

Development of Model Fermented Fish Sausage from New Zealand Marine Species

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Abstract

Three New Zealand marine species, hoki (*Macruronus novaezealandiae*), kahawai (*Arripis trutta*) and trevally (*Pseudocaranx dentex*) were used to develop model fermented fish sausage. The formulation comprised fish mince, carbohydrate, minced garlic and salt in a mass ratio of 1 (fish): 0.15: 0.05: 0.03, respectively. The carbohydrate source was cooked rice or glucose. (Endogenous lactic acid bacteria (LAB) failed to ferment rice). Folate was also added to the mixture as a factor. The mixtures were extruded into 50 mL plastic syringes, where the needle end of the barrel had been excised by lathe. The lubricated barrel was overfilled to 60 mL, capped with a layer of ParafilmTM and aluminium foil, sealed tightly by rubber band and incubated at 30°C. Over time the piston was progressively advanced to yield samples for microbiological, physical, and chemical analysis.

Over 96 hours an increase in the LAB count was observed with a concomitant decrease in pH. After fermentation was complete, the samples contained around 8.77 log cfu LAB g⁻¹ with the pH range from 4.38 to 5.08. The microbiological and pH behaviour of each species varied between preparations.

Hardness, adhesiveness, springiness and cohesiveness of the treatments increased with fermentation, except for hoki. The treatments showed different colour characteristics with fermentation. The light reflectance (L* values) of the trevally and kahawai treatments increased, while the a* (redness) and b* (yellowness) values decreased. Hoki exhibited smaller colour changes except for yellowness, which increased markedly.

Proteolysis, measured colorimetrically by soluble peptide bonds, was greatest for trevally. Lipid oxidation, measured by the thiobarbituric acid method, was least for hoki, notably the species with the lowest fat content. Biogenic amines, which are a general quality indicator of fermented products, increased during fermentation. The trevally treatment generated the highest concentration of amines, but these values were lower than those reported for fermented fish sausage in Southeast Asia.

Notably there were no important difference between folate treatments and those without folate.

The results point to commercial opportunities and further research with New Zealand marine species, especially trevally. To improve the product quality and to show geographical exclusivity, further research could be done by using starter culture, and a New Zealand staple

carbohydrate source such as kumara and potato, and spices and herbs which are commonly used in New Zealand, such as rosemary, thyme and sage or specific to New Zealand, such as horopito. In addition, sensory studies should also be performed before the products could be tested in the market.

Statement of Originality

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institutions of higher learning, except where due acknowledgement is made in the acknowledgement”.

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Chapter 1

Introduction

“If you know the history of man’s food, you know the history of man” Keith H. Steinkraus, Cornell University, Ithaca, New York, U.S.A.

History and current occurrence food preservation

Preservation is the means by which food is able to be kept for longer periods without significant deterioration. To be stable for future consumption, the food must be free from deterioration in quality from a wide range of reactions that can be physical, chemical, enzymatic or microbiological, or combinations of these (Gould, 2000). Table 1 lists the principal quality deterioration reactions of foods. The various forms of spoilage caused by microorganisms, both pathogenic and non-pathogenic are preventable to a large degree by a number of preservation techniques, most of which act by preventing or slowing microbial growth.

Table 1. Principal quality deterioration reactions of foods

Microbiological	Enzymatic	Chemical	Physical
Growth or presence of toxinogenic microorganisms	Hydrolytic reactions catalysed by lipases, proteases, etc.	Oxidative rancidity	Mass transfer, movement of low molecular weight compounds
Growth or presence of infective microorganisms	Rancidity catalysed by lipoxygenases	Oxidative and reductive discolouration	Loss of crisp textures
Growth of spoilage microorganisms	Enzymatic browning	Non-enzymatic browning Destruction of nutrients	Loss of flavours Freeze-induced structural damage

From Gould (1989)

Microbial colonisation is one of the most important factors contributing to the spoilage of food and or development of pathogenicity. One obvious example is the spoilage of fish where the earliest sign of spoilage may be noted by examining the gills for the presence of off-odours. Fresh iced fish are spoiled by bacteria, where the bacterial biota of spoilage is found to consist of the asporogenous, Gram-negative rods of the *Pseudomonas* and *Acinetobacter-Moraxella* types (Jay, 2000). In addition to the action the microorganisms, endogenous enzyme activity can also

cause deterioration in the quality of food, especially fruits and vegetables. For example, once the tissues of fruits such as apple and banana are damaged, browning occurs due to the oxidation of polyphenolic substances, which are usually contained within the vacuole of the plant cells, and are physically separated from the enzyme polyphenol oxidase (Coultae, 2002). Food is comprised of chemicals that are reactive to varying degrees, leading to quality deterioration. One example is the reaction induced by sunlight on milk, which can produce off-flavour due to light-induced fat oxidation (Potter & Hotchkiss, 1998). The last factor that contributes to the deterioration of food quality is physical appearance. An example of physical damage is frozen meat that exhibits air pockets between meat fibres due to the evaporation of water from the tissues (Rahman, 2007).

Table 2. Major existing technologies for food preservation

Techniques that slow or prevent the growth of microorganisms	
Technique	Example
Reduction in temperature	Chill storage, frozen storage
Reduction in water activity	Drying, curing with added salt, nitrate, smoke, conserving with added sugar
Reduction in pH	Acidification (e.g. use of acetic, citric acid etc.), Fermentation
Removal of oxygen	Vacuum or modified atmosphere packaging
Modified atmosphere packaging	Replacement of air with CO ₂ ; O ₂ ; N ₂ mixture
Addition of preservatives	Inorganic (e.g. sulphite, nitrite) Organic (e.g. propionate, sorbate, benzoate, parabens) Bacteriocin (e.g. nisin) Antimycotic (e.g. natamycin) In water-in-oil emulsion foods

Techniques that inactivate microorganisms	
Technique	Example
Heating	Pasteurisation Sterilisation including canning

Techniques that restrict access of microorganisms to products	
Technique	Example
Canning	Canning of food
Aseptic processing	Aseptic packaging

From Gould (2000)

To cope with problems of food deterioration discussed above, many techniques have been developed to prevent the growth of microorganisms, or to delay the chemical, enzymatic or physical changes. Table 2 presents the major current methods of food preservation and each is subsequently described in brief.

Techniques that slow or prevent the growth of microorganisms

Reduction in temperature

Reduction in temperature by refrigerators and freezers is the most common method of preserving almost any kind of foodstuff. Russell & Gould (2003) reported that when the temperature of the food is low, fewer types of microorganisms are able to multiply and the growth rates are generally slowed. A good example is the chilling of the meat after slaughter. The multiplication of the bacteria that populate the surface of meat is increasingly delayed as the temperature falls from body temperature (37°C) past 30°C and except for psychrotrophs it slows markedly at refrigeration temperatures (Ingram, 1972). For truly longer term protection against multiplication of bacteria, freezing to well below the freezing point of meat is required. The suggested maximum holding time for frozen foods is not based on the microbiology of such foods but on such factors as texture, flavour, tenderness, colour, and nutritional quality upon thawing and subsequent cooking (Jay, 2000).

Reduction in water activity

The preservation of food by drying is based on the fact that microorganisms and enzymes need water in order to be active. In preserving food by this method, the moisture content has to be reduced to a point where the growth and general metabolism of food-spoilage and pathogenic microorganisms are inhibited. The water needed to support these activities is not the water content as can be measured by drying at above 100°C , but rather the water that is ‘available’ to the microorganisms. This is called water activity, the values of which lie between 0 and 1. The growth of the majority of food spoilage bacteria can be prevented by reduction of water activities to values between about 0.65 and 0.86 to 0.90 in intermediate moisture foods (Gould, 1996). This can be achieved by curing (the addition of salt) or smoking or conserving (the addition of sugar) or by drying (partial or complete), or freezing. (Freezing not only prevents growth by lowering temperature, but also locks up potentially available water in ice, unusable by the microorganisms.)

Salt curing to lower water activity is a widely-used method of preserving meat and fish (Goulas & Kontominas, 2005). In its common modern application, curing salt in solution is used instead of solid salt. Using salt solution provides several advantages. It permits much more

rapid and uniform distribution of curing salt. Salt curing also results in fluid retention such that water can be added to the product (Ingram, 1972). Traces of nitrate and nitrite are routinely used in modern salt curing of meat. Originally these anions were contaminants of salt. Since salt purity has ‘improved’ it has become necessary to add these contaminating anions – more specifically nitrite – to curing salt. Honikel (2008) reported nitrate and nitrite acts as inhibitors for some microorganisms, and enhance the preservative action and yield the red/pink colour typical of cured meats.

