 8.0 (Ray, 1995).

Pediococcus spp. are commonly found on a great variety of plants. These microbes multiply rapidly and constitute a large part of the LAB present in the early stages of cucumber and olive fermentation (Prescott *et al.*, 1996; Weiss, 1992). Pediococci have also been isolated from fresh fish, fresh and cured meat, sausages, rice and vegetables (Prescott *et al.*, 1996; Weiss, 1992). Pediococci isolated from Thai fermented fish sauce products include *Pediococcus halophilus* (Sands & Crisan, 1974), *P. pentosaceus* and *P. acidilactici* (Tanasupawat *et al.*, 1988).

Staphylococcus

Staphylococcus species are Gram-positive, facultative anaerobic, non-motile cocci, which usually form irregular clusters. *Staphylococcus* spp. are

robust, salt-tolerant microbes (Olarte *et al.*, 1999) and have the ability to survive in air, dust and water (Martin & Landolo, 2000). These microbes are oxidase negative and can utilize glucose anaerobically to produce lactate, acetate and pyruvate (Prescott *et al.*, 1996). Acetate and CO₂ are predominant end-products under aerobic growth conditions (Kloos *et al.*, 1992).

Staphylococcus spp. are usually present on marine and terrestrial mammals as a non-aggressive member of the normal skin population but may be associated with skin infections (Harvey & Gilmour, 2000). *Staphylococcus saprophyticus*, *S. carnosus* and *S. piscifermentans* have been isolated from fermented fish products by Tanasupawat *et al.* (1992). Sanchez & Klitsaneephaiboon (1983) also isolated *Staphylococcus epidermis* from Nampla, as well as other fermented fish sauces. Saisithi *et al.* (1966) reported that the *Staphylococcus* strains isolated from fermented fish products had the ability to produce large amounts of volatile acids, which could contribute to the aroma of the end-product.

Micrococcus

The genus *Micrococcus* consists of Gram-positive cocci that divide in more than one plane to form pairs, tetrads or irregular clusters of non-motile cells. These microbes are aerobic, catalase positive and exert a strictly respiratory metabolism (Kocur *et al.*, 1992; Prescott *et al.*, 1996). Colonies of these saprophytes can be yellow, orange or red due to the production of carotenoid pigments (Kocur *et al.*, 1992). Their optimum growth temperature varies between 22° and 37°C. These microbes have the ability to exhibit proteolytic, lipolytic and esterolytic activity and can produce methanethiol, which contributes greatly to fermentation (García-Lopez *et al.*, 2000).

Species in this genus produce variacin, a bacteriocin that can act antagonistically towards several spoilage microbes, including *Listeria monocytogenes*. However, variacin can also inhibit beneficial microbial starter species like *Lactobacillus* spp. (García-Lopez *et al.*, 2000). *Micrococcus* spp. are commonly included in the initial microbial populations of raw foods, especially foods from animal origin, such as meat and cheese fermentation starters (Bhowmik & Marth, 1990; García-Lopez *et al.*, 2000; Hammes & Hertel, 1998; Kocur *et al.*, 1992). *Micrococcus varians*, *M. colpogenes* and *M. roseus* have also

been isolated from Nampla (Saisithi *et al.*, 1966; Sanchez & Klitsaneephaiboon, 1983).

Other microbes isolated from fermented fish sauces and pastes

These isolated microbial species present in fermented fish products include *Serratia* spp. (Sands & Crisan, 1974), *Clostridium* spp. (Sands & Crisan, 1974), Coryneform bacteria (Saisithi *et al.*, 1966) and *Halobacterium salinarium* (Thongthai *et al.*, 1992). Sands & Crisan (1974) also isolated *Pseudomonas* spp. from fermented fish roe, while *Moraxella* spp. have been isolated during the production of Bakasang (Ijong & Ohta, 1996) (Table 2).

Candida clausenii (Crisan & Sands, 1975), *Debaromyces hansenii* (Sands & Crisan, 1974) and *Hansenula anomala* (Sands & Crisan, 1974) are the only three yeast species that were isolated from any fermented fish products. The mycelial fungus, *Penicillium notatum* (Crisan & Sands, 1975), was isolated from Koami fish sauce, while two other mycelial fungi, *Cladosporium herbarum* (Crisan & Sands, 1975) and *Aspergillus fumigatus* (Crisan & Sands, 1975), were isolated from other fermented fish products.

F. IDENTIFICATION OF MICROBES IN FOOD

The development of fast and accurate procedures for the detection and identification of pathogenic, spoilage and starter microbes in food has become very important to the fast expanding food industry (Van der Vossen & Hofstra, 1996). The identification of microbes present in food has in the past been based mainly on morphological and physiological techniques (Grant *et al.*, 1993). However, these traditional methods of identification have limitations in that they can be time consuming and may not give an accurate reflection of the microbial composition due to the inability to culture many microbial strains (Borrell *et al.*, 1997; Ridley & Saunders, 1993). Furthermore, the phenotypic or physiological characteristics of microbes often vary under different environmental conditions (Gamage *et al.*, 1998) or may be highly variable between different strains of a species (Scheu *et al.*, 1998; Tanasupawat *et al.*, 1992).

The rapid identification of microbial species can also be performed using a variety of molecular techniques (Cocolin *et al.*, 2000; Eldar *et al.*, 1997; Masneuf *et*

Table 2. Microbial genera (Ijong & Ohta, 1996) identified during the laboratory fermentation and production of Bakasang.

Fermentation time (d)	Genera
0	<i>Pseudomonas, Enterobacter, Moraxella, Micrococcus, Streptococcus</i>
4	<i>Lactobacillus, Pseudomonas, Enterobacter, Moraxella, Micrococcus, Staphylococcus, Streptococcus</i>
10	<i>Streptococcus, Pediococcus, Micrococcus, Pseudomonas, Enterobacter</i>
20	<i>Streptococcus, Pediococcus, Micrococcus</i>
30	<i>Streptococcus, Pediococcus, Micrococcus</i>
40	<i>Streptococcus, Pediococcus, Micrococcus</i>
Commercial Bakasang	<i>Staphylococcus, Micrococcus</i>

al., 1996; Nishimura *et al.*, 1996). The advantages of these techniques include the accurate identification of specific microbes as soon as the isolates are available, with the results available within 24 h (Gautier *et al.*, 1996; Johnson *et al.*, 1995; Van der Vossen & Hofstra, 1996). Molecular techniques are also useful in the detection of potentially harmful microbes in food, which may lead to severe illnesses or food spoilage (Harsono *et al.*, 1993; Johnson *et al.*, 1995; Scheu *et al.*, 1998; Van der Vossen & Hofstra, 1996). These techniques can, therefore, be implemented at control points in food processing plants (Hielm *et al.*, 1998b) in order to limit or prevent product wastes and economical losses during production (Van der Vossen & Hofstra, 1996).

A variety of molecular techniques are available to identify microbes from a range of food products. Criteria such as the type of organism, the minimum time required for the identification process, the reproducibility, the cost and the ease of interpreting the results must be considered when selecting an appropriate identification technique (Kristjánsson *et al.*, 1994). The DNA based techniques that are often used in the identification and detection of microbes in food products will be discussed in the following sections.

Polymerase Chain Reaction (PCR)

The PCR technology has been successfully established as a highly specific microbial identification technique (Roy *et al.*, 1996; Sabat *et al.*, 2000; Sawada *et al.*, 2000). The reproducibility and simplicity of interpreting results makes it an essential tool in the detection of microbes in food (Louie *et al.*, 1996). The PCR technique has also been used for the quantification of the contaminating microbes in food (Hyman *et al.*, 2000; Mäntynen & Lindström, 1998) and the prediction of the initial counts of specific microbes present in food products, making it possible to predict the spoilage potential and shelf-life of a product (Pin *et al.*, 1999). The PCR technique can, furthermore, be used to detect very small amounts of microbial DNA (<50 ng) (Nishimura *et al.*, 1996; Van der Vossen & Hofstra, 1996) as was shown by the detection of one to five cells of *Listeria monocytogenes* in 5 g of a food product (Agersborg *et al.*, 1997).

The PCR technique has been used to identify specific microbes in meat (Cocolin *et al.*, 2000), milk products (Wan *et al.*, 2000), seafood (Jeyasekaran *et al.*, 1996; Lees *et al.*, 1994), cold-smoked salmon (Simon *et al.*, 1996), raw oysters

(Vickery *et al.*, 2000) and fermented maize dough (Hayford & Jakobsen, 1999). *Listeria monocytogenes* was also easily identified from smoked mussels (Brett *et al.*, 1998), shrimp (Destro *et al.*, 1996), mozzarella cheese, lettuce, tap and wastewater (Salzano *et al.*, 1995). *Brucella* species were identified from soft cheeses (Serpe *et al.*, 1999), while *Campylobacter* species were identified from water, sewage and meat samples by using the PCR technique (Waage *et al.*, 1999).

The specificity of the PCR reaction depends on the choice of primers, which enables the amplification of a specific region of the microbial genome (Van der Vossen & Hofstra, 1996). Different target DNA sequences have been used in the past, including genes coding for the production of toxins (Hopkins & Hilton, 2000; Mäntynen & Lindström, 1998), specific virulence factors (Dupray *et al.*, 1997; Wang & Hong, 1999), specific conserved proteins (Fujimoto *et al.*, 1994), outer membrane proteins (Fields *et al.*, 1997) and different regions of the ribosomal DNA operon (Dang & Lovell, 2000). The PCR technique also enables the detection of plasmids involved in the pathogenicity of the microbe (Cubero *et al.*, 1999) and viral pathogens that are non-cytopathic or non-culturable (Shieh *et al.*, 1999). The PCR amplification of messenger RNA (mRNA) through reverse transcriptase PCR (RT-PCR), can be used to detect viable microbes, but the short half-life and the difficulty of obtaining intact RNA limit the success of this method (Scheu *et al.*, 1998).

The PCR technique can also be applied to the identification of specific canned seafood and fish products (Sotelo *et al.*, 1993). The PCR detection of these fish species is of great value since the type of fish used determines the properties, quality and price of the final product (Mackie *et al.*, 1999). The identification of specific fish species has in the past been based on the separation of fish proteins, but the protein patterns obtained are not of use when distinguishing between closely related species or when the proteins have been destroyed during heat processing (Rehbein *et al.*, 1997). These limitations can be overcome by using the PCR techniques to amplify the DNA of canned fish, which can be severely degraded to 100 base pair (bp) fragments during the processing steps (Bossier, 1999; Rehbein *et al.*, 1999).

The PCR technique is also currently being used to detect genetically modified food products (Gachet *et al.*, 1999). Due to recent consumer concerns, it

has become important to detect the presence of foreign protein or DNA in a product to ensure the correct labelling of the product (MacCormick *et al.*, 1998). Foreign proteins in genetically modified food products are not detectable using certain standard methods of identification especially after severe heat treatments, whereas degraded foreign DNA sequences can still be detected and amplified by PCR (Gachet *et al.*, 1999). Sequences recognised in genetically modified foods include the gene coding for neomycine resistance (Beck *et al.*, 1982) and glyphosate resistance in soybeans (Meyer & Jaccaud, 1997).

The success of the PCR reaction depends on the food sample being tested, the DNA extraction method and the reaction conditions since different substances present in food can inhibit the DNA polymerase enzymes (Wilson *et al.*, 1993). These inhibitory substances include phenols, cresols, aldehydes, sucrose, ovalbumin and collagen (Kim *et al.*, 2000; Simon *et al.*, 1996). The inhibitory action of these substances include the sequestering of Mg^{2+} -ions that are essential for the activity of the *Taq* DNA polymerase (Serpe *et al.*, 1999) or binding to the target DNA thereby rendering it unavailable for enzymatic amplification (Wilson *et al.*, 1993). Enzyme inhibition can, however, partly be reversed by adjusting the concentration of the Mg^{2+} -ions in the reaction mixture and using phenol DNA extraction methods to remove the inhibitory substances (Abu Al-Soud & Rådström, 1998; Kim *et al.*, 2000). The PCR reaction can also be enhanced by the addition of different compounds, including dimethyl sulfoxide, Tween 20 and polyethylene glycol 6000, which could sensitise the ability of the technique to detect microbes in the presence of inhibitory substances (Simon *et al.*, 1996; Wang & Hong, 1999). A higher concentration of the microbes present in the food if inhibitory substances are present, also contributes to the successful identification of the microbes (Scheu *et al.*, 1998).

The PCR technique can detect the presence of a specific microbe whether the microbe is alive or dead (Scheu *et al.*, 1998). However, the detection of dead microbes in food products is undesirable and can be avoided by cell purification, concentration and culturing methods, including pre-enrichment treatment of the microbes or the dilution of the food components (Salzano *et al.*, 1995). These enrichment steps, may prove to be time consuming when quick identifications have to be made (Salzano *et al.*, 1995) and it cannot be applied to microbes that are difficult to culture (Lees *et al.*, 1994) or when toxin producing bacteria damage

the cells during isolation (Picard *et al.*, 1992). Enrichment can also be achieved by the separation of the microbes from the environment prior to PCR amplification by using immunomagnetic beads or beads coated with monoclonal antibodies targeting the microbial surface antigens (Salzano *et al.*, 1995; Van der Vossen & Hofstra, 1996). This step may also be time-consuming (Salzano *et al.*, 1995) and it has been found that non-viable microbes with intact surface antigens may also be enriched (Scheu *et al.*, 1998).

PCR-based Restriction Fragment Length Polymorphism analysis (RFLP)

PCR-based restriction fragment length polymorphism (RFLP) analysis is based on the digestion of PCR products with specific restriction enzymes to produce different sized fragments due to the number and distribution of the restriction enzyme recognition sites on the target sequence (Yamagishi *et al.*, 1999). This method is highly discriminative and is able to distinguish between microbial species and even strains that differ by only one nucleotide within the restriction site of the enzyme used (Harrington *et al.*, 1991; Nguyen & Gaillardin, 1997; Ridley & Saunders, 1993). The PCR-based RFLP technique is also technologically simple and affordable (Céspedes *et al.*, 2000) and does not require any microbial culturing steps (Studer *et al.*, 1998). It can, therefore, be used to prevent the spread of disease causing microbes by being able to rapidly locate the origin of infection (Eldar *et al.*, 1997).

The RFLP technique has been successfully applied in the fermented beverage and meat industries (Yamagishi *et al.*, 1999) by enabling the determination of the relationship between yeast starters and yeast hybrids (Masneuf *et al.*, 1998), distinguishing among brewing and non-brewing yeast strains in the wine industry (Yamagishi *et al.*, 1999) and distinguishing between psychrotrophic and psychrophilic clostridia associated with 'brown pack' spoilage of vacuum-packed products in the meat industry (Broda *et al.*, 2000). RFLPs have also been applied in epidemiological studies by providing information on the source and route of specific food epidemics (Grimm *et al.*, 1995), which then enabled the removal of the contamination and prevented the occurrence of new cases of illness (Ayling *et al.*, 1996; Carlotti & Funke, 1994; Nassar *et al.*, 1996; Rajora & Mahon, 1997; Shah & Romich, 1997). The RFLP technique has also been applied in the control regulations of the export and import of fish between different

countries, thereby restricting the distribution of fish pathogens, which include *Streptococcus iniae* (Eldar *et al.*, 1997) and *Vibrio parahaemolyticus* (Wong *et al.*, 1999).

Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is an effective electrophoresis technique with alternating electrode pairs used for the separation of high molecular weight DNA molecules (Wilson *et al.*, 1990). PFGE is used for the separation of large DNA molecules of over 1 000 kilobase pairs (kb) in size (Lortal *et al.*, 1997; Wilson *et al.*, 1990) and usually involves the embedding of bacterial cells in agarose, followed by the stripping of the cell walls to obtain intact DNA (Baxter *et al.*, 1998) and then cutting the DNA with rare cutting restriction enzymes (Gamage *et al.*, 1998; Rehberger, 1993). These fragments move through the gel while the orientation of the electrical field is continuously changed, which causes the larger fragments to take longer than the smaller fragments to realign. The fingerprints obtained are then used to identify various microbial species by reflecting variation over the entire genome of the microbe as apposed to techniques relying on only a selected DNA region (Dalsgaard *et al.*, 1996; Laconcha *et al.*, 1998; Mahalingam *et al.*, 1994; Van der Vossen & Hofstra, 1996).

PFGE-based RFLPs have also shown to be a highly sensitive and reproducible method of identification (Baqar *et al.*, 1994; Brett *et al.*, 1998; Buchrieser *et al.*, 1994; Filetici *et al.*, 1997; Mitsuda *et al.*, 1998; Proctor *et al.*, 1995) and has been used for the successful identification of specific microbial species in complex dairy products, including *Lactobacillus helveticus* from Swiss cheese (Lortal *et al.*, 1997), *Listeria monocytogenes* from an ice cream plant (Miettinen *et al.*, 1999), *Salmonella berta* associated with soft cheeses (Ellis *et al.*, 1998) and the DNA fingerprinting of dairy propionibacteria (Gautier *et al.*, 1996). In the meat and poultry industry, *Campylobacter* spp. were identified from meat and poultry (Gibson *et al.*, 2000; Madden *et al.*, 1998; Wassenaar *et al.*, 1998), *Escherichia coli* from chickens (Kariuki *et al.*, 1999), *Leuconostoc carnosum* from spoiled ham products (Björkroth *et al.*, 1998), *Campylobacter coli* from pigs (On, 1998), *Stapylococcus aureus* from broiler flocks (McCullagh *et al.*, 1998) and *Salmonella javiana* from chicken (Lee *et al.*, 1998). In the fish industry, *Listeria monocytogenes* was detected in smoked rainbow trout (Autio *et al.*, 1999) and

Clostridium botulinum was identified from trout farms (Hielm *et al.*, 1998a) using this technique. Other microbes identified using PFGE include *Vibrio vulnificus* from the environment (Tamplin *et al.*, 1996) and *Leuconostoc* strains from fermented rice cakes (Kelly *et al.*, 1995).