In parallel to or in addition to salt/nitrite curing, exposure to wood smoke results in deposition of chemicals into the meat and fish in order to add more preservative effects. Smoke is produced by the process of incomplete combustion of wood which produces a variety of compounds with preservative effects such as aldehydes, ketones, alcohols, acids, hydrocarbons, esters, phenols, ethers etc. (Guillen & Errecalde, 2002). These components are transferred to the smoked foods by deposition on their surface and subsequent penetration into their flesh. Smoking increases the storage life of the flesh food as a result of the combined effects of dehydration, antimicrobial and antioxidant activity of the smoke constituents (Leroi & Joffraud, 2000; Rorvik, 2002).

Reduction in pH

Reduction in pH can be achieved in two ways. Edible food acids like benzoic, sorbic, propionic (propanoic), acetic (ethanoic), lactic, citric, and malic acids can be added directly to the food. Pickled onions made principally of onions and vinegar (acetic acid) is a good example. But in this thesis the acidification is achieved by fermentation. As will be discussed in much more detail later in this chapter, fermentation results in the accumulation of food acids from carbohydrate sources.

Russell & Gould (2003) reported that most of the pathogenic microorganisms such as *Clostridium* species, *Staphylococcus aureus*, *Listeria monocytogenes*, most *Bacillus*, and *Salmonella* species and *Escherichia coli* cannot growth below the pH value of 4.5.

Whether added directly or through fermentation, organic acids exist in a pH-dependent equilibrium between the undissociated and dissociated state when used as food preservatives (Brul & Coote, 1999). The uncharged, undissociated state of the molecule is primarily responsible for the antimicrobial activity and is most prevalent at low pH. Organic acids, therefore, have optimal inhibitory activity at a low pH (Aslim, Yuksekdag, Sarikaya, & Beyatli, 2005; Nazer, Kobilinsky, Tholozan, & Dubois-Brissonnet, 2005). The inhibitory action of the

organic acid is due to the fact that the undissociated compounds are able to freely cross the plasma membrane to enter the bacterial or fungal cell. Once inside the cell, the molecule encounters a higher pH and will then dissociate, resulting in the release of the charged anions and protons that could not have crossed the plasma membrane on their own (Brul & Coote, 1999). Russel (1992) found that these accumulating anions are toxic and able to inhibit metabolic reaction. The other mechanisms that have also been proposed for the inhibition of microbial growth by weak acid preservatives include membrane disruption and stress on intracellular pH homeostasis (Stratford & Anslow, 1998). Organic acids demonstrate both bactericidal and bacteriostatic properties, while the salts of lactic and sorbic acid appear to act primarily bacteriostatically (Smulders & Greer, 1998).

Vacuum packaging

Vacuum packaging is typically packaging of a product in a sealed oxygen-impermeable barrier bag from which air is removed to prevent the growth of aerobic spoilage organisms, oxidation, and colour deterioration. It is also an example of modified atmosphere packaging (see next) because the removal of air from the environment is a modification of the atmosphere, although the composition of residual air is unchanged immediately after the vacuum is applied.

Modified atmosphere packaging (MAP)

The main expression of modified atmosphere packaging is the exclusion of oxygen by modifying the gaseous environment surrounding the products (Rao & Sachindra, 2002). The use of modified atmosphere for food packaging was noted as early as 1882 that carbon dioxide has a preservative effect on food. A good example of this packaging is the continuing large scale transoceanic shipments of (unfrozen) meat under modified atmosphere from Australia and New Zealand to Britain that began in the 1930s. Studies on the effect of carbon dioxide on the slowdown of growth and metabolism of microorganisms under anaerobic condition provided the scientific basis for development of this technology (Rao & Sachindra, 2002).

Principally, gases used in modified atmosphere packaging include oxygen (only for meat on retail display), nitrogen, and carbon dioxide, although trace gases such as carbon monoxide, nitrous oxide, and sulphur dioxide are also used. These gases can be applied individually or in combination in different proportions in order to extend the storage life of the food. Their use in MAP is mainly to replace oxygen to delay oxidative rancidity and inhibit growth of aerobic microorganisms (Rao & Sachindra, 2002; Sivertsvik, Jeksrud, & Rosnes, 2002). However, the presence of some oxygen is desirable to inhibit strict anaerobes.

Carbon dioxide extends the shelf-life of perishable food by retarding bacterial growth. According to Sivertsvik et al. (2002) a combination of the following activities account for the bacteriostatic effect of CO₂:

- Alteration of cell membrane function including effects on nutrient uptake and absorption
- Direct inhibition of enzymes or decreases in the rate of enzyme reactions
- Penetration of bacterial membranes, leading to intracellular pH changes
- Direct changes in the physicochemical properties of proteins

Addition of preservatives other than organic acids

Parabens are alkyl esters of *p*-hydroxybenzoic acid and inorganic sulphites are different ionic forms of sulphur dioxide. They are strongly antimicrobial and can be added to food in tightly controlled concentrations. Sulphite inhibits many of the NAD⁺ dependent dehydrogenases of yeast and bacteria and is a particularly effective inhibitor of the glyceraldehydes dehydrogenase of yeast and the malate dehydrogenase of *Escherichia coli* (Coulgate, 2002).

Techniques that inactivate microorganisms

Heating

The use of heat to preserve food is based on the fact the metabolic activity and growth of microorganisms are inhibited as temperatures rise significantly above the temperature range to which each microorganism is adapted. There are two temperature categories in common use, pasteurisation and sterilisation. In the case of the pasteurisation of milk, for example, the process is designed to destroy the vegetative cell of all disease-producing organisms. The temperature applied to pasteurise the milk is sufficient to destroy the most heat resistant of the non spore-forming pathogenic organisms such as *Mycobacterium tuberculosis* and *Coxiella burnetii* (Jay, 2000).

Sterilisation

Sterilisation of food including canning is the destruction of all viable organisms that are commonly measured by appropriate plating or enumerating techniques. Sterilisation destroys yeasts, mould, vegetative bacteria, and spores (Rahman, 2007). For example, sterilisation of milk can be achieved by ultra high temperature treatment in the temperature range of 140 to 150°C for a few seconds (Jay, 2000).

Techniques that restrict access of microorganisms to products

Canning

Canning is the oldest and most important means of preparing ambient temperature-stable, long storage-life foods. Originally developed in response to military needs in the Napoleonic Wars (Hui, Nip, Rogers, & Young, 2001), the canning of food remains the dominant means of preservation that is fundamental to the food trade between countries. Canned fish, for example, is supplied to the United Kingdom from countries such as Peru, Thailand and Indonesia and nearer home from North Africa and Europe (Footitt & Lewis, 1995). In outline, food canning requires permanent hermetic closure, heating to 120°C, and the prevention the recontamination of the can (Footitt & Lewis, 1995). The heating process kills the microorganisms that would be a health hazard or spoil the food; only very heat resistant spores of spoilage bacteria can survive. Canning also inactivates the enzymes that cause the food to spoil (Berkel, Boogaard, & Heijnen, 2004). Canning of acid foods is conducted under a milder heating regime – the low pH is relied upon to inhibit the germination of *Clostridium botulinum* spores (Simpson, Figueroa, Llanos, & Teixeira, 2007).

Aseptic packaging

The only difference between canning and aseptic packaging is that in canning, non-sterile food is placed in non-sterile metal or glass containers, followed by container closure and sterilisation, whereas in aseptic packaging, sterile food under aseptic conditions is placed in sterile containers and the packages are sealed under aseptic conditions as well (Jay, 2000). Generally aseptic packaging is applied to liquid food such as fruit juices and a variety of single-serve products of this type because these foods can be pumped through a heat exchanger and can be aseptically packaged.

New emerging technologies to preserve food

In addition to the above described commonly used techniques to preserve food, there are some other emerging food preservation technologies, all of which aim at preventing growth or killing microorganisms that cause disease or spoil the food. These are briefly described below.