The availability of typing systems with a high degree of discrimination ensures the correct identification of microbes based on their ability to cause disease in epidemiological investigations (Lorenz *et al.*, 1998; Louie *et al.*, 1996; Rehbein, 1999; Tsen *et al.*, 2000). This technique can also be used to determine the distribution of various epidemiological or spoilage isolates by determining strain relatedness between different isolates with similar genotypes (Baxter *et al.*, 1998; Björkroth *et al.*, 1998; Brett *et al.*, 1998; Buchrieser *et al.*, 1994; Chachaty *et al.*, 1994; Dalsgaard *et al.*, 1996; Filetici *et al.*, 1997; Gamage *et al.*, 1998; Gautier *et al.*, 1996; Khambaty *et al.*, 1994; Lee *et al.*, 1998; Mahalingam *et al.*, 1994; Proctor *et al.*, 1995; Rehberger, 1993; Roy *et al.*, 1996; Tamplin *et al.*, 1996; Tsen *et al.*, 1999; Wassenaar *et al.*, 1998).

This technique is clearly a powerful tool for genomic characterisation and subtyping of isolates from food (He & Luchansky, 1997; Hielm *et al.*, 1998b; Filetici *et al.*, 1997; Rehberger, 1993), but the difficulty in reproducing the results (Kühn *et al.*, 1995), the requirement for technical skill to perform the experiments, the cost of the apparatus and the long time required for the analysis of the results have to be considered (Mitsuda *et al.*, 1998).

Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a PCR-based technique that randomly amplifies DNA fragments by using short, non-specific PCR primers (Destro *et al.*, 1996; Williams *et al.*, 1990). This amplification of random DNA sequences without knowledge of the genomic DNA sequence is a considerable advantage of the RAPD technique (Quicke, 1993). Results are obtained and interpreted relatively easily (Destro *et al.*, 1996; Lin *et al.*, 1996), but it is difficult to reproduce results (Dowling *et al.*, 1996).

RAPDs have been used in a wide variety of studies, including the differentiation between *Salmonella enteritidis* strains (Wilson *et al.*, 1993) and enterotoxigenic *Escherichia coli* O25:NM as the source of a food-borne outbreak (Mitsuda *et al.*, 1998), the typification of *Clostridium difficile* strains (Chachaty *et*

al., 1994) and the classification and characterisation of different cultivars of bananas, enabling the identification of the different naturally occurring variants (Bhat *et al.*, 1995). In the meat and poultry industry, *Campylobacter jejuni*, isolated from meat and chicken was typified (Hanninen *et al.*, 2000; Ono & Yamamoto, 1999), while *Listeria monocytogenes* was identified from pork products (Giovannacci *et al.*, 2000). In the dairy industry, *Bacillus cereus* was detected (Andersson *et al.*, 1999; Svensson *et al.*, 2000), a *Lactobacillus* community present in Mozzarella cheese was characterised (Morea *et al.*, 1999) and *Listeria monocytogenes* was identified from milk products (Wagner *et al.*, 2000).

G. CONCLUSION

The more productive use of fermentation technologies can lead to the stimulation of the development of a variety of new food products in developing countries (Battcock & Azam-Ali, 1998). The fermentation of a perishable raw product cost effectively preserves and provides enhanced flavour and nutritional value to these products, but it can be time consuming to ferment certain raw products since the complete proteolysis of especially fish tissue can take up to 18 months, thereby limiting the production efficiency. This problem can be overcome by the acceleration of the fermentation process by the addition of proteolytic enzymes or selected microbial species with the ability to rapidly produce high concentrations of proteolytic enzymes. The accelerated process produces an end-product with characteristics and quality similar to the traditionally fermented fish products.

The microbial content of fish mixtures is essential to the successful acceleration of the fermentation. The identification of the added microbes can be accomplished by using various morphological and physiological techniques, but these traditional techniques are normally time consuming and various microbial populations can not easily be isolated from the fermentation mixture. However, molecular techniques are highly specific and have been known to facilitate the detection and identification of microbes in fermenting food products. These rapid and reliable techniques, therefore, could be useful in monitoring the accelerated production of fermented fish products.

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CHAPTER 3

PCR-RFLP ANALYSIS OF BACTERIA ISOLATED FROM FERMENTED FISH (NUOC-NAM TYPE) PRODUCTS

Abstract

Fermented fish products are traditionally produced in the East Asian countries and include products such as Nuoc-Nam, which is characteristically a salty, clear brown fish sauce with a distinctive aroma. These products are produced by the degradation of fresh fish tissue, partly due to the action of endogenous fish proteases, as well as proteases secreted by the microbial populations present in the mixture. The microbial composition of the fermenting mixture is essential to the time required for the production and the quality of the product. The ability to identify these microbes within a few hours after isolation can assist in the development of starters that accelerate the fermentation. The bacteria present in fermented fish products were identified by the PCR amplification of a 1 300 bp fragment of part of the 16S rRNA gene directly from the bacterial cells, followed by the digestion of the amplified PCR product with the restriction enzyme, *AluI*. The DNA fingerprints for *Bacillus cereus* and *B. mycoides* and for *Staphylococcus caprae* and *S. schleiferi* proved identical, but unique in respect to the DNA fingerprints of the other species. Unique PCR-RFLPs were obtained for *Bacillus amyloliquefaciens*, *B. coagulans*, *B. megaterium*, *B. firmus*, *B. lentus*, *B. licheniformis*, *B. subtilis*, *Staphylococcus hominus*, *S. simulans*, *S. aureus*, *Sphingomonas paucimobilis*, *Kocuria varians*, *Brevibacillus brevis*, *Cryseomonas luteola*, *Vibrio alginolyticus*, *Stenotrophomonas maltophilia* and *Agrobacterium radiobacter*. This newly developed PCR-RFLP technique for the identification of bacteria present in fermented fish products will be useful in the rapid determination of the microbial quality of the end-product.

Introduction

Traditional fish products are produced by fermenting salted fresh fish (Steinkraus, 1995). The fermentation is initiated by the dehydration and proteolysis of the fresh

fish tissue by the action of endogenous and microbial proteases that are active at high salt concentrations (Beddows, 1998). These proteases are released during the initial stages of the fermentation and they attack the internal cell membranes and fish tissue to produce a product rich in amino acids (Stefansson, 1993). The microbial composition of the fermenting fish, therefore, partly determines the rate of the degeneration of the fish tissue (Crisan & Sands, 1975). The microbes present are also responsible for the aromatic quality of the fermented fish products by producing volatile compounds such as methyl ketones, organic acids and carbonyl compounds (Østergaard *et al.*, 1998; Steinkraus, 1995).

The ability to determine the microbial composition of a food product is essential in monitoring the quality of a product which can be done by using various morphological and biochemical techniques (Tanasupawat *et al.*, 1992). Conventional identification techniques are, however, time consuming and include the enrichment, isolation and subsequent identification of the microbes present in the product (Grant *et al.*, 1993). These results may not provide a true representation of the complex microbial content of fermenting products due to the inability to culture some microbes in commercially available media (Borrell *et al.*, 1997; Gamage *et al.*, 1998; Yamagishi *et al.*, 1999). The use of specific molecular techniques for identification, in contrast to conventional methods, enables the rapid determination of the microbial content of a food product with reliable results available within a few hours (Cocolin *et al.*, 2000; Gautier *et al.*, 1996; Johnson *et al.*, 1995; Masneuf *et al.*, 1998). One such technique is the polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLPs) and has successfully been used to identify microbes present in fermented food products (Bell *et al.*, 1999). The 16S ribosomal RNA (rRNA) genes are essential to protein synthesis and have a conserved function. These genes are, therefore, useful in the identification of bacterial species (Broda *et al.*, 2000; Studer *et al.*, 1998; Woese, 1987; Zumárraga *et al.*, 1999). The aim of this study was to identify bacteria isolated from fermented fish (Nuoc-Nam type) products using PCR-RFLP DNA fingerprinting.

Materials and methods

Bacterial strains and culturing conditions

The bacteria used in this study, previously isolated from fermented fish (Nuoc-Nam type) products (Lubbe, 2000), were obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC), Stellenbosch, South Africa (Table 1). The isolates were cultivated on Plate Count Agar (PCA) (Biolab) and incubated at 35°C for 2 - 3 d.

PCR-RFLP analysis

The PCR amplifications were performed directly on the bacterial cells of all the isolates studied. This approach, however, was unsuccessful in the amplification of the *Bacillus amyloliquefaciens* (1191) and *B. coagulans* (1208) strains and the PCR amplification of these isolates was then rather performed on extracted DNA.

The DNA was extracted using the method of Van Elsas *et al.* (1997) from two of the isolates, *Bacillus amyloliquefaciens* (1191) and *B. coagulans* (1208) by adding sterile glass beads (diameter = 0.1 – 0.11 mm), 120 mM phosphate buffer (1 part 120 mM NaH₂PO₄ : 9 parts 120 mM Na₂HPO₄, pH 8.0) and 20% (w/v) sodium dodecyl sulphate (SDS) to the bacterial cells. The mixture was vortexed for 1 min and incubated at 60°C for 20 min. This step was repeated twice. The mixture was centrifuged at 4 000 rpm for 5 min, the water phase extracted with phenol and then with a phenol:chloroform:isoamylalcohol (25:24:1) mixture until the interphase was clean. The DNA was precipitated with 0.1 volume 3 M NaAc (pH 5.5) and 0.6 volume isopropanol. The DNA was then pelletised by centrifugation at 12 000 rpm for 10 min, washed with 70% ethanol, dried and redissolved in 100µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

A part of the 16S rRNA gene of all the isolates was amplified using the primers VIS 1 (5' GGC GGA CGG GTG AGT AA '3) and VIS 2 (5' GGG CGG TGT GTA CAA G '3). The PCR reactions (50 µl) contained 1 x PCR *Taq* DNA polymerase buffer (Bioline Technologies), 2 mM MgCl₂, 1 mM dNTPs (Promega) and 0.5 µM of each primer. Cells of each isolate were added to the PCR reaction before 1U *Taq* DNA polymerase (Bioline Technologies) was added. The extracted DNA (1 µl) of both *B. amyloliquefaciens* and *B. coagulans* was used in the PCR

Table 1. Bacteria isolated from fermented fish (Nuoc-Nam type) products (Lubbe, 2000) and the respective predicted PCR and RFLP fragments after digestion with *AluI*, based on the 16S rRNA gene sequence available on the GenBank database and the actual PCR and RFLP fragments obtained.

Isolated Microbes	USFSCC ¹ number	GenBank Accession Numbers	PCR product (bp)	Predicted fragment sizes (bp) after <i>AluI</i> digestion	Observed fragments sizes (bp) after <i>AluI</i> digestion ²
<i>Bacillus mycoides</i>	1200	Z84591, AF144645	1314	553, 224, 186, 154, 70, 47, 38, 21, 21	550, 220, 180, 150
<i>Bacillus cereus</i>	1198	Z84590, Z84589, X55060	1310	551, 224, 186, 154, 69, 58, 47, 21	550, 220, 180, 150
<i>Bacillus amyloliquefaciens</i>	1191	X60605	1307	632, 265, 186, 154, 70	600, 220, 180, 150
<i>Bacillus coagulans</i>	1208	D78313, D16267	1314	617, 334, 209, 154	620, 280, 210
<i>Bacillus megaterium</i>	1210	AB022310, Y15052	1312	615, 265, 209, 154, 69	620, 240, 180, 150
<i>Bacillus firmus</i>	1045	D78314, D16268	1314	824, 335, 104, 51	280, 410, 220, 180, 150
<i>Bacillus lentus</i>	1242	AB022661, D78315	1314	824, 207, 128, 89, 66	420, 280, 210, 190, 150
<i>Bacillus licheniformis</i>	1202	Y15047, AF035431	1313	823, 335, 155	280, 250, 190, 170
<i>Bacillus subtilis</i>	1190	AJ288308, AF047177	1412	430, 265, 207, 186, 154, 100, 70	400, 280, 200, 180
<i>Staphylococcus caprae</i>	1235	Z26890, Y12593	1312	615, 214, 209, 87, 70, 66, 51	620, 220, 180, 150
<i>Staphylococcus hominus</i>	1194	X66101, Z26905	1310	615, 214, 207, 87, 70, 66, 51	620, 220, 180
<i>Staphylococcus schleiferi</i>	1211	AB009945, D83372	1312	429, 214, 209, 186, 153, 70, 51	620, 220, 180, 150
<i>Staphylococcus simulans</i>	1195	AF128291, U90027	1312	615, 214, 160, 153, 70, 51, 49	620, 220, 190, 150
<i>Sphingomonas paucimobilis</i>	1226	D38428, D38422	1345	+212, 370, 334, 211, 144, 32	620, 380, 220, 190, 150
<i>Staphylococcus aureus</i>	1193	-	-	-	620, 280, 220, 180, 150
<i>Kocuria varians</i>	1043	X87754	1289	310, 279, 210, 207, 148, 133	310, 210, 150
<i>Brevibacillus brevis</i>	1046	AF106606, D78457	1311	403, 335, 212, 161, 154, 46	620, 210
<i>Cryseomonas luteola</i>	1241	D84002, D84003	1324	385, 231, 207, 144, 137, 128, 58, 34	620, 230, 190, 150
<i>Vibrio alginolyticus</i>	1231	D11290, D11292	1345	599, 338, 222, 144, 42	610, 280, 220, 180, 150
<i>Stenotrophomonas maltophilia</i>	1214	AJ293474	1304	338, 276, 207, 169, 144, 128, 42	610, 280, 250, 180, 150
<i>Agrobacterium radiobacter</i>	1222	-	-	-	800, 280, 180, 100

1 – University of Stellenbosch Food Science Culture Collection, Department of Food Science, University of Stellenbosch, Stellenbosch.

2 – Fragments smaller than 100 bp were not taken into consideration.

amplification reactions. The PCR reactions were performed on a Mastercycler (Eppendorf, Germany) with initial denaturation at 95°C for 2 min. Further denaturation was performed at 95°C for 1 min, primer annealing took place at 58°C for 1 min, followed by chain elongation at 72°C for 2 min. These three steps were repeated for 35 cycles. Final chain elongation was performed at 72°C for 10 min and the PCR reactions were then cooled to 4°C. The PCR products were separated on a 1% (w/v) agarose gel containing ethidium bromide using 0.5 x TBE electrophoresis buffer and visualised under UV light.

The PCR products were digested by adding 1U *AluI* (Promega) to 5 µl of the PCR product followed by the incubation at 37°C for 3 h. Digestion products were then separated on 2.5% (w/v) agarose gels containing ethidium bromide. Restriction patterns were visualised under UV light and the separated PCR-RFLP fragments were analysed. Fragments smaller than 100 bp were not taken into consideration.

Results and discussion

PCR-RFLP analysis

The specific PCR primers (VIS 1 and VIS 2) were designed based on aligned 16S rRNA gene sequences of specific reference strains obtained from the GenBank database (Table 1). These primers amplify a 1 300 bp fragment (base 93 to base 1 400) of the 16S rRNA gene of the studied isolates (Table 1). The targeted 1 300 bp PCR fragment of all the isolates studied were successfully amplified (Fig. 1).

The expected PCR fragment sizes for *Staphylococcus aureus* and *Agrobacterium radiobacter* could not be determined, as no sequence data of the 16S rRNA genes of these species are available in the GenBank database. The amplification of the *Staphylococcus aureus* isolate produced a fragment (1 500 bp) that was larger than the predicted size of the PCR fragments for the other isolates. Only a part of the 16S rRNA gene sequence of the target fragment of *Sphingomonas paucimobilis* was available on the GenBank database (accession numbers D38428, D38422) and only one primer annealing site could be allocated. However, the PCR amplification of the 1 300 bp fragment of *Sphingomonas*

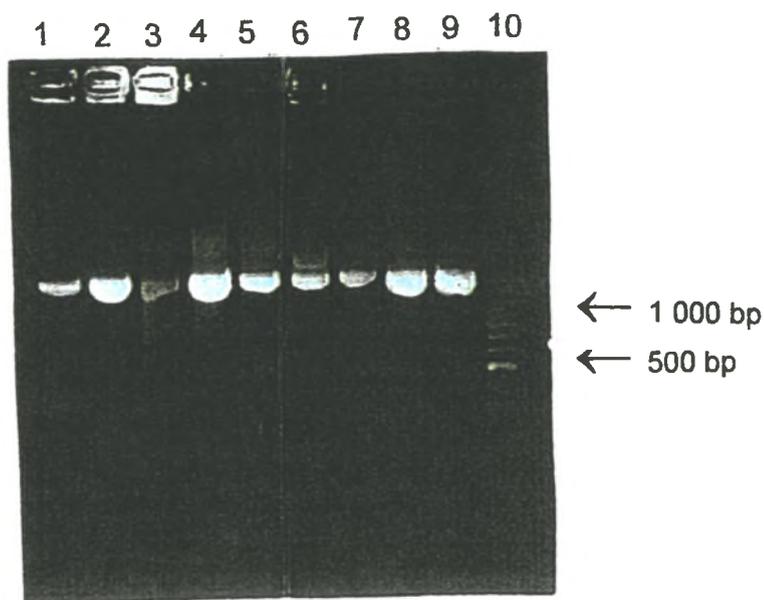


Figure 1. The PCR amplification of a 1 300 bp fragment of the 16S rRNA gene of the *Bacillus* strains isolated from fermented fish (Nuoc nam type) products (Lubbe, 2000). The fragments were separated on a 1% (w/v) agarose gel. In lane 1 – *Bacillus mycoides* (1200), lane 2 – *B. cereus* (1198), lane 3 – *B. amyloliquefaciens* (1191), lane 4 – *B. coagulans* (1208), lane 5 – *B. megaterium* (1210), lane 6 – *B. firmus* (1045), lane 7 – *B. lentus* (1242), lane 8 – *B. licheniformis* (1202), lane 9 – *B. subtilis* (1190) and lane 10 – molecular marker – 100 bp ladder (Promega).

paucimobilis was successful. The PCR amplification of *Bacillus amyloliquefaciens* and *B. coagulans* from their extracted DNA also produced 1 300 bp fragments after the PCR amplification of the isolated DNA (Fig.1). The failure of the PCR amplification performed directly on these bacterial isolates could have been either due to insufficient denaturation of the cells at the initial stages of amplification or to the ability of the outer membrane of these two species to endure high temperatures (Maede & Stark, 2000).

The PCR-RFLP technique proved useful in distinguishing the isolates from the Nuoc-Nam type products by producing unique patterns for 17 of the bacterial isolates. Identical patterns were obtained for two *Bacillus* strains (1200 and 1198) and two *Staphylococcus* strains (1235 and 1211). The predicted PCR-RFLP fragments based on the 16S rRNA gene sequences obtained from GenBank (Table 1) and the actual fragments obtained after the digestion of the PCR product with the restriction enzyme, *AluI* (Table 1), are presented in Fig. 2. Restriction digests using *AluI* produced unique patterns (given in bp) for *B. amyloliquefaciens* strain 1191 (600, 220, 180, 150), *B. coagulans* strain 1208 (620, 280, 210), *B. megaterium* strain 1210 (620, 240, 180, 150), *B. firmus* strain 1045 (410, 280, 220, 180, 150), *B. lentus* strain 1242 (420, 280, 210, 190, 150), *B. licheniformis* strain 1202 (280, 250, 190, 170), *B. subtilis* strain 1190 (400, 280, 200, 180), *Staphylococcus simulans* strain 1195 (620, 220, 190, 150), *S. aureus* strain 1193 (620, 280, 220, 180, 150), *Sphingomonas paucimobilis* strain 1226 (620, 380, 220, 190, 150), *Kocuria varians* strain 1043 (310, 210, 150), *Brevibacillus brevis* strain 1046 (620, 210), *Cryseomonas luteola* strain 1241 (620, 230, 190, 150), *Vibrio alginolyticus* strain 1231 (610, 280, 220, 180, 150), *Stenotrophomonas maltophilia* strain 1214 (620, 280, 250, 180, 150) and *Agrobacterium radiobacter* strain 1222 (800, 280, 180, 100) (Fig. 3 and 4; Table 1).

All the isolates were expected to have unique RFLP patterns based on the DNA sequence data as submitted to GenBank, with the exception of *B. mycoides* strain 1200 and *B. cereus* strain 1198. The PCR-RFLPs of both of these species produced fragments of 550, 200, 180 and 150 bp in size (Table 1). The similar sequence data of these strains indicates the very close phylogenetic relatedness of the two species (Lubbe, 2000; Sanz *et al.*, 1998). The inability to distinguish between these strains of the genus *Bacillus* based on their 16S rRNA genes can be attributed to the possible phylogenetic divergence of these species (Fox *et al.*,

Figure 2. The predicted *AluI* restriction enzyme maps of the isolates based on the GenBank database (above the line) and the observed PCR-RFLP fragments larger than 100 bp (below the line).

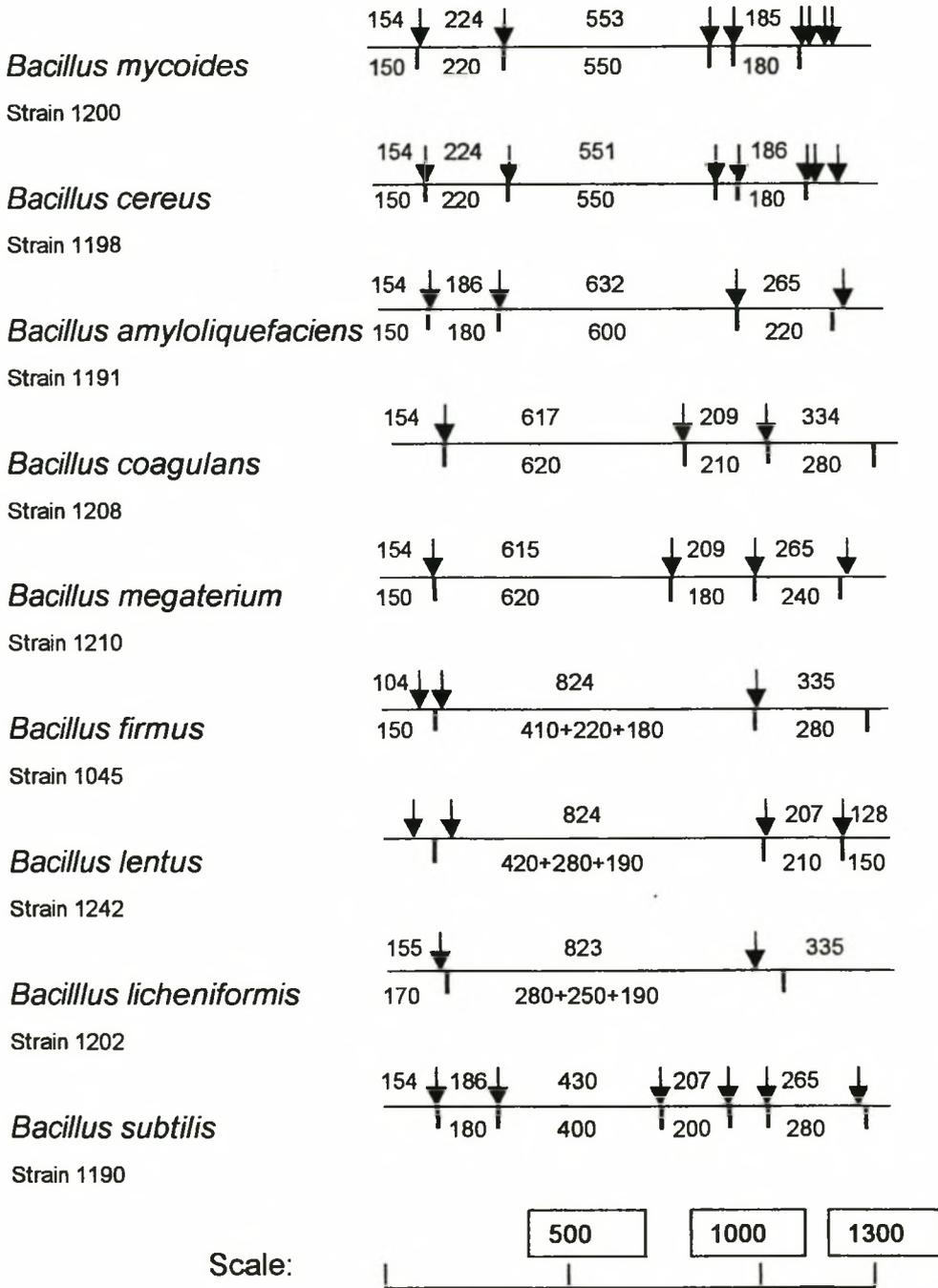
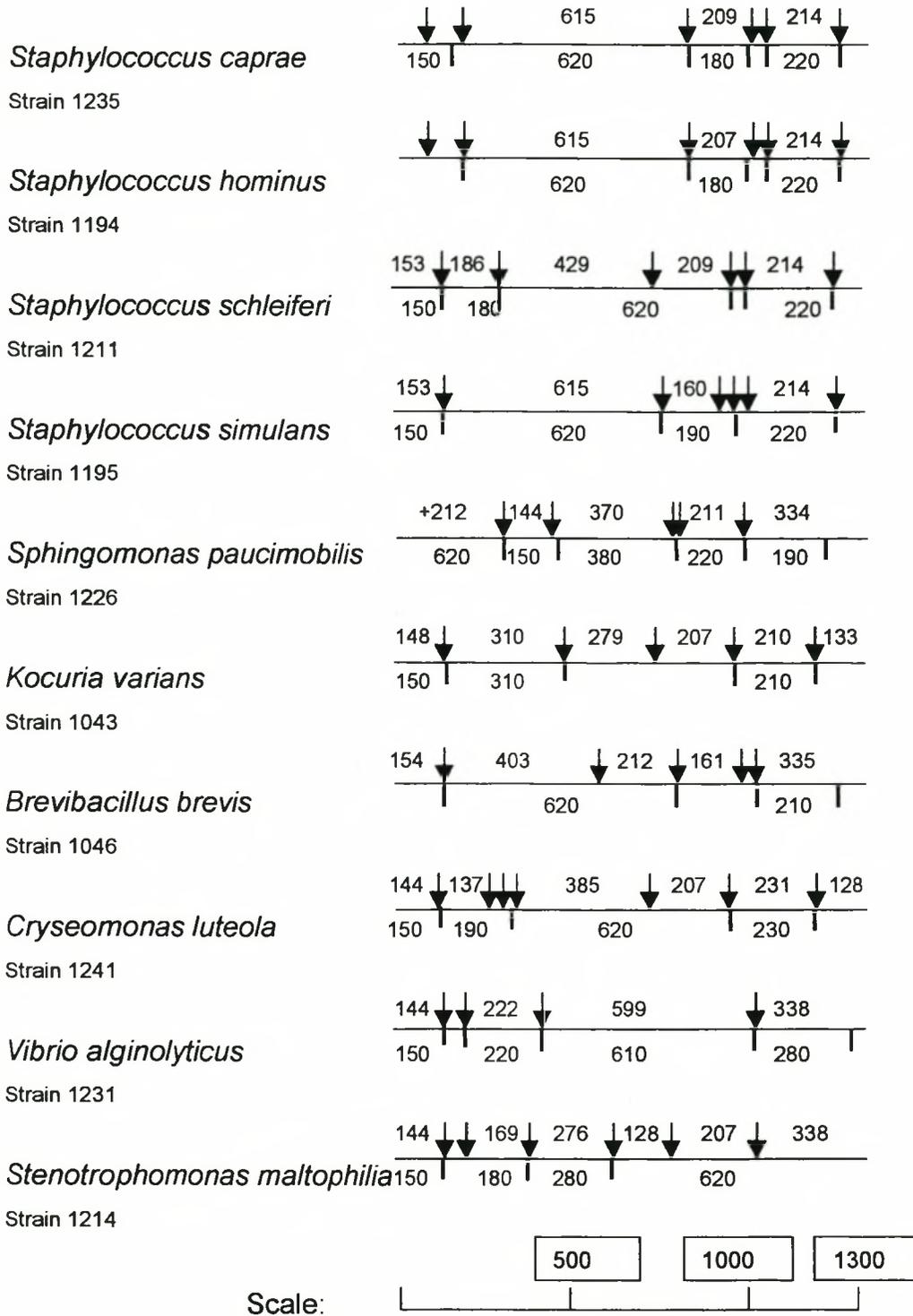


Figure 2. (continue)



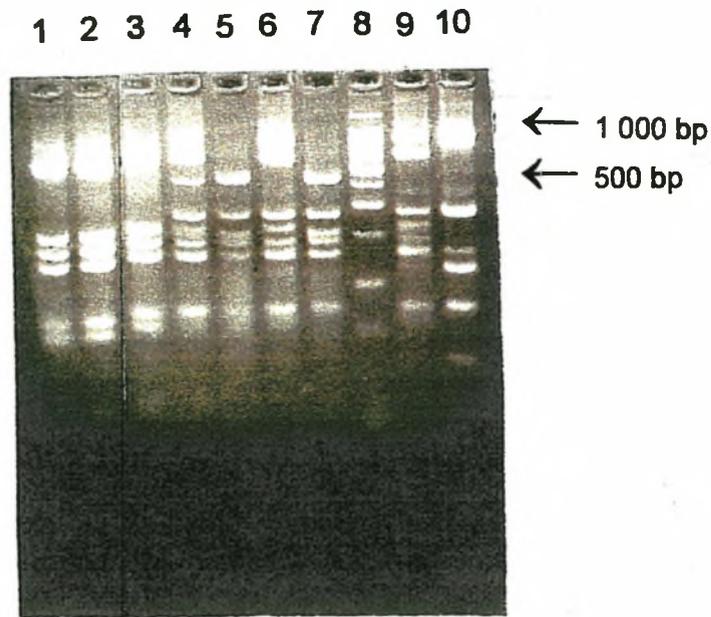


Figure 3. The PCR-RFLPs of the bacteria isolated from fermented fish (Nuoc-Nam type) products. The fragments were separated on a 2.5% (w/v) agarose gel and scored against a 100 bp ladder (Promega). In lane 1 – *Bacillus mycoides* (1200), lane 2 – *B. cereus* (1198), lane 3 – *B. megaterium* (1210), lane 4 – *B. firmus* (1045), lane 5 – *B. lentus* (1242), lane 6 – *B. licheniformis* (1202), lane 7 – *B. subtilis* (1190), lane 8 – molecular marker - 100 bp ladder, lane 9 – *Stenotrophomonas maltophilia* (1214) and lane 10 – *Agrobacterium radiobacter* (1222).

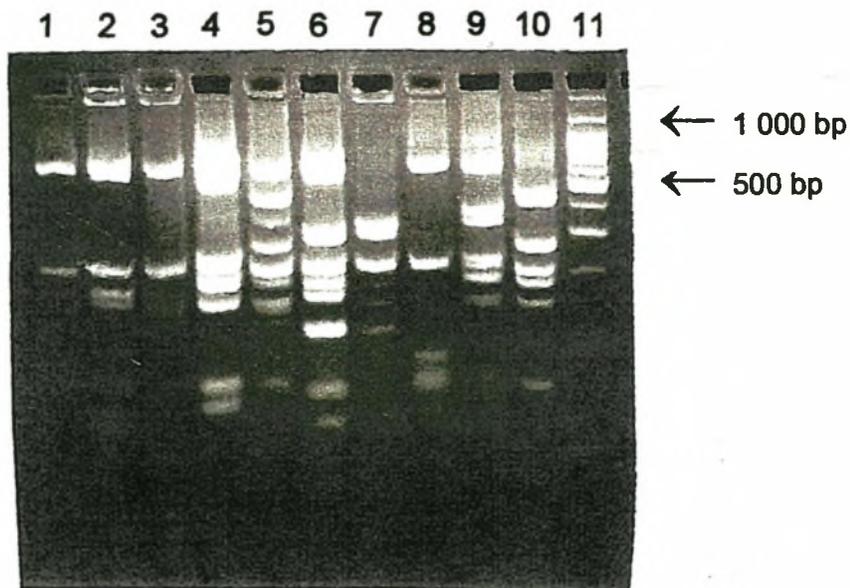


Figure 4. The PCR-RFLPs of the bacteria isolated from fermented fish (Nuoc-Nam type) products. The fragments were separated on a 2.5% (w/v) agarose gel and scored against a 100 bp ladder (Promega). In lane 1 – *Staphylococcus caprae* (1235), lane 2 – *S. hominis* (1194), lane 3 – *S. schleiferi* (1211), lane 4 – *Staphylococcus simulans* (1195), lane 5 – *Sphingomonas paucimobilis* (1226), lane 6 – *S. aureus* (1193), lane 7 – *Kocuria varians* (1043), lane 8 – *Brevibacillus brevis* (1046), lane 9 – *Cryseomonas luteola* (1241), lane 10 – *Vibrio alginolyticus* (1231), lane 11 – molecular marker - 100 bp ladder.

1992). Identical PCR-RFLP patterns were also obtained for *Staphylococcus caprae* strain 1235 and *S. schleiferi* strain 1211 (620, 220, 180, 150 bp), which also suggests that these two species are phylogenetically closely related.

After the restriction enzyme digestion of the amplified PCR fragments of the fingerprints were found to differ from the predicted PCR-RFLP fragments based on the DNA sequences obtained from GenBank. The restriction fragments were either larger or smaller than expected or completely absent. This could be due to DNA sequence difference in the 16S rRNA genes between strains. These differences in DNA sequences could also be due to incorrect sequences as submitted to GenBank. The following isolates gave PCR-RFLP fragments larger than expected from the sequence data due to the loss of a restriction site and included *S. caprae* strain 1235 (150 bp vs 66 bp and 87 bp fragments), *S. schleiferi* strain 1211 (620 bp fragment vs 429 bp and 209 bp fragments), *S. simulans* strain 1195 (190 bp vs 160 bp and 49 bp fragments), *Brevibacillus brevis* strain 1046 (620 bp vs 403 bp and 212 bp fragments), *Cryseomonas luteola* strain 1241 (190 bp vs 137 bp and 58 bp fragments; 620 bp vs 385 bp and 207bp fragments) and *Stenotrophomonas maltophilia* strain 1214 (620 bp vs 207 bp and 338 bp fragments). Smaller fragments were observed in the PCR-RFLPs of *B. firmus* strain 1045 (410 bp, 220 bp and 180 bp fragments obtained vs the predicted 824 bp fragment), *B. lentus* 1242 strain (420 bp, 220 bp and 190 bp fragments vs 824 bp fragment), *B. licheniformis* strain 1202 (250 bp and 190 bp fragments vs 823 bp fragment) and *Brevibacillus brevis* strain 1046 (210 bp vs 335 bp fragment). The absence of predicted fragments larger than 100 bp was also observed during the PCR-RFLP analysis of *Bacillus coagulans* strain 1208 (154 bp), *B. subtilis* strain 1190 (154 bp and 100 bp), *Kocuria varians* strain 1043 (279 bp and 133 bp), *Brevibacillus brevis* strain 1046 (154 bp and 161 bp) and *Cryseomonas luteola* strain 1241 (128 bp).