High hydrostatic pressure

High hydrostatic pressure or ultra-high pressure is a cold pasteurisation method employed for storage- life extension and pathogen reduction while retaining the food's inherent colour, flavour, nutrients and texture (Morris, Brody, & Wicker, 2007). This method has been applied commercially to refrigerated delicatessen meats, pressurised cooked ham, and as a post-

packaging lethality step for the inactivation of *Listeria monocytogenes* on ready-to-eat meats such as sliced ham, and deli meat (Devlieghere, Vermeiren, & Debevere, 2004; Morris et al., 2007). However, one of the major drawbacks of this process for the food industry is the occurrence of pressure resistant vegetative bacteria after successive pressure treatments, and the high capital cost of equipment.

Ohmic heating

Ohmic heating, also known as Joule heating, electric resistance heating, direct electric resistance heating, electroheating, and electroconductive heating, is a process in which alternating current is passed through food material. Principally, ohmic heating can kill microorganisms due to the conduction of electric current (De Alwis & Fryer, 1990; Rahman, 2007). This process has been used to a variety of foods, including liquids, solids, and fluid-solid mixtures. It is used commercially to produce liquid egg product in the United States and to process whole fruits such as strawberries in the United Kingdom and Japan. In 1997, there were 19 plants operating worldwide using ohmic heating technology (Rahman, 2007).

Light energy in food preservation

The principal mode of action of this technique is that ultraviolet radiation contributes to the killing of bacteria on the surface of the food. Application of ultraviolet light at 254 nm and doses of 300 mW cm^{-2} resulted in a surface microbial reduction on mackerel by 2 to 3 log cycle (Huang & Toledo, 1981). These authors also affirmed that the shelf life of Spanish fresh mackerel was extended by 7 days over the untreated sample when the skin surface was treated with high-intensity ultraviolet light and stored in ice at -1°C . In addition, high energy pulsed visible light has also been found to destroy microorganisms (Morris et al., 2007).

Irradiation

Food irradiation is a method of preserving food using ionising radiation. Although the discovery of this process was a century ago, there have been a lot of controversies about the health effects (if any) on the use of the irradiation on food. Irradiation of food needs an approval from governmental food regulators. For example, in the United States, irradiation of meat and meat products requires prior approval not only by the Food and Drug Administration, but also by the US Department of Agriculture's Food Safety and Inspection Service (Diehl, 2002). The first commercial package of irradiated beef reached the retail consumer market in 2000 (Mermelstein, 2001).

Hurdle technologies

The utilisation of combinations of several food preservation methods is called hurdle technology. Hurdle technologies include the use of modified atmosphere packaging, active packaging, cryogenic cooling, antioxidants, ozonation and enzymes in conjunction with the aforementioned and other technologies (Morris et al., 2007).

Modified atmosphere packaging has been discussed above. Active packaging is the addition of absorbing or emitting agents that limit product degradation or microbial growth by controlling oxygen, moisture, carbon dioxide and odours (Brody, 2005). Cryogenic cooling and freezing may be employed to rapidly chill a product, thus extending shelf life. Antioxidants as hurdles minimise the fat oxidation.

Although there are many methods to preserve food, there is no single method that can preserve the quality of all food. Each preservation method discussed above has its own benefits and deficiencies.

History of fermented foods and principles of preservation by fermentation

As noted in the section on the **Reduction in pH**, organic acids can be directly added to food to lower the pH; however, the reduction in pH discussed in this section is achieved by fermentation.

It is widely believed that the change from hunting and gathering societies to the production and cultivation societies occurred between 10,000 and 15,000 before present (BP), in the Middle East (Bamforth, 2005; Campbell-Platt, 1987). With increasing population concentrations, the devising of methods to preserve food was crucial to meet nutritional demands. Drying of foodstuffs was probably the first development in this direction, followed by smoking, and fermentation (Toldra, 2007).

The word fermentation, derived from the Latin (to boil), means among other things to simmer or bubble, or to leaven as a process. It was not well understood, except that the effect was certainly used as an antecedent to baking, in wine making, beer brewing, and in the production of dairy products and certain muscle food products. Over the centuries, fermentation techniques have been refined and diversified. But as a preservation technique, fermentation was probably the least understood because it is almost certain that prior to the work of Pasteur no one had any idea of what was actually happening during fermentation. Fermentation of food is currently practised by every ethnic group in the world (Hui et al., 2004). Such food products play an important role in cultural identity, local economy and gastronomic delight.

The knowledge of alcoholic beverages production from barley was known to the Egyptians and other Middle Eastern cultures as is clear from an inscription on Babylonian brick dating from 4800 BP. By 2800 BP, Europeans were making flat sour-dough bread, and it is estimated that around 2100 BP there were 250 bread bakeries operating in Rome. The drinking of wine was common in the Roman Empire. Records of soy sauce and *miso* production in China go back to around 3000 BP, with the transfer of knowledge of these production processes to Japan occurring around 1500 BP (Campbell-Platt, 1987). Accounts of production of fermented dairy products can be found in early Sanskrit and Christian works, while recipes of both sweet and savoury fermented milks were given in Roman times around 1900 BP. The preparation of fish sauce by fermentation was practised by the Romans around 1900 BP in Europe and North Africa, but today Southeast and East Asia are the main regions of its production. Figure 1 shows important chronological events in food fermentation and preservation.

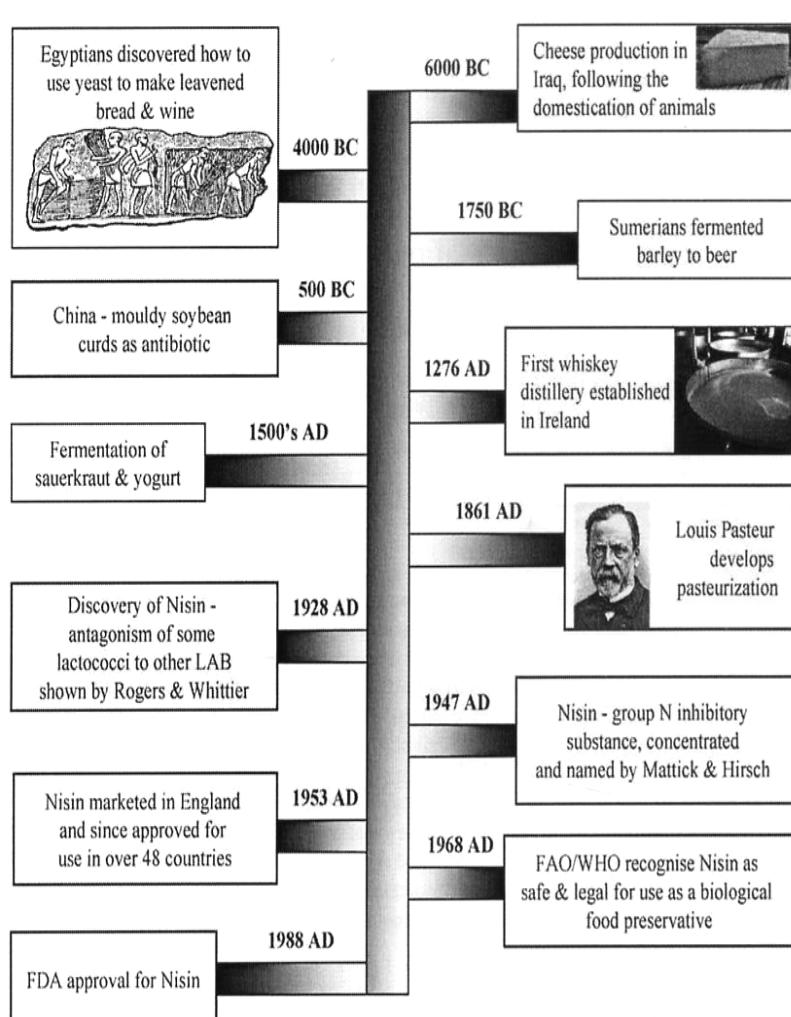


Figure 1. Major events in food fermentation and preservation through the years. Dates in this figure are expressed in the Christian calendar, but as before present (BP) in the text. From Ross, Morgan, & Hill (2002)

Fermented food is now produced all over the world according to the availability of the food substrate and food consumption patterns. For example, fermented dairy products are common in Europe, while the fermented fish products are common in South and Southeast Asia. Table 3 lists the production of classes of fermented food by geographical region.