Conclusions

The PCR-RFLPs developed in this study enabled the rapid and reproducible identification of a diverse group of bacterial isolates that were obtained from fermented fish (Nuoc-Nam type) products. The use of PCR-RFLPs in the food fermentation industry can possibly assist in the commercial production

of high quality end-products by rapidly and accurately determining the specific microbes present in the fermentation mixture during the different stages of the production (Lubbe, 2000). The rapid identification of potential spoilage or pathogenic microbes present in fermented products may prove economically beneficial to the fish fermentation industry. The sensitivity and simplicity of this technique may also be useful in food safety control in a variety of other food processing technologies. However, this technique must still be evaluated in at least laboratory scale level.

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CHAPTER 4

ACCELERATED FISH FERMENTATION BY THE INOCULATION WITH SELECTED STARTER BACTERIA

Abstract

The production of fermented fish products is a time consuming process, which can be accelerated by the addition of selected bacteria to the fermenting mixtures. In this study macerated trout was inoculated with selected bacteria, including lactic acid bacteria (LAB) and bacteria with high proteolytic activity, on days 0, 8, 15 and 23 of a 30 d fermentation. The quality of the fermenting product was determined by measuring the pH, titratable acidity (%TA) and free amino nitrogen (FAN) on days 0, 15 and 30. The LAB included a combination of *Lactobacillus plantarum*, *Lactococcus diacetylactis* and *Pediococcus cerevisiae*. These bacteria contributed to the fermentation by resulting in an increase in the %TA. The presence of LAB did, however, appear to limit the production of FAN in the product and had no apparent effect on the decrease in the pH as compared to the control samples. The concentration of LAB present in the fermenting fish products decreased during the fermentation, regardless of the continual inoculations. Macerated fish was also inoculated with bacteria with high proteolytic activity, which included strains of either *Kocuria varians*, *Bacillus subtilis*, *B. amyloliquefaciens* strains or a combination of the strains. The addition of these bacterial species resulted in a slightly accelerated decrease in the pH and increase in the %TA of the inoculated samples when compared to the control samples. The inoculation also resulted in a considerable acceleration of the FAN production in the fermenting product. The concentration of the inoculated bacteria with high proteolytic activity either decreased or increased in conjunction with the inoculation intervals. This was observed in all the samples except for the samples inoculated with *Kocuria varians* where a dramatic decrease in the concentration of *K. varians* was observed early in the fermentation followed by an almost total elimination at the end of the study. The LAB and bacteria with high proteolytic activity were successfully identified in the fermenting fish using a PCR-RFLP technique. Results from this study show that the bacteria with high proteolytic

activity can be useful in the development of industrial starters to accelerate the production of fermented fish products.

Introduction

The production of fermented fish products is initiated by the dehydration of the fish tissue at a high salt concentration (Sanceda, 1996). This enhances cellular osmosis and lowers the moisture content of the product to a level where spoilage is minimised (Beddows, 1998). During the fermentation, the fish proteins are hydrolysed to amino acids and other low molecular weight products (Van Veen, 1965). The degradation of fish tissue and the subsequent production of cellular liquid is mainly due to the activity of endogenous hydrolytic proteases or proteases produced by microbes present in the fermenting fish mixture (Steinkraus, 1995; Stefansson, 1993). Microbes such as *Pseudomonas* and *Acinetobacter-Moraxella* types, usually present on the gills and the intestines of the fish, have the ability to produce high concentrations of proteases (Herbert, 1971; Shaw & Shewan, 1968). These enzymes are active at variable salt concentrations and a wide range of temperatures varying from 30° - 40°C (Chaiyanan *et al.*, 1999; Steinkraus, 1995).

The production of fermented fish products is time consuming (Steinkraus, 1995) and the acceleration of the fermentation would result in the production of a more cost-effective product (Stefansson, 1993). This could be achieved by the addition of proteolytic enzymes to the fish mixtures during fermentation (Al-Jedah & Ali, 2000; Cha & Cadwallader, 1998; Steinkraus, 1995). The fermenting fish could also be inoculated with bacteria that have the ability to produce high concentrations of extracellular proteases (Crisan & Sands, 1975; Lubbe, 2000; Prochaska *et al.*, 1999). Bacteria with the additional ability to cause a rapid decrease in the pH of the fish during the early stages of the fermentation will ensure the microbial safety of the end-product (Beddows, 1998; Østergaard *et al.*, 1998).

The aim of this study was to accelerate the laboratory production of fermented fish sauce and paste produced from fresh water rainbow trout by the inoculation of the fish tissue with lactic acid bacteria (LAB) and other bacteria with high proteolytic activity. The progress of the fermentation was monitored over a

period of 30 d by determining the pH, titratable acidity (%TA) and free amino nitrogen (FAN) of the fermenting fish, and the survival rates of the inoculated bacteria at different stages during the production. PCR-RFLPs were used to confirm that the inoculated bacteria were still present in the fish fermentation mixture by day 30.

Materials and methods

Fish preparation

Fresh rainbow trout (*Oncorhynchus mykiss*) was obtained from the Jonkershoek Trout Farm, University of Stellenbosch, Stellenbosch, South Africa. The fish were gutted, skinned and filleted and the fish tissue minced in a chemically sterilised mincer. A 100 g of macerated fish tissue was mixed with salt (100 g coarse sea salt per kg), glucose (50 g per kg) and 150 ml sterile distilled water, mixed in a sterile Warring commercial blender (Warring Products Corporation, USA) and placed in sterile 250 ml containers.

Bacterial inoculations

The bacterial strains used in this study were obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC), Stellenbosch, South Africa. The LAB strains included *Lactobacillus plantarum* (strain 226), *Lactococcus diacetylactis* (strain 140) and *Pediococcus cerevisiae* (strain 407), while the isolates with high proteolytic activity included *Kocuria varians* (strain 1043), *Bacillus subtilis* (strain 1190) and *B. amyloliquifaciens* (strains 1191 and 1239). The LAB strains were incubated in MRS broth (Biolab) at 30°C for 1 to 2 d and the bacterial strains with high proteolytic activity were incubated in Nutrient Broth (NB) (Biolab) with 2% (m/v) D-glucose (Biolab) added, at 35°C for 2 d. The cells were centrifuged and resuspended in sterile MRS and NA, to give a concentration of $\pm 1 \times 10^8$ cfu.ml⁻¹. These concentrations were determined using a spectrophotometer (Spectronic 20, Genesys, USA) according to the method described by Lubbe (2000). The correct microbial cell suspensions were then centrifuged at 1.5 g for 10 min and the cells were washed and resuspended in 100 µl saline solution (0.85% (w/v) NaCl).

Macerated triplicate fish samples of 100 g each were inoculated with 100 µl of a combination of the LAB strains (*Lactobacillus plantarum*, *Lactococcus diacetyllactis* and *Pediococcus cerevisiae*) (1:1:1) on days 1, 8, 15 and 23 of the fermentation. One sample was not inoculated with any starter organisms and served as the control. The study was repeated twice.

Additional 100 g duplicate macerated fish samples were inoculated with 100 µl of each of the bacteria with high proteolytic activity (*K. varians*, *B. subtilis* and *B. amyloliquefaciens*, respectively). One sample was also inoculated with 100 µl of a combination of the three bacterial strains. Inoculations were done on days 1, 8, 15 and 23 of the fermentation. Uninoculated control samples were included.

Inoculum survival

A dilution series (10^{-1} - 10^{-8}) of samples from each inoculated and control fish mixture was plated out in duplicate a day before and after each inoculation. The dilutions of the samples inoculated with LAB were plated on MRS and incubated at 30°C for 1 to 2 d, while the samples inoculated with the bacteria with high proteolytic activity were plated on Plate Count Agar (PCA) (Biolab) and incubated at 35°C for 12 – 18 h.

pH, titratable acidity (%TA) and free amino nitrogen (FAN)

The pH of 30 ml of decanted fermented fish samples was measured on days 0, 15 and 30 using a Knick pH meter (pHB-4, Merck). The %TA was determined by adding phenolphthalein (Roche) to 10 ml of the sauce and then titrating with 0.1 N NaOH until a colour change was observed and the %TA calculated according to the method of Dickson (1973). The FAN content of each sample was determined by adding neutralised phenolphthalein-phenol solution to a 20 ml sample and titrating with 0.2 N Ba(OH)₂ until a colour change was observed, as described in the AOAC (1975) method.

PCR-RFLPs

Part of the 16S ribosomal RNA gene was amplified from the pure cultures of *L. plantarum*, *L. diacetyllactis*, *P. cerevisiae*, *K. varians*, *B. subtilis* and *B. amyloliquefaciens* (as described in Chapter 3 of this thesis). PCR amplifications were also done on the bacterial colonies isolated from the dilution series of the

LAB and the bacteria with high proteolytic activity after 30 d of fermentation. This amplification was accomplished with primers VIS 1 (5' GGC GGA CGG GTG AGT AA '3) and VIS 2 (5' GGG CGG TGT GTA CAA G '3). The PCR reactions (50 μ l total volume) contained 1 x *Taq* DNA polymerase buffer (Bioline Technologies), 2 mM $MgCl_2$, 1 mM dNTPs (Promega) and 0.5 μ M of each primer. Cells of each isolate were added to the PCR reaction without the extraction of DNA, before 1U *Taq* DNA polymerase (Bioline Technologies) was added. The PCR amplifications were performed on a Mastercycler (Eppendorf) with initial denaturation at 95°C for 2 min. Further denaturation was performed at 95°C for 1 min, primer annealing took place at 58°C for 1 min, followed by chain elongation at 72°C for 2 min. These amplification steps were repeated for 35 cycles. Final chain elongation was performed at 72°C for 10 min and the PCR reactions were then cooled down to 4°C. The PCR products were separated using a 1% (w/v) agarose gel containing ethidium bromide and 0.5 x TBE electrophoresis buffer and visualised under UV light.

The PCR products were digested by adding 1U *AluI* (Promega) to 4 μ l of the PCR product followed by the incubation at 37°C for 3 hours. The digested PCR products were separated on 2.5% (w/v) agarose gels containing ethidium bromide. Restriction patterns were visualised under UV light and scored. Fragments smaller than 100 bp were not taken into consideration.

Results and discussion

First control samples (not inoculated with LAB)

The LAB present in the control samples (not inoculated) decreased from 75×10^6 to 1×10^6 cfu.ml⁻¹ during the first 2 days of fermentation, increased to 6×10^6 cfu.ml⁻¹ by day 7 and then decreased to low final viable counts of 1×10^2 cfu.ml⁻¹ towards the end of the fermentation (Fig. 1).

pH - The pH of the control samples decreased from 5.8 to 3.8 by day 15 and slightly increased to 3.9 by day 30 (Fig. 2 and Table 1). The slight increase in pH could be due to the inability of the microbial population present in the fermenting fish mixture to maintain a low pH in the product.

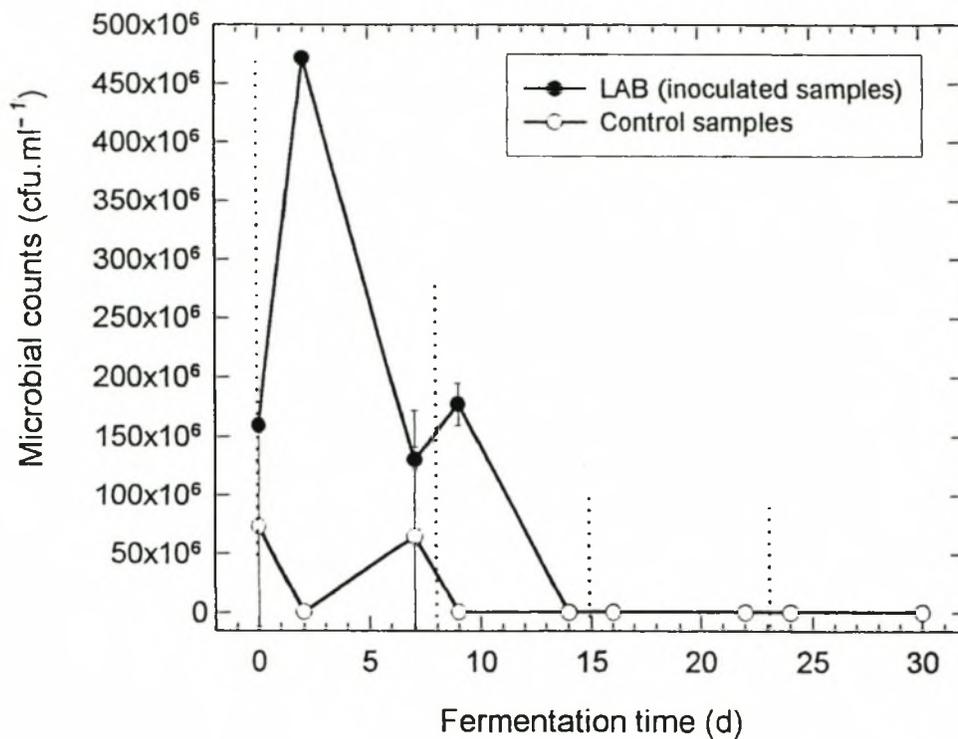


Figure 1. Survival rate (cfu.ml⁻¹) of LAB during the 30 days of fermentation. The fish mixtures (in triplicate) were inoculated on days 0, 8, 15 and 23 with a combination of *Lactobacillus plantarum* (226), *Lactococcus diacetylactis* (140) and *Pediococcus cerevisiae* (407). The dotted lines indicate reinoculations. The standard deviation was used as the error bar length.

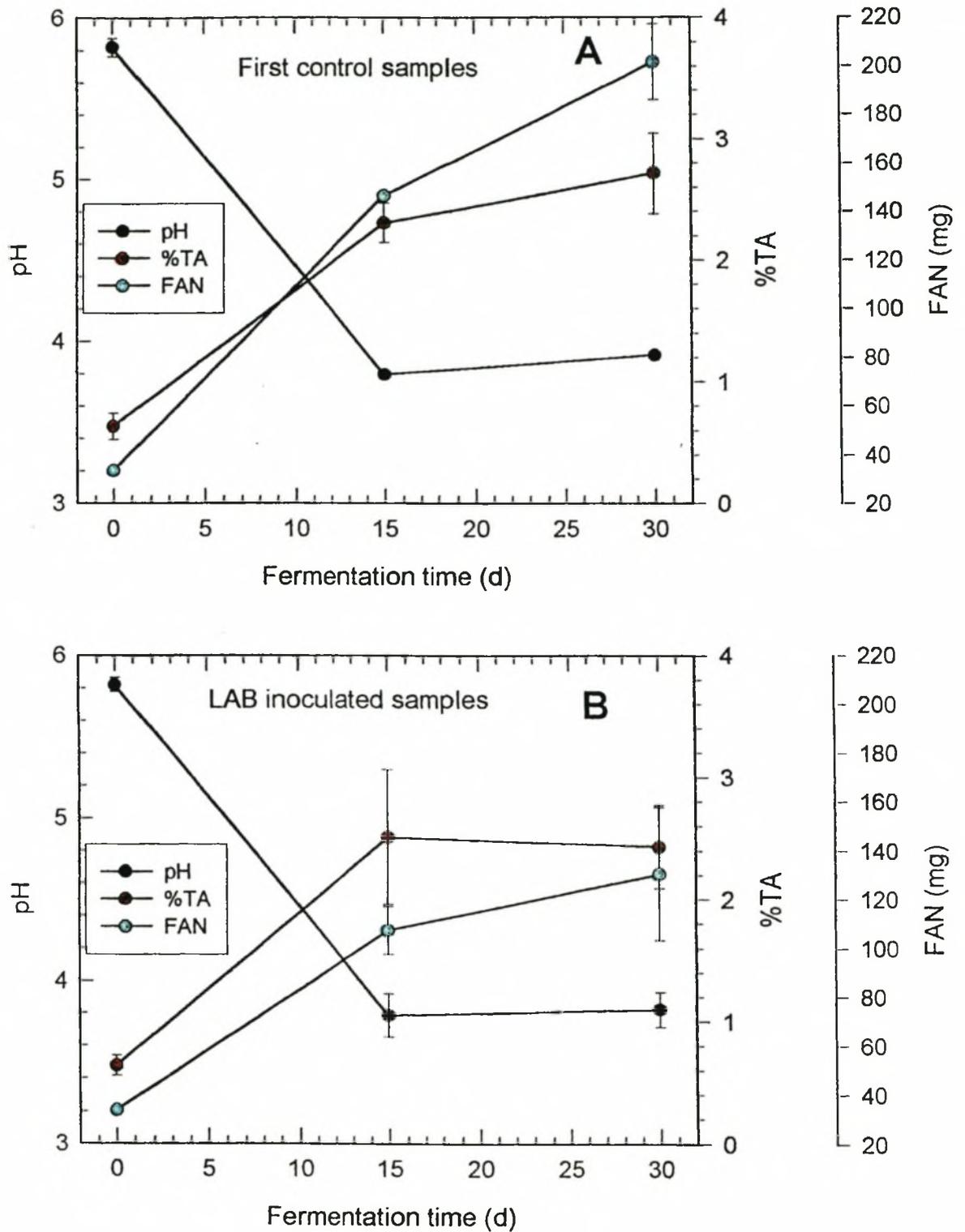


Figure 2. The changes in pH, %TA and FAN of the fermenting fish mixtures over the fermentation period of 30 days. **A** – first control samples (not inoculated with LAB) and **B** – samples inoculated with LAB on days 0, 8, 15 and 23.

Table 1. The changes in pH, %TA and FAN of the triplicate macerated fish batches (A, B, C) inoculated with LAB over a 30 d fermentation period (repeated twice).

	Day	pH		%TA		FAN	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Control	0	5.86	5.78	0.56	0.71	32.9	34.2
	15	3.81	3.78	2.19	2.42	147.0	146.2
	30	3.92	3.91	2.95	2.48	212.8	190.7
Batch A	0	5.86	5.78	0.56	0.71	32.9	34.2
	15	3.87	3.68	2.41	2.00	96.0	100.2
	30	3.83	3.72	2.15	2.43	100.8	153.2
Batch B	0	5.86	5.78	0.56	0.71	32.9	34.2
	15	3.89	3.63	2.96	3.38	115.4	108.6
	30	3.88	3.69	3.05	2.39	120.96	159.9
Batch C	0	5.86	5.78	0.56	0.71	32.9	34.2
	15	3.95	3.69	2.29	2.02	101.9	102.5
	30	3.98	3.84	2.09	2.47	150.9	115.92

%TA - The %TA of the control samples increased from 0.64 on day 0 to 2.3% by day 15 and increased slightly further to 2.7% by day 30 of the fermentation. The %TA values in the control samples were higher on day 30 than the values detected in the LAB inoculated samples. This could suggest that a higher concentration of convertible substrates are available in the absence of viable LAB bacteria. This could be justified by the increased production of fatty acids during the initial stages of the fermentation when the concentration of LAB in the product is higher and the more gradual increase in %TA up to the end of the fermentation when the viable counts of LAB in the product were drastically reduced to 1×10^2 cfu.ml⁻¹ (Fig. 1).

FAN - The FAN content of the control samples was considerably higher than that found in the inoculated LAB samples and increased from 33.6 mg to 150 mg by day 15 and 200 mg on day 30 (Fig. 2A).

LAB inoculated samples

Inoculum survival - The viable counts (Fig. 1) of the inoculated LAB samples increased from 160×10^6 to 470×10^6 cfu.ml⁻¹ after the first inoculation on day 0 and then decreased to 130×10^6 cfu.ml⁻¹ by day 7. After the second inoculation on day 8, the viable counts of the LAB increased to 180×10^6 by day 9. Despite further inoculation on days 15 and 23, the viable counts of the inoculated LAB decreased to 1.3×10^2 cfu.ml⁻¹ by day 30 of the fermentation (Fig. 1). It was thus concluded that the inoculated LAB did not survive well in the fish fermentation environment.

The low numbers of LAB present in the fermenting fish mixtures towards the end of the fermentation may have been due to the sensitivity of the LAB strains to the high salt content or the low final pH of the fermenting product, or a combination of both. It has previously been reported that LAB did not easily survive in other fermented fish products, possibly either due to the unavailability, absence or depletion of essential nutrients or fermentable sugars during the fermentation process (Lubbe, 2000; Tanasupawat *et al.*, 1998).

The low survival rate of the LAB, as was found in this study, corresponds to the LAB levels observed in fermented fish products with a high salt concentration (20 – 30% w/v) (Cooke *et al.*, 1993). However, the salt concentration of the fish mixture used in this study was only 6.5% (w/v), which rather corresponds to other

low salt fermented fish products that are normally characterised by increased LAB counts during the fermentation (Tanasupawat & Komagata, 1995). These low salt concentration products are supplemented with other carbohydrates, lactose or garlic, which serve as additional fermentable substrates and could, therefore, account for the enhanced survival rates of the LAB reported in these studies (Paludan-Mueller *et al.*, 1999). It is, therefore, possible that the survival of the LAB in fermenting fish is dependent rather on the presence of additional fermentable substances and not the salt concentration of the fermenting product. However, a high salt concentration does play a critical role in the preservation of the fermented fish product.

pH - The average pH of the macerated trout samples inoculated with LAB strains was reduced from 5.8 to 3.8 during the fermentation, which is similar to the values obtained in the control samples (Table 1; Fig. 2A and B). The inoculation of the fish tissue with the selected LAB strains, therefore, did not have a considerable effect on the decrease of the pH of the fish samples. Lubbe (2000) observed similar results when fermenting fish tissue was inoculated with selected single strains and combinations of LAB.

A rapid pH decrease to values below 4.5 during the first 48 h of the fermentation is critical in ensuring the quality and microbial safety of the fermented product (Adams *et al.*, 1987). Adams *et al.* (1987) reported that fish tissue has a high buffering capacity, which could possibly prevent the initial rapid decrease in the pH. This high buffering capacity of the fish tissue could possibly be overcome by the addition of selected bacterial strains that will be able to produce various acidic substances, including lactic acid, ensuring the required decrease in pH. The addition of other fermentable carbohydrates to the fermenting product could also increase the production of acidic substances in the product and thereby lower the pH of the fermented product (Twiddy *et al.*, 1987).

%TA - During the first 15 days of fermentation the %TA increased strongly in the samples inoculated with LAB, which was followed by a more gradual increase or a slight decrease in the %TA from 0.64 (day 0) to an average of 2.51% by day 15 and 2.4% by day 30. The increased %TA values measured in the LAB inoculated samples on day 15 correspond to the high survival rate of these inoculated bacterial strains during the initial stages of fermentation (Fig. 1). The high concentration of these bacteria during this period could be responsible for the

enhanced production of fatty acids, including lactic acid, from the glucose and other substrates present in the fermenting mixture, thereby causing an increase in the %TA of the fermented product. In another study done by Lubbe (2000), fermenting fish mixtures were inoculated with combinations of LAB, with the obtained %TA values (2.2%) similar to the values found in this study.

FAN -The sufficient conversion of insoluble protein to soluble protein in a fermenting fish product is indicated by a high FAN content and subsequently increased nutritional value and expanded shelf-life of an end-product (Chang *et al.*, 1994; Ijong & Ohta, 1995; Sanceda *et al.*, 1996). The FAN values of the samples inoculated with LAB strains gradually increased from 33.6 to 104 mg by day 15 and 133 mg by day 30 (Fig. 2B and Table 1). These values are considerably lower than the values determined for the control samples (Fig.2A). It could be possible that the inoculated LAB limited the FAN production in the fish products, by utilising part of the substrates necessary for high FAN production. The increase in the FAN content of the fermenting fish used in this study correlates to data previously obtained by Faid *et al.* (1997). They recorded a decrease in total nitrogen and an increase in non-protein nitrogen and total volatile nitrogen during the LAB fermentation of fish waste. The FAN values (97 – 116 mg) obtained in a previous study done by Lubbe (2000) when fish mixtures were inoculated with the same combination of LAB were lower than the values obtained in this study.

Second control samples (not inoculated with bacteria with high proteolytic activity)

None of the selected bacteria with high proteolytic activity was detected in the control sample pour plates on day 0. However, the presence of yeasts and mycelial growth was evident on the pour plates of the control fish samples up to day 30 of the fermentation, which can possibly be ascribed to the presence of fungal spores on the non-sterile fish tissue.

pH - The pH of the second control samples was found to decrease from 5.70 to 4.00 by day 15 and 3.80 by day 30 (Fig. 3). These results correlate with the pH values obtained in the first control samples (not inoculated with LAB).

%TA - The %TA of the second control samples increased from 0.47 to 1.6% by day 15 with a further increase to 2.9% during the last 15 days. The %TA values in these control samples on day 30 are slightly higher than the values

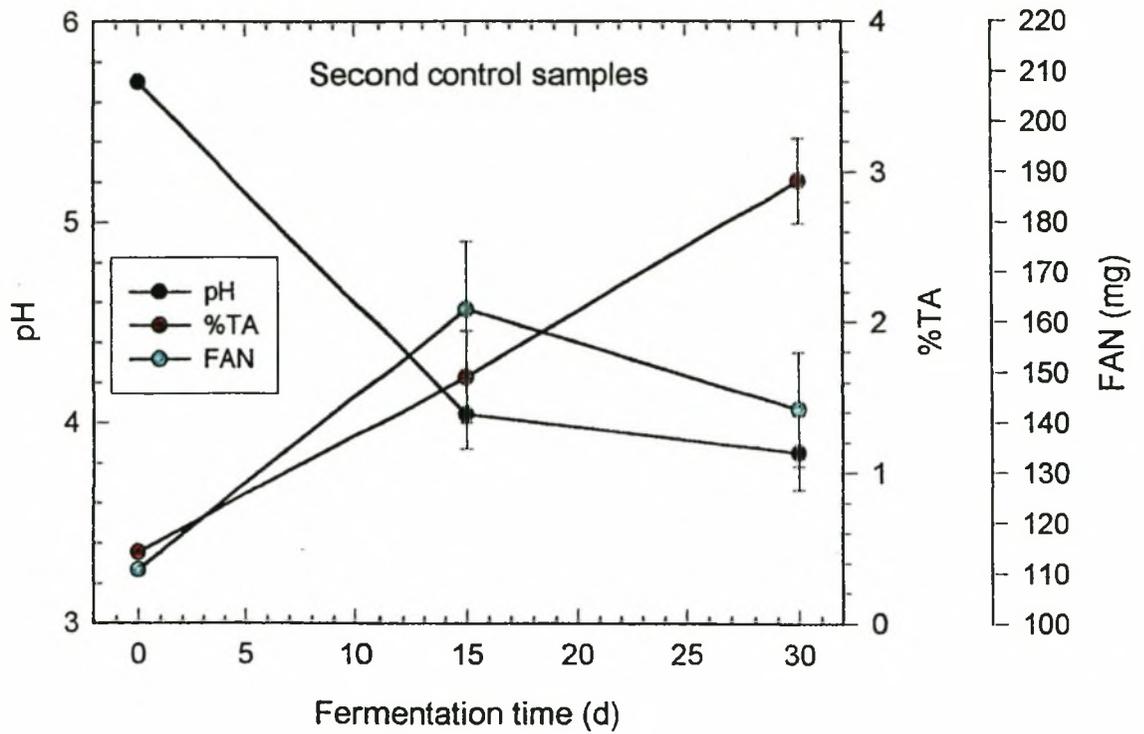


Figure 3. The changes in pH, %TA and FAN of the fermenting fish mixtures over the fermentation period of 30 days (second control samples not inoculated with bacteria with high proteolytic activity).

obtained previously, which could be due to the extensive growth of a wider variety of microbes in the fermenting product.

FAN - The FAN content of the second fermenting fish mixtures increased from 110.6 to 162 mg by day 15 and then decreased to 138 mg by day 30 (Table 2). The decrease in the FAN after the initial increase to day 15 (Fig. 3) differs from the data obtained in the first control samples (not inoculated with LAB) where the samples exhibited a continuous increase in FAN production up to day 30 (Fig. 2A). The decrease in the FAN content in the second control samples in the later stages of the fermentation could possibly be ascribed to the depletion of protein substrates necessary for FAN production by the observed moulds and yeasts present in the fish mixtures. It is also possible that these microbes could have utilized part of the proteinaceous substrates, which may explain the subsequent lower FAN values produced at the end of the fermentation.

The initial FAN values of the second control samples were considerably higher (110.6 mg) on day 0 than the initial FAN values of the first control samples (33.5 mg) (Tables 1 and 2). This initial difference could also possibly be ascribed to either the difference in age, percentage fat, levels of the endogenous fish enzymes present in the fish tissues or the varying microbial populations present in the fish mixtures, which singly or in combinations could have affected the breakdown of the fish proteins during the preparation stages. In the second control samples, the fish was older, fatter and subsequently larger than the young smaller fish that was used for the first set of control samples that had not been inoculated with the LAB. This difference in age and percentage fat appears to have influenced the rate of proteolysis of the fish proteins.

Kocuria varians (strain 1043)

Inoculum survival – In this study the viable counts of *K. varians* were much lower than in the case of the LAB studies. The presence of *K. varians* on the pour plates were microscopically confirmed. The viable counts of *K. varians* (1043) were found to increase after the first inoculation on day 0 to 1×10^6 cfu.ml⁻¹ by day 2 (Fig. 4 and Table 2). By day 7 the microbial counts decreased to 1×10^3 cfu.ml⁻¹ and then again increased to 50×10^3 cfu.ml⁻¹ after the second inoculation on day 8. The microbial counts then increased after the third inoculation on day 15 to reach 60×10^3 cfu.ml⁻¹ by day 16 and then remained unchanged up to day 22

Table 2. The changes in pH, %TA and FAN of duplicate samples of macerated fish inoculated with bacteria with high proteolytic activity over a 30 d fermentation period.

	Control		<i>K. varians</i> (1043)		<i>B. subtilis</i> (1190)		<i>B. amyloliquefaciens</i> (1191)		<i>B. amyloliquefaciens</i> (1239)		Combination starter	
	pH											
Sample	1	2	1	2	1	2	1	2	1	2	1	2
0	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70
15	4.16	3.92	4.13	3.86	3.95	4.11	3.71	3.88	3.97	3.97	3.90	4.26
30	3.98	3.72	4.06	3.72	3.89	4.06	3.81	3.78	3.98	3.88	3.80	3.95
	%TA											
Sample	1	2	1	2	1	2	1	2	1	2	1	2
0	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47
15	1.85	1.42	1.98	1.94	2.06	1.19	2.31	2.35	1.79	1.91	2.02	1.84
30	3.14	2.74	2.61	2.86	3.25	2.94	2.85	3.01	3.24	3.15	3.14	2.94
	FAN											
Sample	1	2	1	2	1	2	1	2	1	2	1	2
0	110.6	110.6	110.6	110.6	110.6	110.6	110.6	110.6	110.6	110.6	110.6	110.6
15	172.2	152.9	139.2	151.8	184.2	168.3	181.4	166.6	190.7	198.8	196.0	185.6

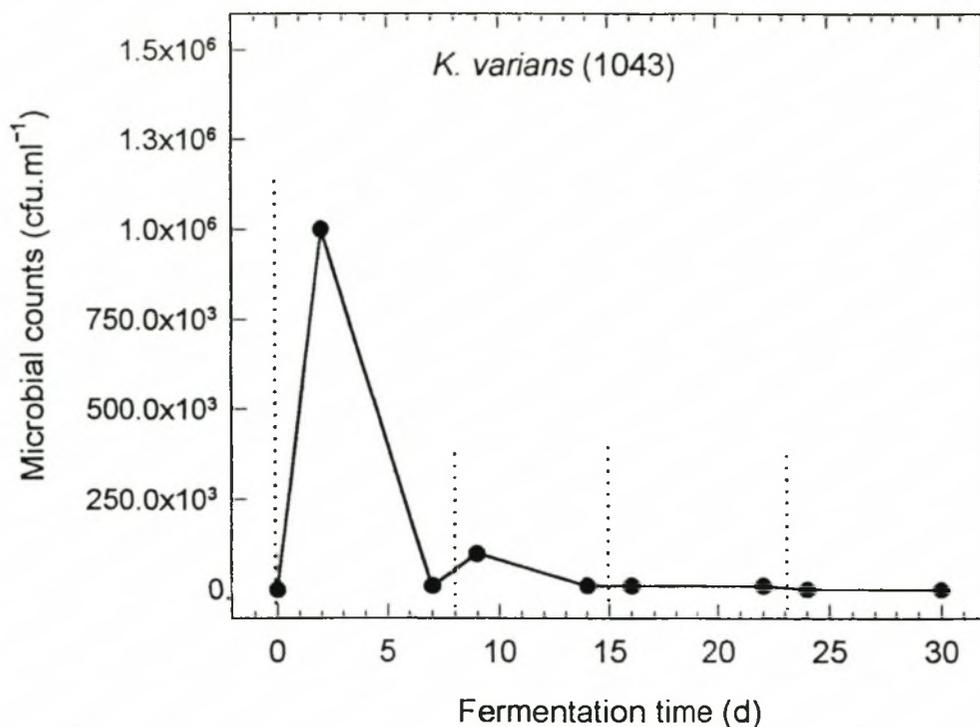


Figure 4. Survival rate (cfu.ml⁻¹) of *Kocuria varians* (1043) during the 30 days of fermentation. The dotted lines indicate reinoculation on days 0, 8, 15 and 23.