Table 3. Classes of fermented food by geographical region

World production	Region	Dietary importance	
		Major	Minor
High	Europe	Dairy; beverages; cereals; meat	Legumes; starch crops
	North America	Beverages; dairy; meat	Fish; legumes, starch crops
Medium	South Africa	Starch crops; cereals; beverages	Dairy
	South America	Beverages; dairy	Legumes
	Middle East	Dairy	Legumes; meat
	Indian Subcontinent	Cereals; legumes	Meat
	East Asia	Fish; legumes	Dairy
	Southeast Asia	Fish; legumes	Dairy
Low	Oceania	Dairy	Legumes
	North Africa	Dairy	Legumes

From(Campbell-Platt, 1987)

Europe produces the largest quantity of fermented food such as fermented dairy products, cereals, beverages and meat products, but produces only a small quantity of fermented legumes and starch crop products. The second largest producer of fermented food is North America, presumably stemming from European settlement, but only minor quantities of fermented legumes, starch crops and fish products are produced. Africa is the third largest fermented food producer. There, the significant fermented products are starch crops, many of which are tropical crops, but Africa is only a minor producer of fermented dairy products because of low milk production.

The less producing region of fermented food reflects the less populated areas of Oceania and North Africa. However, some fermented commodities are produced all over the world, such as dairy products (cheese, butter and yoghurt), meat products (sausages), and other cereal products such as bread, buns etc.

Looking beyond fermented products by country or region, different authors have classified products in different ways. Campbell-Platt (1987) classified fermented food into nine classes: beverages, cereal products, dairy products, fish products, fruit and vegetable products, legumes,

meat products, starch crop products, and miscellaneous products (like mushrooms). Steinkraus (1997) classified fermentations according to the type of fermentation, such as alcoholic wines and beer, and alkaline Nigerian *dawadawa*. Rahman (2007) grouped the fermentations in terms of the biochemical products used to transform the food, for example, production of lactic acid, acetic acid, ethanol, and CO₂. In this chapter, the principle of food preservation by fermentation will be described with the greatest emphasis on the preservation by lactic acid as the end product.

According to Steinkraus (1997) there are five basic principles behind the safety of fermented foods, fermentation of plant matter producing a meat-like texture food, fermentation yielding ethanol, alkaline fermentation of plant matter, high-salt savoury-flavoured amino acid/peptide sauces and pastes from animal and plant matter, and lactic acid fermentation. Some of these processes are dominated by enzymatic hydrolyses, which by some definitions are not strictly fermentations. However Steinkraus' classification is adopted here and discussed below.

Classes of fermented food according to Steinkraus (1997)

Fermentation producing a meat-like texture food

The food substrates are overgrown with desirable, edible microorganisms and become resistant to invasion by spoilage or pathogenic microorganisms. One example based on this principle is Indonesian *tempe* (Steinkraus, 1997). The soybeans are soaked, dehulled and partially cooked. During soaking, the soybeans undergo an acid fermentation that lowers the pH to 5.0 or below, which is the value that can inhibit many microorganisms but not desirable moulds. Lactic acid bacteria dominate the soaking stage (Mulyowidarso, Fleet, & Buckle, 1991). Nout & Kiers (2005) showed that acidification can suppress the natural microflora such as coliforms, *Klebsiella pneumoniae* and yeasts. Challenge tests with pathogenic bacteria such as Enterobacteriaceae, *Listeria monocytogenes* and *Bacillus cereus* were successfully inhibited by the colonising *Lactobacillus plantarum*. The main mould that completes the fermentation is *Rhizopus oligosporus* or related *Rhizopus* species, which knit the bean cotyledons into a compact cake used in recipes as a protein-rich meat substitute. These moulds grow rapidly around 40°C, a temperature that is too high for many bacteria and moulds, and come to dominate the microflora in less than a day.

Fermentation involving production of ethanol

Ethanol is the end product for foods produced based on this principle. These include Western styles wines and beers and a wide range of equivalent drinks from other cultures (Steinkraus, 1997). These are generally yeast fermentations but they also involve yeast-like moulds such as *Amylomyces rouxii* and mould-like yeasts such as *Endomycopsis* and sometimes

bacteria such as *Zymomonas mobilis*. The substrates for fermentation in this class are simple sugars usually derived from more complex carbohydrates. Ethanol is both flavour compound and food preservative. It prevents the growth of spoilage and pathogenic microorganisms as long as the drink is maintained in an anaerobic state (Haard et al., 1999).

In an aerobic environment, bacteria of the genus *Acetobacter* can oxidise ethanol producing acetic acid more commonly called vinegar. As foods, vinegars are generally safe. Acetic acid is bacteriostatic to bactericidal depending upon the concentration (Steinkraus, 1996).

Alkaline fermentation

Alkaline fermentations involve the genus *Bacillus*, typically *Bacillus subtilis*. These fermentation products – made from a variety of seeds including soy, locust bean, cucurbit seeds, sesame, and the nominally highly poisonous castor bean (Odunfa, 1988; Steinkraus, 1991) – are common in Africa. Biochemical changes during the fermentation include the release of ammonia resulting in the increase of pH up to 8 or higher and the release of free amino acids. Ammonia is produced by deamination of amino acids (Hui et al., 2001). The combination of high pH and free ammonia along with very rapid growth of the colonising microorganisms at above 40°C makes it very difficult for other microorganisms to compete. These are safe foods even though usually made in an unhygienic environment (Steinkraus, 1997).

High-salt savoury-flavoured amino acid/peptide sauces and pastes

Fermented foods with the salt content of around 13% w/v or higher prevent putrefaction, and prevent the development of pathogens like those responsible for botulism. The substrates for these fermentations include soy and other grains (fermented by *Aspergillus oryzae*), or small fish.

The latter class is prepared by protein hydrolysis, principally using the proteolytic gut enzymes of the fish and shrimp (Killinc, Cakli, Tolasa, & Dincer, 2006; Steinkraus, 1997), with fermentation by halophilic pediococci.

Whether prepared from grains or fish, these sauces and pastes contain a mixture of savoury, amino acids and peptides that are important condiments, particularly for those unable to afford much meat in their diet (Steinkraus, 1996). In a study on the chemical, microbiological and sensory changes associated with fish sauce processing over a period of 57 days, no pathogenic bacteria such as *Staphylococcus aureus* were detected during the whole period of fermentation (Killinc et al., 2006). Fermentation by this principle usually takes quite a long time to complete, usually from a month to one year (Buisson, 1978) (Table 4).

Table 4. Examples of some high-salt fermented fish products in Southeast Asia

Country	Name of product	Fish species	Fermentation time
Cambodia	<i>Prarhok</i>	<i>Channa micropeltes</i> spp.	1 year
	<i>Phaork</i>	Small fishes	1 to 3 months
Thailand	<i>Nam-pla</i> (sauce)	<i>Stolephorus</i> spp. <i>Rastrelliger</i> spp. <i>Cirrhinus</i> spp.	5 months- 1 year
	<i>Pla-chao</i>	<i>Wallago, Belodontichthys,</i> <i>Kryptopterus, Puntius,</i>	1 month
	<i>Nuoc-nam</i> (sauce)	<i>Stolephorus</i> spp. <i>Engraulis</i> spp.	3 months- 1 year will keep indefinitely
Vietnam	<i>Mam-nem</i>	<i>Stolephorus</i> spp.	1 month

From Buisson (1978)

Lactic acid fermentation

Lactic acid fermentations became known to humans as soon as domesticated animals were milked. People stored the milk in a container, and one of the earliest containers was the stomachs of slaughtered animals, which would become colonised by lactic acid bacteria, which as the name suggests are capable of fermenting the milk disaccharide lactose to lactic acid. Sour milk became one of the first fermented foods and exists in the form of yoghurts to this day. The pH declines as lactic acid accumulates – in stomach or any other container – and the caseins precipitate (curdle) leaving the whey proteins in colloidal suspension in a lactic acid/lactose/salts solution. The precipitate is primitive cheese and for millennia, cheeses have been an important part of the diet of human and they remain so today (Hui et al., 2004).

Lactic acid fermentations include those in which the fermentable sugars – not necessarily lactose – are converted to lactic acid by organisms such as *Leuconostoc mesenteroides*, *Lactobacillus. brevis*, *L. plantarum*, *L. bulgaricus*, *L. acidophilus*, *L. citrovorum* *Pediococcus cerevisiae*, *Streptococcus thermophilus*, *S. lactis*, and *Bifidobacterium bifidus* (Steinkraus, 1997). This type of fermentation is responsible for preserving vast quantities of human foods. In addition, lactic acid fermentations provide the consumer with a wide variety of flavours, aromas and textures, thereby helping to determine unique product characteristics (Caplice & Fitzgerald, 1999). Fermentations involving production of lactic acid are generally safe and can also prevent pathogenic conditions (Steinkraus, 1997). The specific antimicrobial mechanisms of lactic acid bacteria exploited in the biopreservation of foods include the production of organic

acids, hydrogen peroxide, carbon dioxide, diacetyl, broad-spectrum antimicrobials such as reuterin and the production of bacteriocins (Caplice & Fitzgerald, 1999).

The direct antimicrobial effects of organic acids including lactic, acetic and propionic (propanoic), all of which can be produced in varying quantities by lactic acid bacteria, are well known (Davidson & Taylor, 1997). Eklund & Gould (1989) suggested that the antagonism results from the action of the acids on the bacterial cytoplasmic membrane which interferes with the maintenance of membrane potential and inhibits active transport (see section on **Reduction in pH**).

Lactic acid bacteria lack catalase to break down the hydrogen peroxide generated in the presence of oxygen. Condon (1987) proposed that hydrogen peroxide can accumulate and have an inhibitory effect on some competing microorganisms. Inhibition is mediated through the strong oxidising effect on membrane lipids and cell proteins (Lindgren & Dobrogosz, 1990).

Carbon dioxide formed from heterolactic fermentation can directly create an anaerobic environment that is toxic to some microorganisms through its action on cell membranes and its ability to reduce internal and external pH (Eklund & Gould, 1989) (see section on **Modified atmosphere packaging**).

Diacetyl is a product of citrate metabolism and is partly responsible for the aroma and flavour of butter and some other fermented milk products (Lindgren & Dobrogosz, 1990). Many lactic acid bacteria including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Lactobacillus* may produce diacetyl, although production is repressed by the fermentation of hexoses. Gram-negative bacteria, yeasts and moulds are more sensitive to diacetyl than Gram-positive bacteria and its mode of action is believed to be due to interference with the utilisation of arginine (Jay, 2000).

Reuterin (2-hydroxypropionaldehyde) is produced during the stationary phase in the anaerobic growth of *Lactobacillus reuteri* on a mixture of glucose and glycerol or glyceraldehydes. It has a general antimicrobial spectrum affecting viruses, fungi and protozoa as well as bacteria. Its activity is thought to be due to inhibition of ribonucleotide reductase (Chung, Axelsson, Lindgren, & Dobrogosz, 1989).

Bacteriocins are secreted low-molecular mass peptides or proteins (between 30 and 60 amino acids), which have a bacteriostatic or bactericidal effect on other bacteria, either in the same genus (narrow spectrum) or across genera (broad spectrum) (Cotter, Hill, & Ross, 2005). Bacteriocins target the cell envelope, and with the exception of the larger bacteriocins (>20 kDa)

that degrade the murein layer (e.g. lysins and muramidases), use non-enzymatic mechanisms to cause the depolarisation of the target cell membrane and/or inhibit cell wall synthesis (Settanni & Corsetti, 2008).

Because foods fermented by lactic acid bacteria have long been recognised as safe, there is a huge range of food produced based on this principle throughout the world. The profile of products in each country depends on culture and availability of the raw materials. Among these product categories of food fermentation are mammalian meats in the Western world and fish in Southeast Asia.

Preservation of meat and fish by lactic fermentation

Meat is the most common substrate in lactic-acid fermented flesh foods in Western cultures, salami being the best known example. Principally, fermented meat (broadly known as fermented sausage) consists of muscular tissue, from which fat and thick connective tissue membranes have been trimmed, and fatty tissue that is most often pork back fat, fermentable carbohydrate, salt and spices. Fermentable carbohydrates include sugar (glucose, sucrose, oligosaccharides derived from starch, sometimes lactose) are commonly used, and sometimes occasionally foodstuffs of vegetable origin like tomato or onion (Toldra, 2007). Nitrite (sometimes initially in the form of nitrate) and ascorbate are normally used to retain a bright red colour and to create another microbiological hurdle against spoilage and pathogens. Moreover, nitrite curing, as it is called, minimises fat oxidation and contribute to the cured meat flavour (Hui et al., 2001).

A variety of meats and spices have been used depending on the culture of the producing countries and availability. For example, Sicilian salami is produced from pork and spices such as pepper and wild fennel seeds (Moretti et al., 2004). *Uratan*, a Balinese sausage is made from pork and spices like garlic, aromatic ginger, galangal piñata, pepper and red chilli (Antara, Jujaya, Yokota, Asano, & Tomita, 2004). *Sucuks*, a Turkish fermented sausage is made from beef meat and/or sheepmeat and tail fat from sheep plus spices like garlic, chilli, bell capsicum, pepper and cumin (Soyer, Ertas, & Uzumcuoglu, 2005).

After mixing the ingredients, the sausage is maintained anaerobically to allow fermentation. This may be followed by maturation procedures, such as smoking and drying, depending on the type of sausage required. Fermentation may be effected by either indigenous microflora or by added starter culture. During fermentation and maturation, a plethora of biochemical changes occur including production of lactic acid, proteolysis, and lipolysis, events that are responsible

for the flavour and textural characteristics and keeping qualities. Table 5 lists different types of fermented sausages and areas of production along with meat from which it is made.

It is clear from Table 5 that the focus in Europe is on beef and pork. But in many parts of Asia, fish is the focus of fermentation.

Typical fish skeletal muscle is a very high protein food with a pH just below neutral, and there is little glycogen reserve to lower pH through glycolysis before and during the onset of rigor.

Table 5. Types of fermented sausages and areas of production

Type	Area	Sausage name	Meat
Dry fermented, mixed culture	Southern and Eastern Europe	Italian salami Spanish <i>salchichon</i> and <i>chorizo</i> French <i>saucisson</i>	Made from comminute pork, Usually with starter culture
Semidry	U.S.A.	Summer sausage Lebanon <i>bologna</i> Sweet <i>bologna</i> Snack sticks	Made from beef, pork or a mixture of the two. Poultry may sometimes be included Usually made with starter culture
	Africa	<i>Biltong</i> <i>Beirta</i>	Made from young and lean carcasses from cattle or game Made from goat meat with offal
	China	<i>La cheong</i> <i>Aap gon cheong</i> <i>Gam ngan cheong</i>	Made from pork and pork fat Made from duck liver instead of pork Made from pork liver
	Thai	<i>Sai Ua</i> <i>Nham</i>	Made from pork with Thai curry paste Made from ground pork and pork skin
	Korea	<i>Sundae</i>	Made from minced pork, pig blood

From Toldra (2007)

Therefore, fish muscle – more commonly called fish – is very susceptible to spoilage by microorganisms (Campbell-Platt, 1987). Fermentation is one way of preventing deterioration by spoilage and/or pathogenic microorganisms.

There are two categories of fermented fish products – high salt products (discussed above, *High-salt savoury-flavoured...*) and low salt products. The latter group is characterised by a

marked increase in the microbial count of lactic acid bacteria whose growth is supported by the addition of fermentable carbohydrate. As an example of such a product, fermented fish mince (FFM) comprises minced fish, minced steamed rice, minced garlic and salt. In its Southeast Asian expression for example, the mixture is tightly packed in banana leaves or plastic bags to exclude air and left to ferment for 2 to 5 days at 30°C (Riebroy, Benjakul, & Visessanguan, 2008; Riebroy, Benjakul, Visessanguan, & Tanaka, 2007) (Figure 2, Figure 3). It is important to note that in these essentially ‘cottage-industry’ examples of FFM, the active LABs are those occurring naturally on the fish and other ingredients, and on hands and equipment. Failure to ferment and thus to generate a low pH could easily result in food poisoning.



Figure 2. FFM packed in banana leaves.
These are nominally airtight



Figure 3. FFM packed with plastic bags. The bags are nominally airtight

Steamed rice and garlic act as the carbohydrate substrates for the fermentation. In addition to the substrate role, garlic is believed to act as an antimicrobial agent particularly against Gram-negative bacteria. This is believed to be due to garlic’s allicin content (Feldberg et al., 1998) and garlic reportedly promotes the growth of lactic acid bacteria (Christine Paludan-Muller, Huss, & Gram, 1999; Zaika & Kissinger, 1984). This growth is fuelled by the approximately 30% content of fructo-oligosaccharides, in the form of inulin. Inulin is a polymer of D-fructose linked by β -(2→1) bonds with an α -(1→2) linked D-glucose at the terminal end of the molecule.