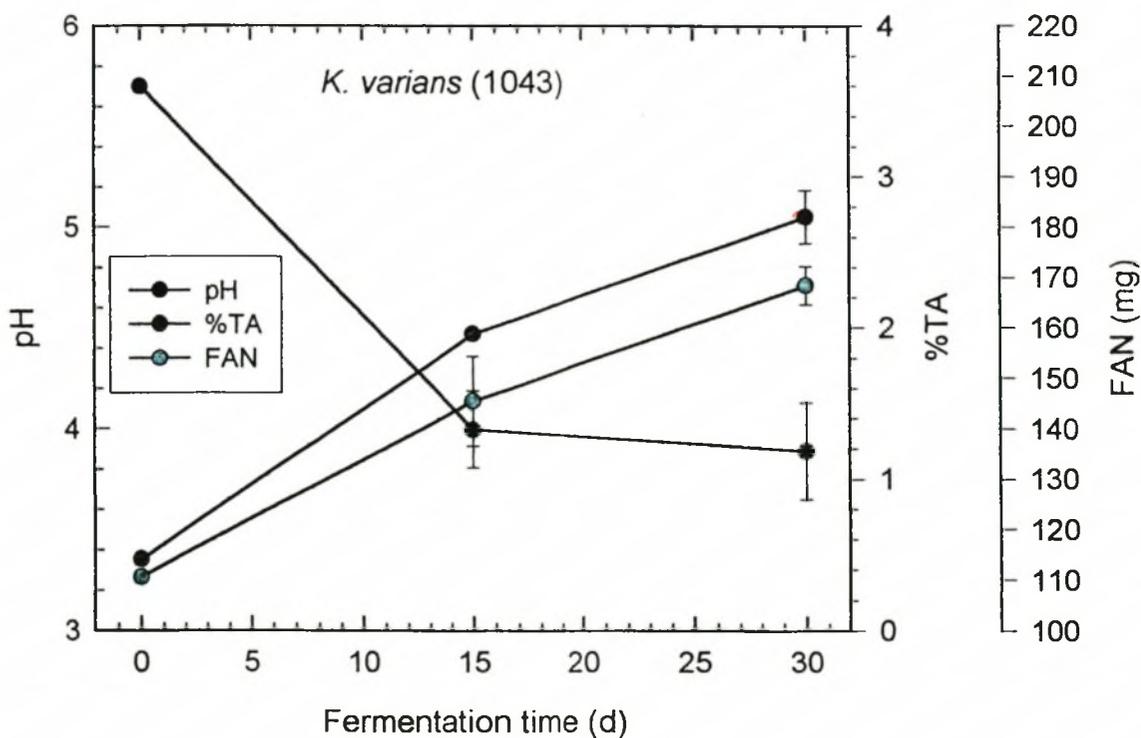


Figure 5. The changes in pH, %TA and FAN of the fermenting fish mixtures inoculated with *Kocuria varians* (1043) over the fermentation period of 30 days.

where after the number decreased to 1×10^3 cfu.ml⁻¹ by day 30. The viable counts of the inoculated *Kocuria varians* decreased to very low numbers during the fermentation process and it was concluded that this organism does not survive well under the fermentation conditions. In this study it was also found that yeasts and mycelial growth were present up to day 30 of the fermentation process.

pH - The pH of the samples that had been inoculated with *K. varians* (Fig. 5) decreased from 5.7 on day 0 to 4.0 by day 15. The pH then further decreased slightly to 3.9 by day 30 of the fermentation.

%TA - The %TA of the samples inoculated with *K. varians* increased from 0.47 to a value of 1.9% by day 15 and reached 2.7% by day 30. This end-value was considerably higher than the values measured on day 15 (Fig. 5).

FAN - The FAN values of the samples inoculated with *K. varians* reached average values of 146 mg by day 15 and 168 mg by day 30. The FAN values of the fish samples inoculated with *K. varians*, therefore, continually increased during the fermentation but did not reach very high values (Fig. 5).

Bacillus subtilis (strain 1190)

Inoculum survival - The viable counts of *B. subtilis* (1190) increased after the first inoculation on day 0 to 1×10^6 cfu.ml⁻¹ by day 2 (Fig. 6). The microbial counts then decreased to 10×10^3 cfu.ml⁻¹ by day 7, then again increased after the second inoculation on day 8 to 150×10^3 cfu.ml⁻¹ and then remained constant up to day 14. After the third inoculation on day 15, the viable counts of *B. subtilis* again increased to 1×10^6 cfu.ml⁻¹ and then remained the same up to day 22. This was followed by an increase to 1.5×10^6 cfu.ml⁻¹ by day 24, which remained constant to day 30.

pH - The pH of the samples inoculated with *B. subtilis* (Fig. 7 and Table 2) decreased from 5.70 on day 0 to 4.0 by day 15. The pH then showed a slight further decrease to 3.98 by day 30 of the fermentation.

%TA - The %TA of the samples inoculated with the *B. subtilis* strain increased from 0.47% on day 0 to 1.6% by day 15 and to a final value of 3.0% on day 30, which was similar to the %TA values of the second control samples (Fig. 7 and Table 2).

FAN - The FAN values of the *B. subtilis* inoculated samples increased from 110.6 to 176 mg by day 15 and 180 mg by day 30. The FAN values did not

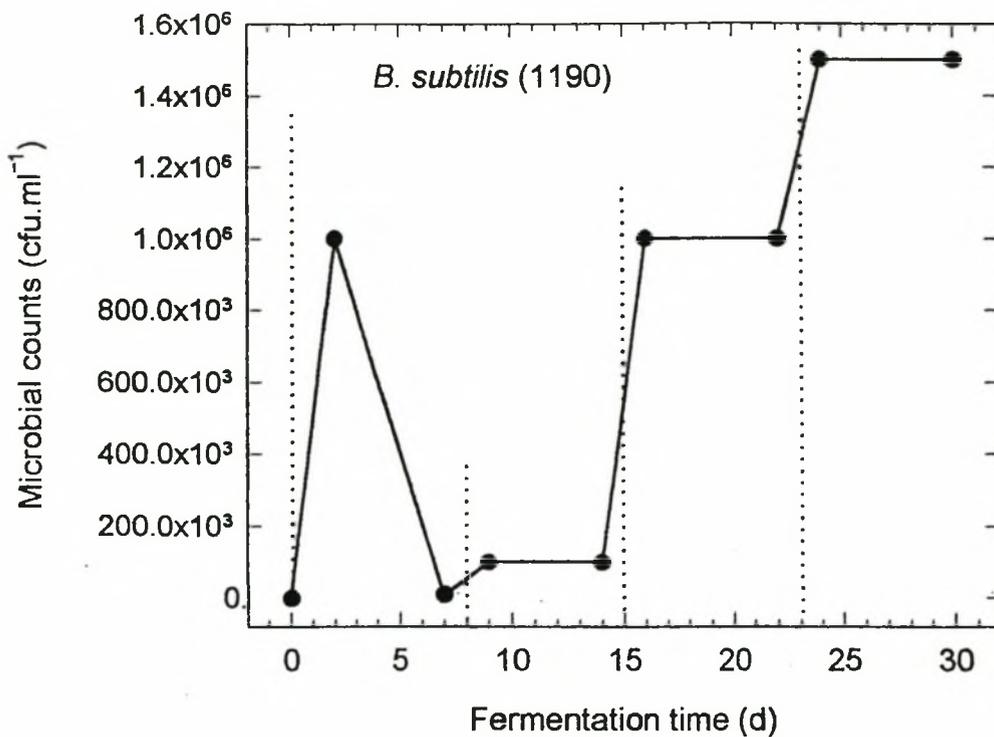


Figure 6. Survival rate (cfu.ml⁻¹) of *Bacillus subtilis* (1190) during the 30 days of fermentation. The dotted lines indicate reinoculation on days 0, 8, 15 and 23.

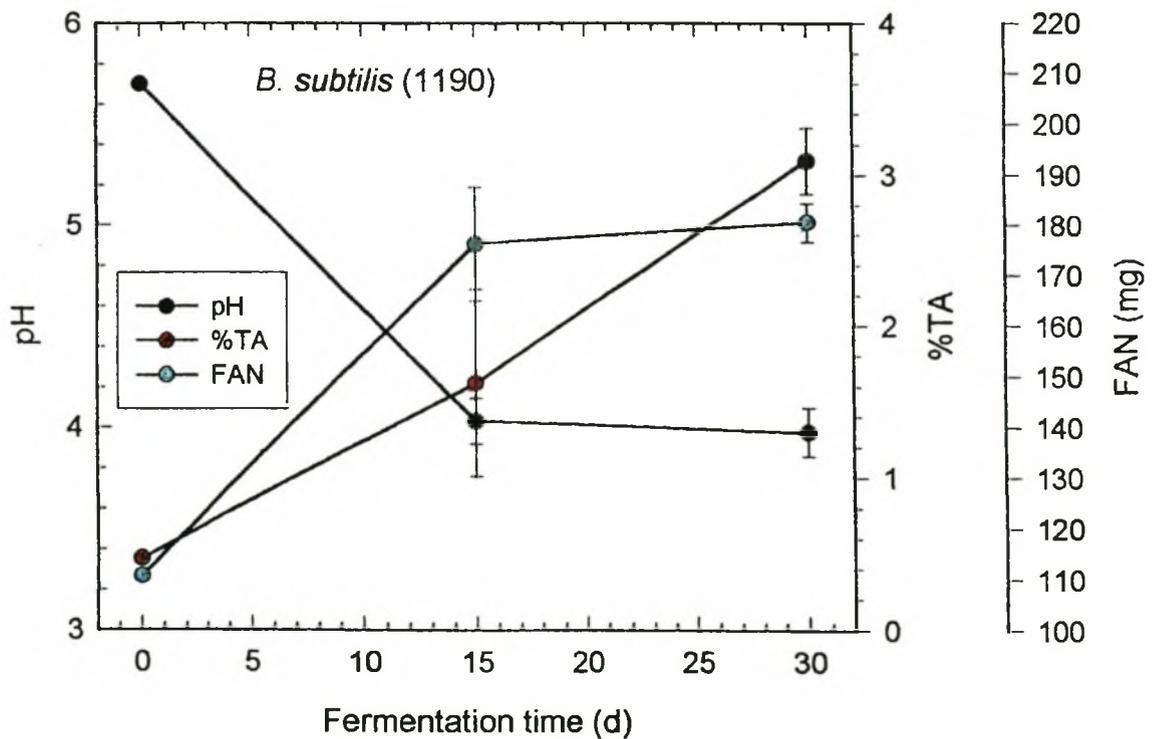


Figure 7. The changes in pH, %TA and FAN of the fermenting fish mixtures inoculated with *Bacillus subtilis* (1190) over the fermentation period of 30 days.

increase much during the last 15 days of the fermentation, which could be due to the depletion of substrates necessary for the conversion to FAN products.

Bacillus amyloliquefaciens (strain 1191)

Inoculum survival - The viable counts of *B. amyloliquefaciens* (1191) increased after the first inoculation on day 0 to 1×10^6 cfu.ml⁻¹ by day 2 (Fig. 8). The microbial counts then decreased to 1×10^3 cfu.ml⁻¹ by day 7 and increased to 55×10^3 cfu.ml⁻¹ after the second inoculation on day 8. The counts decreased to 1×10^3 cfu.ml⁻¹ by day 14 and then increased to 60×10^3 cfu.ml⁻¹ after the inoculation on day 15. The *B. amyloliquefaciens* (1191) counts then increased drastically to 1×10^6 cfu.ml⁻¹ by day 22 and remained constant up to day 30 despite reinoculation on day 23.

pH - The pH of the samples inoculated with *B. amyloliquefaciens* (1191) (Fig. 9 and Table 2) decreased from 5.7 to 3.8 by day 15 and remained the same up to the end of the fermentation.

%TA - The %TA of these samples gave a value of 2.3% by day 15, which increased to 2.9% by day 30.

FAN - The FAN content of the samples inoculated with *B. amyloliquefaciens* (1191) reached average values of 174 mg by day 15 and 194 mg by day 30.

Bacillus amyloliquefaciens (strain 1239)

Inoculum survival - The viable counts of the second strain of *B. amyloliquefaciens* (strain 1239) increased after the first inoculation on day 0 to 1×10^6 cfu.ml⁻¹ by day 2 (Fig. 10). The counts then decreased to 50×10^3 cfu.ml⁻¹ by day 7 and then increased again to 1×10^6 cfu.ml⁻¹ after the second inoculation on day 8. The viable counts of *B. amyloliquefaciens* (1239) then decreased to 100×10^3 cfu.ml⁻¹ by day 14 and increased to 1×10^6 cfu.ml⁻¹ after the third inoculation on day 15. The viable counts of this *B. amyloliquefaciens* strain increased to 1.5×10^6 cfu.ml⁻¹ by day 22, remained the same after the third inoculation on day 23 and then decreased to 1×10^6 cfu.ml⁻¹ by day 30.

pH - The pH of the samples inoculated with *B. amyloliquefaciens* (1239) (Fig. 11 and Table 2) decreased from 5.70 on day 0 to 3.97 by day 15 and 3.93 by day 30 of the fermentation.

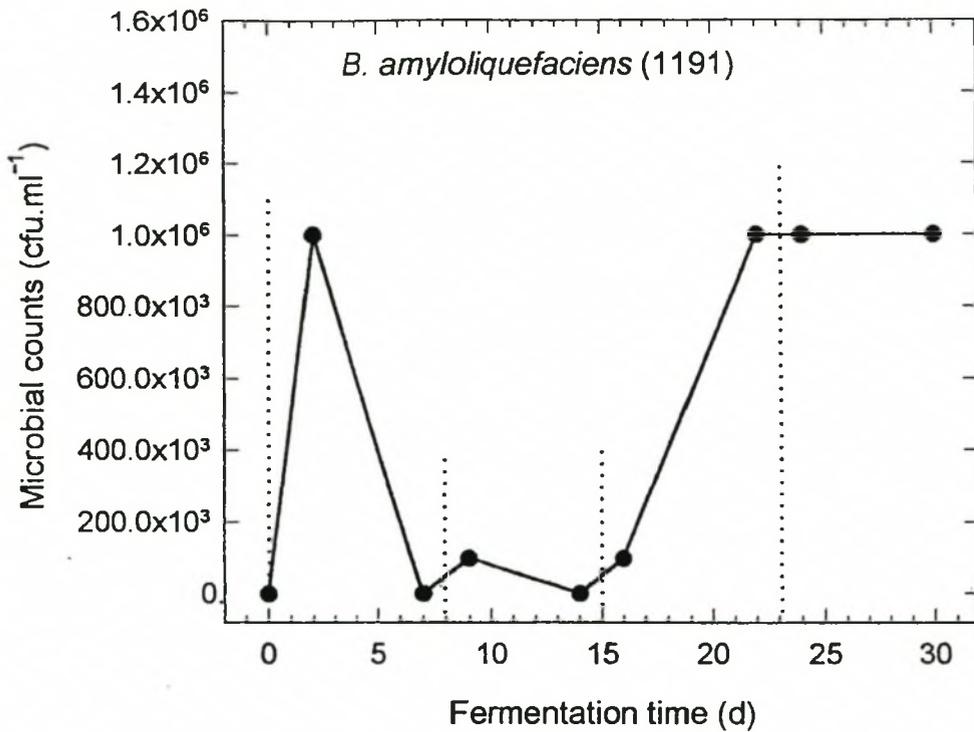


Figure 8. Survival rate (cfu.ml⁻¹) of *Bacillus amyloliquefaciens* (1191) during the 30 days of fermentation. The dotted lines indicate reinoculation on days 0, 8, 15 and 23.

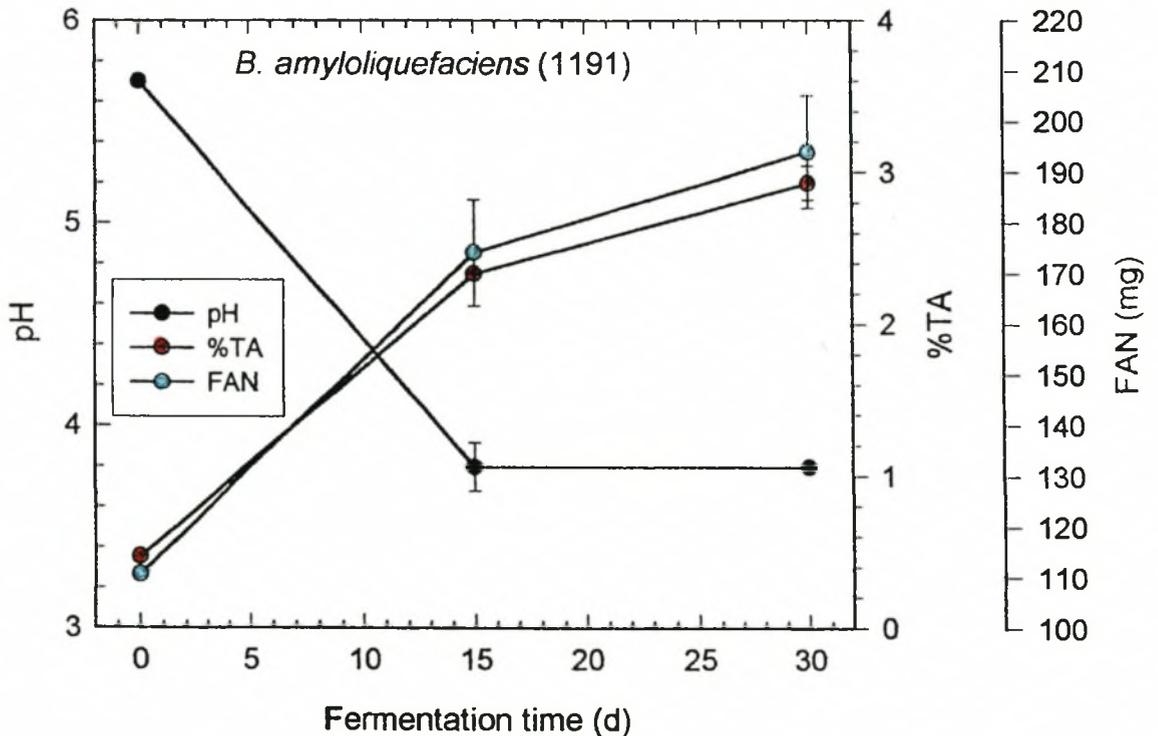


Figure 9. The changes in pH, %TA and FAN of the fermenting fish mixtures inoculated with *Bacillus amyloliquefaciens* (1191) over the fermentation period of 30 days.