Proteolysis is one of the significant biochemical changes occurring during fermentation of fish mince. It strongly affects texture and flavour development owing to the formation of low molecular weight compounds such as peptides, amino acids, aldehydes, organic acids and amines at the expense of the original protein polymers (Visessanguan, Benjakul, Riebroy, &

Thepkasikul, 2004) (Figure 4). The oxidation of fish fats also contributes to the changes in flavour, colour, and nutritive value (Riebroy, Benjakul, Visessanguan, Kijrongrojana, & Tanaka, 2004). It is important that this fat oxidation is controlled, because excessive fish fat oxidation is undesirable due to the unpleasant odours and flavours that develop.

After fermentation is complete, the product is slightly sour and salty and has a relatively firm and springy texture. In addition, there is a fall in pH which not only increases the firmness and mouthfeel because of the acid denaturation of muscle proteins, but also increases desirable flavours. Paludan-Muller, Valyasevi, Huss, & Gram (2002) found that LAB was predominant in FFM. *Lactobacillus* sp. and *Pediococcus* sp. were identified as the dominant LAB in commercial FFM (Tanasupawat, Okada, Komagata, Suzuki, & Kazaki, 1993).

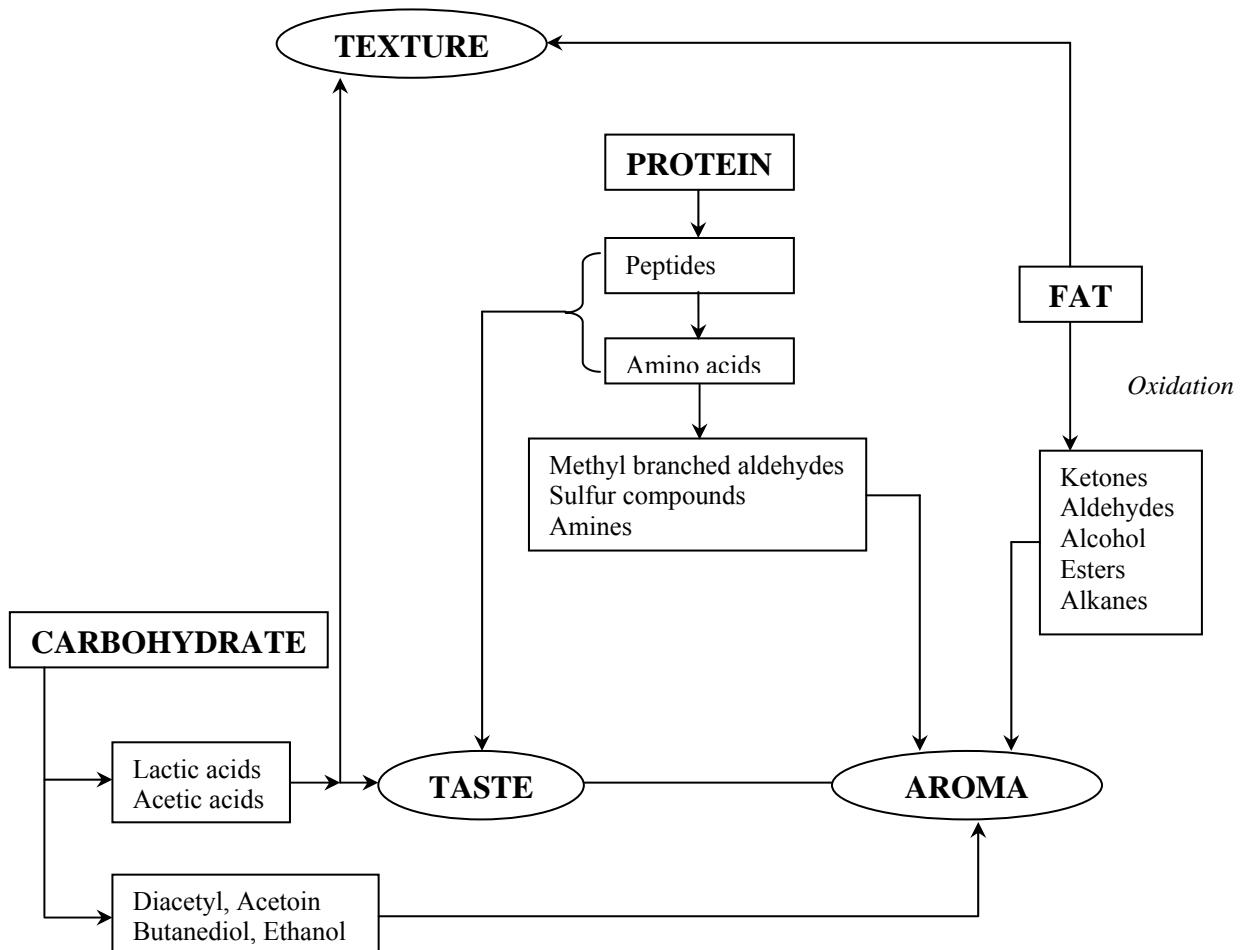


Figure 4. Contribution of fermentation to the sensory attribute of muscle food. From Toldra, Sanz, & Flores (2001)

Generally, LABs produce lactic acid as their main fermentation end-product via glycolysis (the Embden-Meyerhof pathway) or the 6-phosphogluconate pathway and they are named homo-

or heterofermentative LAB, respectively (Axelsson, 1998). The lactic acid provides the typical lactic flavour of these fermentations (Riebroy et al., 2004).

FFM is a good source of protein due to the largely unchanged profile of essential amino acids (Riebroy et al., 2004). A sensory study comparing seven commercial FFMs bought in Thai supermarkets, Riebroy et al. (2004) found some evidence that excess fat oxidation was responsible for inferior flavour; it is well known that fat oxidation can be a major cause of flavour problems. In addition, other parameters might contribute to the effect on flavour such as fish type used in FFM, proteolytic development, the ripening of the products and the amount of lactic acid present in the product.

According to Valyasevi & Rolle (2002), FFM has a colour variability from white to brownish or reddish, depending on the type of fish and the methods used in its production. The product is routinely consumed in countries of Southeast Asia with various names according to the producing countries: *nam* in Cambodia, and *som-fug* in Northeastern Thailand (Campbell-Platt, 1987).

In Southeast Asia the main species used for FFM are fresh water fish. In Thailand for example, fresh water species such as giant snake-head fish (*Ophicephalus micropeltes*), grey featherback (*Notopterus notopterus*), spotted featherback (*Notopterus chitala*), Jullien's golden price carp (*Probarbus jullieni*), and common barb (*Puntius gonionotus*) are used to prepare FMM (Saisithi et al., 1986). In a study to investigate the changes during fermentation of FFM made from several marine species, Riebroy et al. (2007) reported that FFM big-eye snapper (*Priacanthus tayenus*) showed comparable acceptability to the commercial FFM from freshwater species. In another study using big-eye snapper (notably using a starter culture rather than endogenous LABs), Riebroy et al. (2008) observed a reduction in fermentation time and an improvement in product quality.

In this thesis the author has sought to apply Cambodian models of FFM to New Zealand marine species, because the author is a Cambodian national.

Fish preservation in Cambodia

Fish is an important source of animal protein for the population of Cambodia accounting for approximately 75% of the protein intake (Ahmed, Hap, Ly, & Tiongco, 1998). Of the fish landed in Cambodia, 60% is used as fresh, 18% is fermented, 13% salted-dried, 5% smoked, 2% made into high-salt fish sauces and the remaining 2% is made into other derived fish products

(Thuok, Nam, & Sensereyvath, 2001). Most of the non-fresh products are destined for the local market (The Department of Fisheries Cambodia, 2001).

The main products of fish fermentations are high-salt fish sauce, high-salt savoury fermented fish paste (*prarhok*), high-salt fermented fish with roasted ground rice (*phaork*), the low-salt FFM prepared with steamed rice. The high-salted fermented fish products are important food condiments for most households in Cambodia (The Department of Fisheries Cambodia, 2001). The low-salt FFM category is more of a food. Generally, all these products are produced in ‘cottage’-type businesses or are a household activity for family consumption.

Fish preservation in New Zealand

In spite of New Zealand’s huge exclusive economic zone for commercial fishing (Ministry of fisheries, 2007), most fish caught in this zone is marketed nationally and internationally in relatively unprocessed forms, typically unfrozen (stored on ice or at equivalent temperatures) or frozen (Seafood Industry Council, 2009). Smoking is applied to more oily fish, typically salmon and eel (The Coromandel Smoking Co, 2009) and there are emerging methods to preserve fish in New Zealand such as vacuum packaging, preservation by additives, high pressure treatment, ultra-sonication, and strong magnetic fields (Fletcher, 2005).

Inspection of offerings in New Zealand supermarkets and dedicated delicatessens reveals a wide range of fermented mammalian meat products, mostly made from pork. Thus there is wide acceptance of these rather expensive added-value products, but the fermentation technology has (curiously) never been extended to sheepmeat, nor to fish in a modern commercial sense. To the author’s knowledge there are no known commercial examples of FFM-like products made in New Zealand. This suggests there is a gap in the market for FFM, which not only could add value but would require minimal temperature control in longer-term storage. Significantly the FFM approach to preservation was recently identified by Lu (2005) of the then Crop and Food New Zealand.

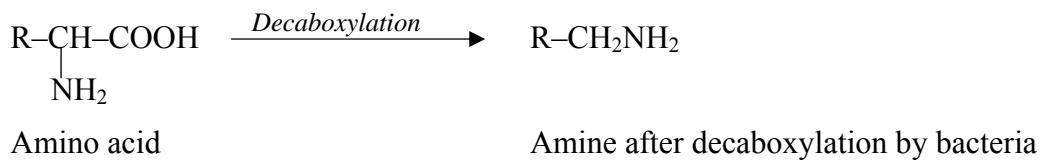
There is an indigenous history of seafood preservation by Maori. In a review of traditional Maori food preparation methods and food safety, Whyte, Hudon, Hasell, Gray, & O'Reilly (2001) described, for example, two methods of preserving kina (sea urchin). First, *kina* was placed in containers of fresh water and held for several weeks. *Kina* was immediately eaten after recovery. Second, *kina* were buried underground for several months. *Kina* prepared in these ways was consumed by removing the ‘teeth’, adding fresh water to the cavity, stirring and then drinking. The authors were not sure if *kina* underwent fermentation during storage because salt

and fermentable carbohydrate were not added at the beginning of the process. The New Zealand kina, *Evechinus chloroticus* reportedly contains up to 5% wet weight of glycogen (Woods, James, Moss, Wright, & Siikavuopio, 2008). Its role in any potential fermentation is not known. Regardless, underground or water storage would lower oxygen concentrations that would be conducive to fermentation. It also seems obvious that generalised hydrolytic activity (proteolysis etc.) would occur due to endogenous or microbial enzymes. Beyond this technology there is no record of fish fermentation ever being employed as a preservation method in New Zealand (Buisson, 1978).

The preservation method by Maori for kina raises an important point. In fermentations where there is inadequate carbohydrate fermentation, microbes present in the fermentation ‘mix’ rely on other macronutrients which are fermented as a source of energy producing biogenic amines (Suzzi & Gardini, 2003). High concentrations of these as a group and of two in particular present a health risk when consumed, and also cause malodours.

Biogenic amines

The term “biogenic amines” represents a group of low-molecular mass organic bases occurring in all organisms. Amines are formed and degraded during normal metabolic processes in living cells and therefore they are ubiquitous in animals, plants, and microorganisms (Halasz, Barath, Simon-Sarkadi, & Holzapfel, 1994). In fermented foodstuffs, biogenic amines are produced by microbial decarboxylation of amino acids (Yongjin, Wenshui, & Xiaoyong, 2007) summarised in equation below.



For example, histidine, tyrosine, and tryptophan generate the biogenic amines histamine, tyramine and tryptamine, respectively (Figure 5).

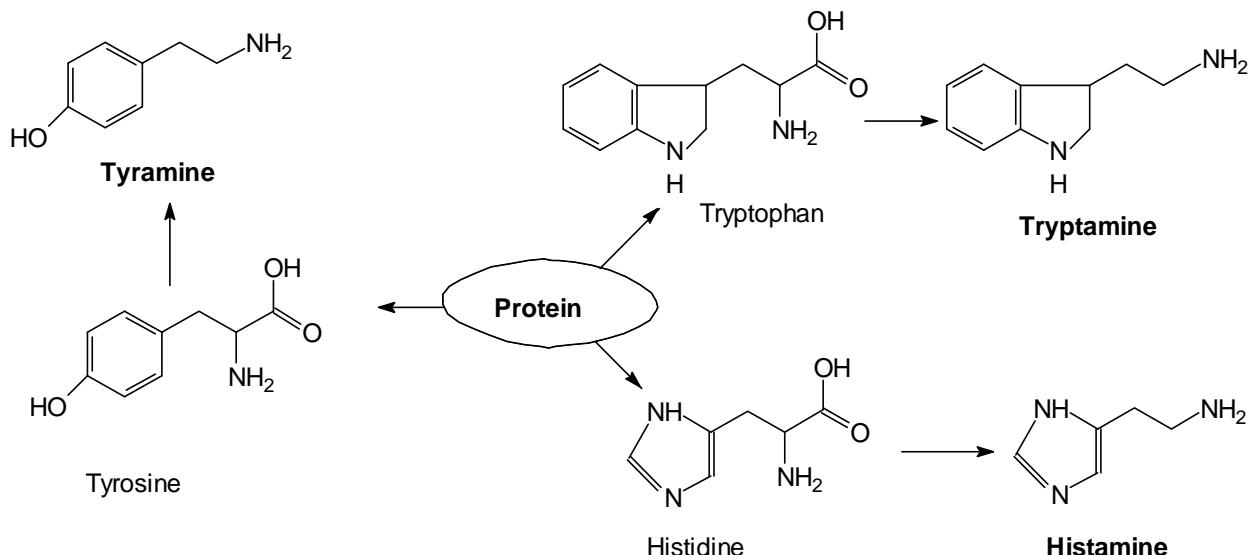


Figure 5. Chemical structure and formation pathway of histamine, tryptamine and tyramine
From Halasz et al. (1994)

Consumption of food containing excessive amounts of biogenic amines, especially histamine and tyramine, were reported to result in nausea, respiratory distress, heart palpitations, headaches and hyper -or hypotension and even intracerebral hemorrhage and death in very severe cases (Smela, Pechova, Komprda, Klejdus, & Kuban, 2003; Yongjin et al., 2007). In addition, tyramine has been reported to cause distress, hot flushes, sweating, a bright red rash, oral burning and a rise in blood pressure (Mah, Kim, & Hwang, 2008). Furthermore, biogenic amines are considered precursors of carcinogenic amines, such as N-nitrosamines (Scanlan, 1983).

Although many studies have shown an increase in the concentration of biogenic amines in fermented meat sausages during fermentation, there have been no reports of health problems caused by biogenic amines in these products (Moore, 2004; Toldra, 2007). However, biogenic amine poisoning, especially histamine poisoning from the ingestion of fish is very common (Mines, Stahmer, & Shepherd, 1997), and occurs usually after the consumption of non-fermented fish where bacteria have had the opportunity to colonise and ferment amino acids. Because histamine food poisoning usually presents with mild symptoms, it is often misdiagnosed as food allergies by medical personnel, resulting in under-reporting. In countries with advanced medical access, those with the most reported incidents of histamine poisoning since 1970 are Japan, the United States and the United Kingdom (Lehane & Olley, 2000).

In Asia, where many countries have limited medical access, various Food and Agriculture Organisation fisheries reports have indicated high concentrations of histamine in fish on sale.

There are also reports of extremely high concentrations of histamine in some salted and dried or salted fermented products in Asia (Lehane & Olley, 2000). Histamine has also been a problem in canned products from Asia. In Japan, histamine fish poisoning was a major cause of illness in the early 1950s, and remains a major foodborn malady in that country. From 1970 to 1980, 42 outbreaks involving 4122 cases were reported by the Ministry of Health and Welfare.

The main source of histamine poisoning is from the Scombridae Family of fish (Lehane & Olley, 2000). This family includes such species as mackerel, tuna, anchovies, sardines, marlin, and the salmonids. In New Zealand, several cases have been reported related to scombroid food poisoning. In 2003, the New Zealand food safety authority issued a recall for smoked kahawai and warned people not to eat the product bought from supermarkets between specific dates in May 2003 because of suspected histamine poisoning in the top half of the North Island. Similarly, in 2002 the same authority also issued a recall for the same products sold in December 2002 (New Zealand Food Safety Authority, 2003). In summary, the cases of histamine fish poisoning outbreaks and associated patient cases in New Zealand are illustrated in Figure 6 below.