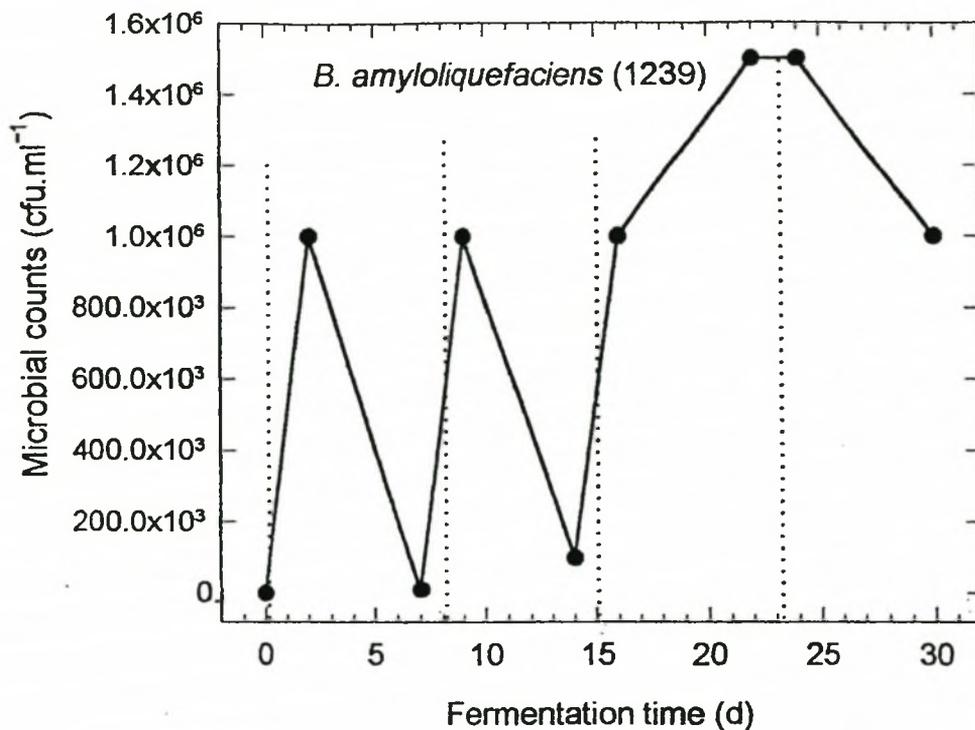


Figure 10. Survival rate (cfu.ml⁻¹) of *Bacillus amyloliquefaciens* (1239) during the 30 days of fermentation. The dotted lines indicate reinoculation on days 0, 8, 15 and 23.

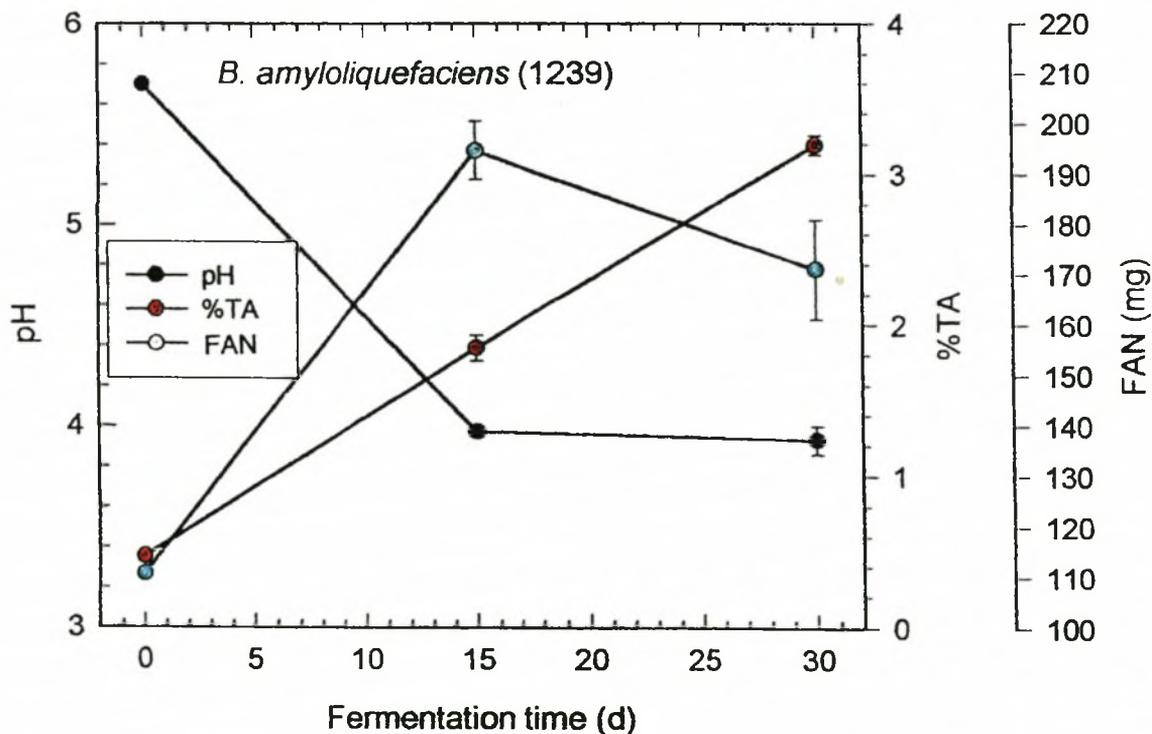


Figure 11. The changes in pH, %TA and FAN of the fermenting fish mixtures inoculated with *Bacillus amyloliquefaciens* (1239) over the fermentation period of 30 days.

%TA - The %TA of the samples inoculated with this *B. amyloliquefaciens* (1239) gave a value of 1.85% by day 15 and 3.2% by day 30. The %TA values obtained in these samples were the highest of all the inoculated samples.

FAN - The FAN values of these samples reached an average value of 195 mg by day 15 and then slightly decreased value to 171 mg by day 30. Samples inoculated with this strain of *B. amyloliquefaciens* (1239) reached the highest FAN values by day 15, but the values then decreased slightly towards the end of the fermentation. This could possibly be due to the rapid breakdown of fish proteins by the proteases released by these bacteria during the first 15 days of fermentation.

Combination of starters with high proteolytic activity

Inoculum survival - The viable counts of the samples inoculated with a combination of bacteria with high proteolytic activity increased to 1.5×10^6 cfu.ml⁻¹ after the first inoculation on day 0 (Fig. 12). The microbial counts decreased to 1×10^6 cfu.ml⁻¹ and then remained the same up to day 9 despite reinoculation on day 8. The viable counts of the inoculum decreased to 1×10^5 cfu.ml⁻¹ by day 14, increased to 1×10^6 cfu.ml⁻¹ by day 16 and day 22 and then drastically increased to 10×10^6 cfu.ml⁻¹ by day 24 after the reinoculation on day 23, which remained constant to day 30. This mixed starter inoculum showed the highest concentration of viable cell counts during the fermentation.

pH - The pH of the samples inoculated with a combination of the bacteria with high proteolytic activity (Fig. 13 and Table 2) decreased from 5.70 to 4.0 by day 15 and further decreased to 3.8 by day 30.

%TA - The %TA of the samples inoculated with the selected bacteria with high proteolytic activity increased to 1.9% by day 15 and 3% by day 30.

FAN - The FAN values of these samples reached a high average value of 190 mg by day 15 and then slightly decreased to 170 mg by day 30. The decrease in the FAN values towards the end of the fermentation is possibly also due to the early utilization of FAN substrates by the microbes.

Overall comparison of inoculums

Inoculum survival – The LAB cultures used did not appear to convincingly survive the fish fermentation conditions as applied in this study. However, the

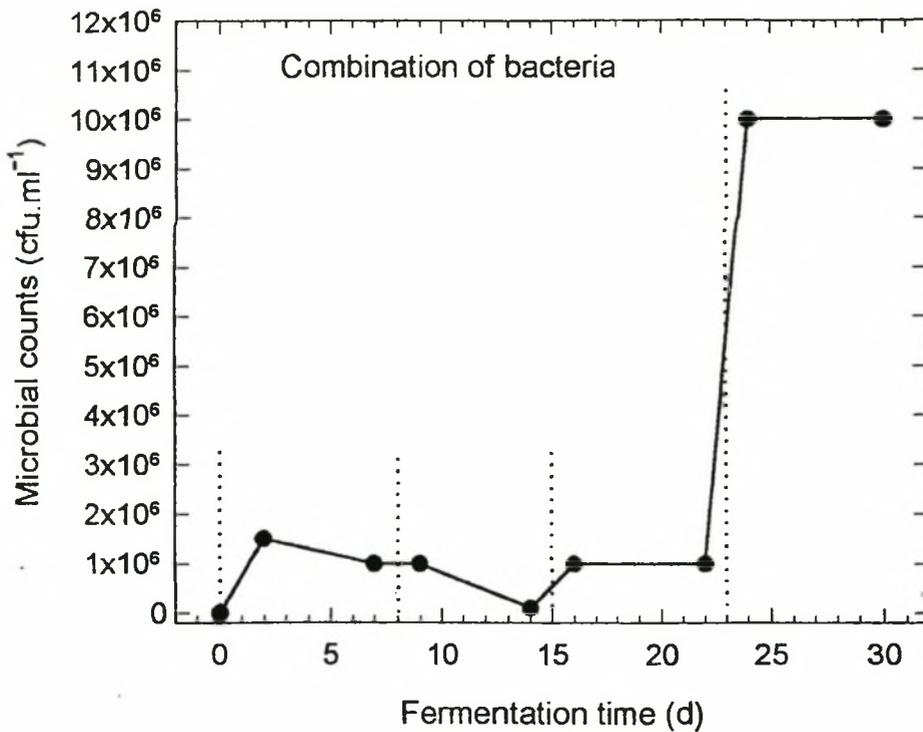


Figure 12. Survival rate (cfu.ml⁻¹) of a combination of bacteria with high proteolytic activity during the 30 days of fermentation. The dotted lines indicate reinoculation on days 0, 8, 15 and 23.

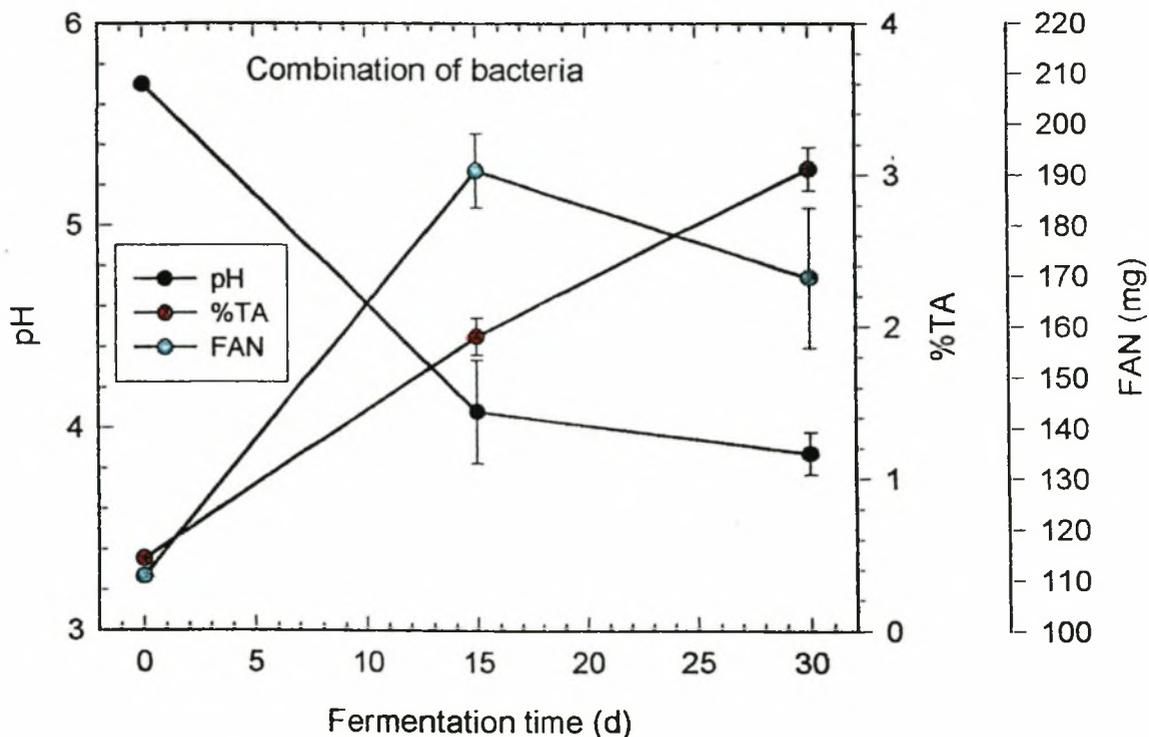


Figure 13. The changes in pH, %TA and FAN of the fermenting fish mixtures inoculated with a combination of bacteria with high proteolytic activity over the fermentation period of 30 days.

bacteria with high proteolytic activity were present in reasonably high concentrations throughout the process, except for the reduced viable counts of *K. varians* (1043). The increase in the concentration of the inoculated *Bacillus* strains during the fermentation process could either be ascribed to the possible germination of endospores formed during adverse conditions or to the increased availability of specific amino acids produced by the degradation of proteins present in the mixture that are essential to the growth of the different *Bacillus* spp. The presence of moulds and yeasts in the fermenting fish samples inoculated with *Bacillus* strains were also observed only during the first 15 days of the fermentation process. The absence of these microbes during the last 15 days of production presents the possibility that the *Bacillus* strains can possibly inhibit the growth of these yeasts and moulds. It is known that these bacteria have the ability to survive, show strong growth and even synthesise degradation enzymes under the rather challenging environmental conditions applied during fish fermentations. This might, therefore, explain why a large number of *Bacillus* spp. were identified from the final fermented fish products in a similar study done by Lubbe (2000). The domination of *Bacillus* strains in the fish tissue during the later stages of the fermentation process also suggests that these bacteria play an important part in the fermentation of the fish tissue due to their proteolytic activity and ability to survive the fermentation conditions.

pH - It is clear that the inoculation of the fish tissue with LAB and bacteria with high proteolytic activity did not have a great impact on the decrease in the pH of the fermenting fish mixture as similar results were obtained in both the inoculated and the control samples. The samples inoculated with *B. amyloliquefaciens* (1191) did, however, seem to allow a more rapid decrease in pH than that what was found for the other inoculated samples.

%TA - The %TA increased in all the fish samples especially during the first 15 days of the fermentation process. This increase correlates with the microbial growth observed in the inoculated samples during the initial stages of the fermentation. The %TA values obtained when inoculating the fish mixtures with LAB presented a more rapid increase until day 15 of the fermentation than the samples inoculated with the bacteria with high proteolytic activity. This could be due to a higher rate of acidic metabolite production by the LAB. The samples inoculated with LAB, however, reached %TA values ranging from 2.09 to 3.05%

on day 30, whereas the samples inoculated with bacteria with high proteolytic activity reached higher values ranging from 2.61 to 3.25% (Tables 1 and 2). The inoculation of macerated fish tissue with selected bacteria with high proteolytic activity, therefore, probably contributes to the slight increase in the %TA of the products throughout the fermentation, although the inoculation with LAB resulted in a more rapid increase in the %TA at the initial stages of the fermentation. The samples inoculated with the *B. subtilis* (1190) and *B. amyloliquefaciens* (1239) strains and a combination of starters reached the highest values obtained during this study. Lubbe (2000) also determined the %TA of a fermenting fish mixture inoculated with selected LAB and bacterial strains with high proteolytic activity and found that the %TA on day 30 of the fermentation averaged 2.4%, which is slightly lower than the values obtained in this study.

FAN - The fish samples inoculated with LAB did not reach values as high as those obtained in the control samples and it is possible that the LAB limited the production of FAN in these samples. The inoculated bacterial strains with high proteolytic activity appeared to increase the levels of FAN production in the fermenting fish samples. The samples inoculated with the *B. amyloliquefaciens* strain 1239 and a combination of starters reached higher values by day 15. However, these values decreased slightly towards the end of the fermentation and the samples inoculated with *B. amyloliquefaciens* strain 1191 reached the highest FAN value by day 30. This could possibly be due to a rapid breakdown of fish proteins by the proteases released by the *B. amyloliquefaciens* (1239) strain during the first 15 days of fermentation. Although Lubbe (2000) reported slightly lower FAN values in the fish samples inoculated with single strains with a high proteolytic activity (112 – 119 mg), it is possible that other selected bacteria with high proteolytic activity could further enhance the FAN production in industrially produced fish products.

PCR-RFLPs

A question that arose during this study was: were the final plate counts after 30 days of fermentation representative of the inoculated bacterial strains? One way to identify the microbial colonies isolated from the fermenting fish mixture was to use the PCR-RFLP identification technique. This reliable and reproducible molecular technique enables the rapid identification of microbial species within a

few hours as was described in Chapter 3 of this thesis. Thus, in this study the PCR-RFLP identification technique was used to confirm the presence of the selected starters in the final fish samples after the 30 d fermentation period. This was achieved by the amplification of a 1 300 bp fragment of the 16S rRNA gene of the pure cultures of *Lactobacillus plantarum* (226), *Pediococcus cerevisiae* (407), *Kocuria varians* (1043), *Bacillus subtilis* (1190) and the two *B. amyloliquefaciens* (1191 and 1239) strains (Table 3 and Fig. 14). The primers successfully amplified the targeted sequences of *L. plantarum* and *P. cerevisiae*, but were unsuccessful in the amplification of *Lactococcus diacetylactis*, which suggests the unavailability of the target primer annealing sites in the 16S rRNA gene sequence of this strain.

The digestion of the PCR products of the pure culture of *L. plantarum* with *AluI*, produced two fragments that were 600 bp and 200 bp in size, thereby producing a unique DNA fingerprint for *L. plantarum*. The PCR amplified fragments of *P. cerevisiae* could not be digested by the restriction enzyme *AluI*, which also suggests the absence of the target restriction sites on the amplified PCR product of this strain. The RFLP fingerprints of *L. plantarum* and *P. cerevisiae* and the inability of the chosen primers to amplify the target sequence in *L. diacetylactis* enabled the successful discrimination among the pure cultures of the different LAB strains.

The PCR-RFLP fragments of the LAB isolated from the fermenting fish product on day 30, presented patterns identical to the fingerprints obtained from the pure culture of *L. plantarum*. It is, therefore, clear that *L. plantarum* was present in the fermenting fish tissue at the end of the fermentation as the dominant LAB strain. However, the conventional counting method only showed very low levels of the LAB at the end of the fermentation period (Fig.1).

The digestion of the PCR products of the bacterial species with high proteolytic activity produced the following fragments (Fig. 15): *K. varians* showed fragments of 650, 200 and 150 bp in size; *B. subtilis* showed fragments of 450, 280, 200 and 180 bp in size; and both strains of *B. amyloliquefaciens* presented fragments 450, 280, 200 and 180 bp in size. The RFLP fragments of *K. varians* could clearly be used to distinguish this strain from the *Bacillus* strains. The discrimination between the three *Bacillus* strains could not be achieved by mere digestion with the *AluI* enzyme. Thus is probably due to the close phylogenetic relationship between these bacterial species (Rössler *et al.*, 1991).

Table 3. The PCR-RFLP fragments obtained after digestion with the restriction enzyme *AluI*.

LAB and proteolytic bacteria	PCR-RFLP fragments
<i>Lactobacillus plantarum</i> (226)	600, 200
<i>Lactococcus diacetylactis</i> (107)	No amplification product
<i>Pediococcus cerevisiae</i> (407)	1 300
<i>Kocuria varians</i> (1043)	650, 200, 150
<i>Bacillus subtilis</i> (1190)	450, 280, 200, 180
<i>Bacillus amyloliquefaciens</i> (1191)	450, 280, 200, 180
<i>Bacillus amyloliquefaciens</i> (1239)	450, 280, 200, 180

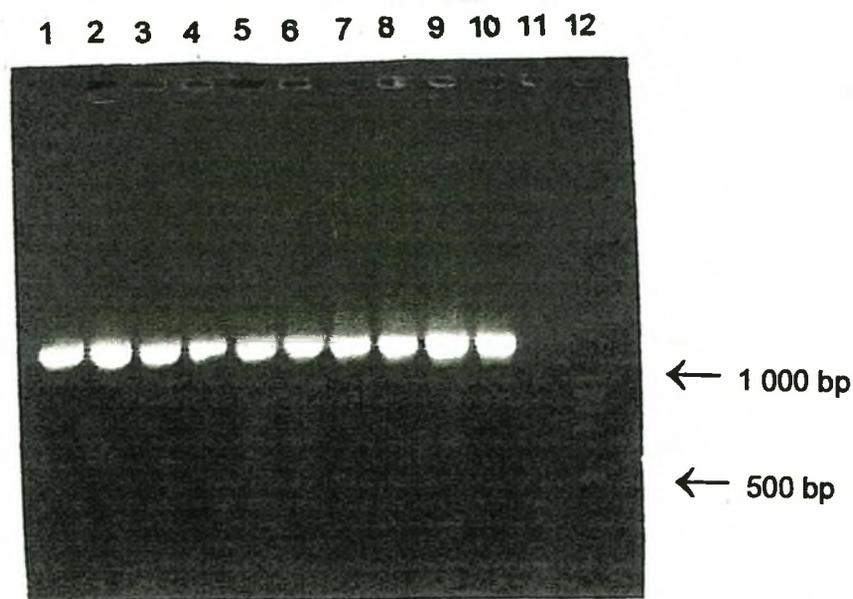


Figure 14. The amplified 16S rDNA gene fragments (1 300 bp) of the inoculated LAB and proteolytic bacteria during a 30 d fermentation process. Lanes 1,2 – *Lactobacillus plantarum* (226), lanes 3,4 – *Lactococcus diacetylactis* (140), lanes 5,6 – *Pediococcus cerevisiae* (407), lane 7 – *Kocuria varians* (1043), lane 8 – *Bacillus subtilis* (1190), lane 9 – *B. amyloliquefaciens* (1191), lane 10 – *B. amyloliquefaciens* (1239), lane 11 – negative control and lane 12 – molecular marker – 100 bp ladder (Promega).

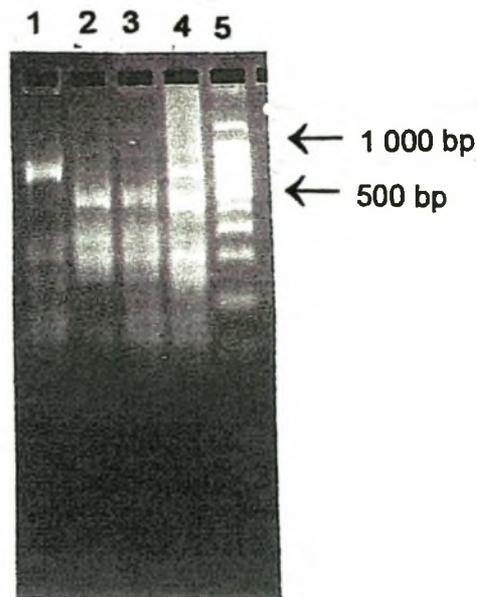


Figure 15. PCR-RFLP of the inoculated pure cultures of bacteria with high proteolytic activity. Lane 1 – *K. varians* (1043), lane 2 – *Bacillus subtilis* (1190), lane 3 – *B. amyloliquefaciens* (1191), lane 4 – *B. amyloliquefaciens* (1239) and lane 5 – molecular marker – 100 bp ladder (Promega).

When a comparison was made, the PCR-RFLP fragments of the microbial colonies isolated from the final fermenting fish samples that had been inoculated with the bacteria with high proteolytic activity showed DNA fingerprints that were identical to the fragments obtained from the pure cultures (Fig. 16). It is also clear from the data obtained and shown in lanes 8 and 9 of Figure 16 that the *K. varians* strain did not survive the fermentation process as part of the inoculated combination of bacterial species.

The ability to identify these starter bacterial strains in the final fermented product proves that the PCR-RFLP technique can be used successfully as a fast and accurate identification method of microbes present in fermented fish products. Based on the data obtained in this study it is recommended that the PCR-RFLP conditions be further improved to enable the identification of spoilage bacteria directly from the fermenting fish samples without the need for bacterial culturing.

Conclusions

Fermenting fish can be inoculated with starter bacteria to ensure a sufficiently controlled microbial activity in the product, alter the characteristics of the product and ensure the safe preservation of the product. The use of bacteria with high proteolytic activity as starter culture leads to the enhancement of the degradation of fish tissue and thus accelerates the fermentation process. This accelerated fermentation, as found in this study, resulted in an increase in the FAN production of the fermented products, as well as a lower pH and slightly higher %TA than in the control samples. The addition of LAB and suitable carbohydrates during the first stages of fermentation could also be beneficial to the fermentation process by allowing a more rapid decrease in the pH of the fermenting product due to the formation of acidic products, possibly including lactic acid from the available added carbohydrate substrate. The resulting low pH in the fermenting product then inhibits the growth of LAB since LAB are known not to be able to successfully tolerate a highly acidic environment.

In this study, the concentration of the starter LAB strains decreased early in the fermentation regardless of further inoculations during the production process and these strains were almost eliminated in the final product. However, the viable counts of the starter bacteria with high proteolytic activity either increased or

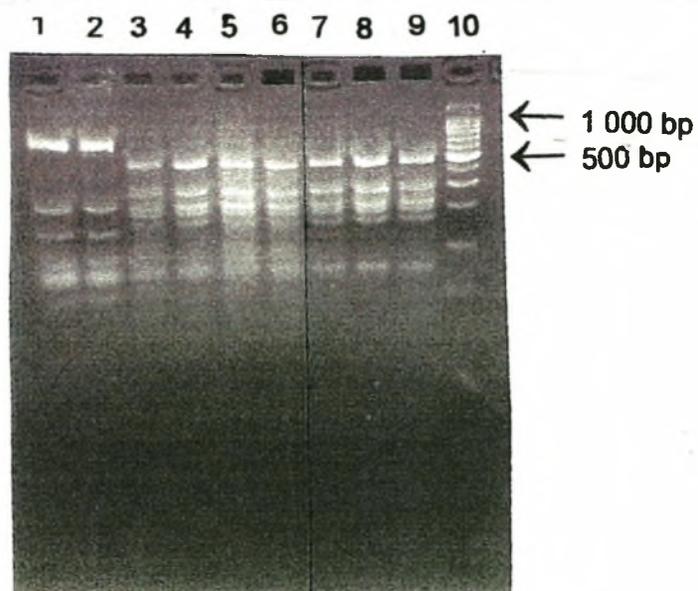


Figure 16. PCR-RFLPs of the bacteria isolated from the fermenting fish tissue on d 30. Lanes 1,2 – *K. varians*, lanes 3,4 – *B. subtilis*, lanes 5,6 – *B. amyloliquefaciens*, lanes 7,8 – *B. amyloliquefaciens*, lanes 8,9 – combination of *Bacillus* spp. and lane 10 – molecular marker – 100 bp ladder (Promega).

decreased concurring with the inoculation intervals during the fermentation. Since the LAB strains were unable to totally survive the fermentation conditions, the use of starter bacterial species with high proteolytic activity can be recommended for the industrial production of fermented fish products.

The inoculated *L. plantarum*, *K. varians*, *B. subtilis* and *B. amyloliquefaciens* strains were successfully detected in the final fermented product (day 30) with the use of the PCR-RFLP technique. These detection results illustrate the specificity, effectiveness and value of the technique in the rapid identification of microbes present in fermented fish even if present in very low concentrations.

The fermented fish products produced in this study were not sensory evaluated, as the presence of certain pathogens has been reported in a previous study (Lubbe, 2000) on similar fermented fish products and the end-products produced in this study were not tested for the presence of potentially pathogenic bacteria. However, it was found that the final fermented product produced in this study had a pleasant fishy aroma and showed a good colour and consistency when compared to other fermented fish products. Furthermore, the addition of *Bacillus* strains to food products as starters is not a common practice and thus the ability of these strains to survive and be classified as GRAS can not be excluded. In future, other possible starter combinations can be developed and tested for the accelerated production of fermented fish products and the sensory evaluation thereof in order to commercialise the process.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The preservation of various fresh fish products is achieved by either smoking, salting, canning, freezing or fermenting a highly perishable raw product. Since many of these facilities are not readily available in remote areas or in many developing countries, the use of fermentation as a means of preserving the product has been extensively practised in the Eastern Asian countries (Battcock & Azam-Ali, 1998). However, the fermentation of fish is a time consuming practise and only by accelerating a standard production process would it be possible to ensure the production of a more cost effective and readily available safe end-product.

The quality of the fermented fish product is partially determined by the fermentation conditions and the metabolite activity of the microbes present in the product. The rapid identification of the microbes present during the fermentation would enable the selection of possible starters to ensure an accelerated production of high quality fermented fish products (Steinkraus, 1995). The use of molecular techniques for microbial identification (Van der Vossen & Hofstra, 1996) has been shown to be highly reliable and reproducible and the results can be available within a few hours, whereas traditional identification techniques rely on morphological and physiological characteristics that can vary under different environmental conditions and are time consuming to determine.

This study was thus undertaken to develop identification fingerprints for bacteria previously isolated from similar fermented fish products (Lubbe, 2000) by using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. A further objective of this study was to accelerate the production of a fermented fish product by inoculating macerated trout with either selected lactic acid bacteria (LAB) or with selected bacteria with high proteolytic activity over a 30 day fermentation period. The quality of the fermented product was then measured during the fermentation process using changes in product pH, whereas the titratable acidity (%TA) and free amino nitrogen (FAN) formation were used as efficiency parameters (Cha & Cadwallader, 1998).

To achieve the above set goals a 1300 bp fragment of the 16S rRNA genes of each of the previously isolated bacteria (Lubbe, 2000) was successfully amplified using the PCR technique. The isolates included strains of *Bacillus cereus*, *B. mycoides*, *B. amyloliquefaciens*, *B. coagulans*, *B. megaterium*, *B. firmus*, *B. lentus*, *B. licheniformis*, *B. subtilis*, *Staphylococcus caprae*, *S. hominus*, *S. schleiferi*, *S. simulans*, *S. aureus*, *Sphingomonas paucimobilis*, *Kocuria varians*, *Brevibacillus brevis*, *Cryseomonas luteola*, *Vibrio alginolyticus*, *Stenotrophomonas maltophilia* and *Agrobacterium radiobacter*.

The fingerprints obtained after the digestion of the amplified PCR products with the restriction enzyme *AluI*, were identical for the *B. cereus* and *B. mycoides* strains and for the *Staphylococcus caprae* and *S. schleiferi* strains, while unique patterns were obtained for the other isolates. The inability to distinguish between the two *Bacillus* species and the two *Staphylococcus* species suggests that the members of the genera have a close phylogenetic relationship. The unique patterns obtained for the other isolates demonstrates the ability of this molecular technique to successfully distinguish among a variety of different bacterial species based on their 16S rRNA gene sequences. The data obtained in this study can, therefore, be used in the identification of these microbes from other similar fermented fish products. The fingerprints could also be used to assist in determining the dominant microbial populations responsible for the characteristic qualitative changes occurring in the fish product during fermentation.

The microbial composition of a fermenting fish product partially determines the quality of the end-product, therefore, the use of selected bacterial starters could result in the accelerated production of a microbial safe fermented fish product. In this study the production of a fermented fish product was initiated by the inoculation of macerated trout tissue with selected lactic acid bacteria (LAB) and bacteria with the ability to produce high concentrations of extracellular proteases. The LAB included a combination of *Lactobacillus plantarum* (USFSCC 226), *Lactococcus diacetylactis* (USFSCC 140) and *Pediococcus cerevisiae* (USFSCC 407) strains, whereas the bacteria with high proteolytic activity (Lubbe, 2000) included strains of *Kocuria varians* (USFSCC 1043), *Bacillus subtilis* (USFSCC 1190), *B. amyloliquefaciens* (USFSCC 1191 and 1239) and a combination of these bacterial species.

The data obtained during the fermentation of the macerated trout which had been inoculated with either the LAB and bacteria with high proteolytic activity showed that the selected starters did not have a significant effect on the pH decrease in the product over a 30 day fermentation period. The LAB strains did not have a significant effect on the %TA of the fermenting fish product, yet the presence of these bacteria appeared to limit the FAN production in the product. The bacteria with high proteolytic activity resulted in slightly enhanced %TA values and a higher FAN content in the fermented product, which indicates the value of the addition of these bacterial strains in the production of a better quality fermented fish product.

The survival rates of the bacterial inoculums were also determined by plating out a day before and after the inoculations took place and results showed that the LAB strains and *Kocuria varians* strain did not survive the fermentation conditions well, as their viable counts decreased to very low numbers early in the fermentation process. The inability of the LAB strains to survive the fermentation conditions could be ascribed to their sensitivity to a low pH environment created by the production of acidic metabolites during the fermentation. However, the viable counts of the inoculated *Bacillus* spp. were found to retain relatively high levels throughout the fermentation process, possibly due to the germination of endospores. The presence of the starter bacteria in the fermenting fish mixture at the end of the fermentation was also successfully determined by the use of the PCR-RFLP technique. Considering the variations in pH, %TA and FAN content of the fermenting fish products inoculated with selected bacterial starters as well as the survival rate of these starters, it appears that the *Bacillus* spp. showed the most potential as commercial starters.

In addition to this, the ability to develop fingerprints for a variety of bacterial species including the inoculated starters, shows the effectiveness of PCR-RFLPs as an identification technique. This molecular technique can, therefore, be used to rapidly identify selected bacteria at any stage during similar fish fermentation processes and would enable the determination of the prevalent dominant microbial species in fermented fish products.

The fermented fish product, obtained at the end of the fermentation period, had a good aroma and compared favourably to similar commercially available fermented fish products (Cha & Cadwallader, 1998). The produced fish paste had

an acceptable yellowish-brown colour and smooth texture, whereas the fish sauce was a clear dark coloured liquid with a buttery aroma, indicating the successful laboratory production of these products. The end-products were not sensory evaluated, since the possibility that certain spoilage or pathogenic organisms could be present in the product could not be safely dismissed.

Concluding remarks

The PCR-RFLP profile data obtained in this research was used in the rapid identification of the bacterial isolates previously identified from similar fermented fish products (Lubbe, 2000). This technique could, therefore, also be used to identify these isolates in other similar fermented fish products and determine the dominant bacterial strains present during the fermentation of fish tissue. These strains could then be considered as essential to the fermentation process and be included in commercial starters.

The presence of spoilage and pathogenic bacteria in commercially available fermented fish products have been observed in previous studies (Lubbe, 2000) and this would indicate the uncontrolled and unhygienic processing of these products. The PCR-RFLP technique could, therefore, also be used to develop fingerprints for potential spoilage or pathogenic organisms, which would enable the screening of fermented fish products for these microbes to ensure the distribution of microbiological safe end-products.

The bacterial starters used in this study resulted in a product with good aroma and the end-product was consistent with other commercially available fermented fish products. The survival of the LAB strains used were, however, unsatisfactory and other LAB strains should be evaluated for their ability to survive in a low pH environment. Since the microbial populations present in the fermenting fish mixture contribute greatly to the characteristics of the fermented product, other microbial starters should in future be considered. This will enable the production of a diverse range of high quality products with acceptable individual tastes and textures, which could also be produced and marketed in South Africa.

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