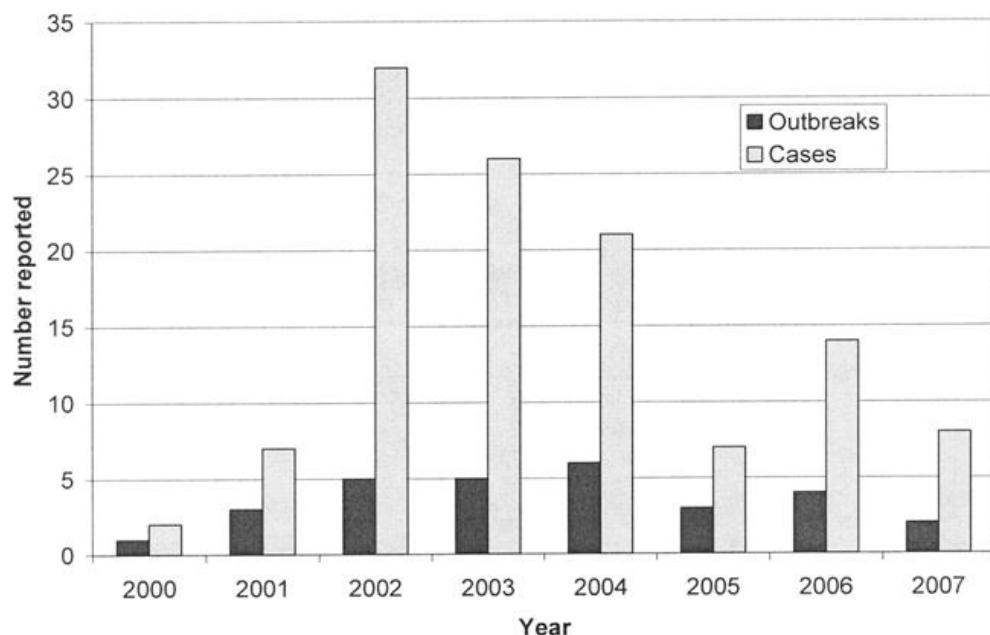


Figure 6. Histamine (scombroid) fish poisoning outbreaks and associated cases reported. From Williman, Cressey, & Pirie (2008)

The concern over food poisoning related to the consumption of foods containing biogenic amines has urged the related authority to set the standard for foods. The Canadian food authority set the histamine limit in seafood at 500 ppm (Gill, 2005) for scombrotoxin, while Food Standards Australia New Zealand has set upper limit of 200 ppm in fish (Food standards

Australia New Zealand, 2006). The U.S. Food and Drug Administration has set 500 ppm as the upper limit for tuna, mahi-mahi and related fish species (FDA, 1998).

Because of the concerns over scombroid fish poisoning, it was thought useful to include analysis for biogenic amines in the present study. This was particularly important in the two species studied here were the scombrids trevally and kahawai.

Food fortification

Shortly before this FFM project was begun, a visiting German Professor Seewald of Anhalt University of Applied Science presented a seminar at the University of Auckland (5 June 2008) reporting his studies on addition of folic acid (as folate) to fermented German-style sausages. The motive for his work was the concern in Germany that the citizens were eating large quantities of meat products at the expense of foods like vegetables and whole grains that are a source of folic acid. (These German concerns echo similar concerns in New Zealand exemplified by the 5+ a day campaign (5aday, 2009). Folate is a B vitamin and among other effects is important for the healthy growth and the development of red blood cell and nerve tissue and the prevention of neural tube defects (Neuhouser & Beresford, 2001).

In Seewald's case, he claimed that folate-fortified sausage enhanced the growth of *Lactobacillus* sp., improved the sausage colour and enhanced the maturation process compared with the non-fortified control. A subsequent literature search has revealed no refereed publications relating to this work. However, an Internet search revealed a patent entitled *Method for production of raw sausage with folic acid and folate* belonging to a German research institute which develops new food processes and agricultural technologies (http://www.invenia.es/tech:06_de_nstt_0fez) . This invention relates to a method for producing raw sausage, comprising an accelerated ripening process based on previously known recipes and basic principles used for producing typical kinds of raw sausage using folic acid and/or folate to support the ripening and also healthy nutrition (Seewald, 2008).

In spite of this lack of scientific validation, it was decided to include a folate treatment in the experimental designs. Therefore, folate was added to some FFM treatments at 10 ppm which is in the range purportedly used by Seewald and coincident with New Zealand Ministry of Health (1999) recommendation for fortification, which is the range 0.4 ppm to 20 ppm.

The planned experiments and the commercial opportunity

The literature study, the Cambodian experience with FFM, the purported advantages of folic acid as an ingredient and the product gap in the New Zealand market have together led to the

following plan: New Zealand marine species are used in models of FFM, with and without folate. Three species were chosen (see below) and the initial carbohydrate source was steamed rice. The fermenting organisms were those naturally occurring on raw materials, cleaned hands and processing equipment. The incubation temperature was arbitrarily set at 30°C, and the fermentation time was four days.

In New Zealand, most commercial fish are marine species. In the development of FFM, three Zealand marine fish were used (shallow water species and deep water species) – hoki (*Macruronus novaezealandiae*), kahawai (*Arripis trutta*), and trevally (*Pseudocaranx dentex*). Hoki is a demersal fish which could be found in the depth between 150 and 1100 meters over the upper continental slope and is also New Zealand's most important commercial fish species with delicate moist white flesh with few bones (Figure 7). The flesh can be flaked easily and formed into a solid block as would occur in FFM production (Seafood Industry Council, n.d-a). Kahawai is a pelagic species in the coastal waters living in the depth between 0 and 50 meters. Its fillets have medium to thick flakes (Figure 8). They have a relatively high oil content and concomitantly a high omega-3 fatty acid content (Seafood Industry Council, n.d-b). Trevally is a pelagic fish living in the depth between 0 and 100 meters over the continental shelf. It has medium to soft fillets with low oil content (Figure 9). The flesh is marbled pink (Seafood Industry Council, n.d-c).

The fermentation outcomes for these three species included textural appearance, colour, smell, pH, LABs, soluble protein, fat oxidation, and biogenic amines, most of which have been outlined in the context of FFM in previous sections of this chapter. The results have then been compared with equivalent published values for fermented fish products from Southeast Asia with the aim of making preliminary commercial assessment in New Zealand market.

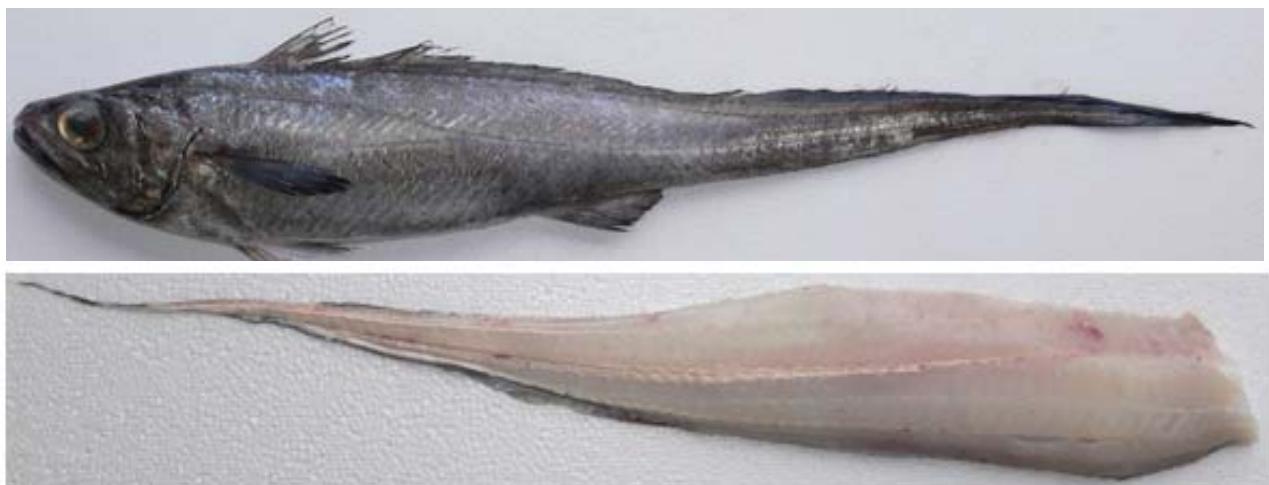


Figure 7. Hoki and its fillet

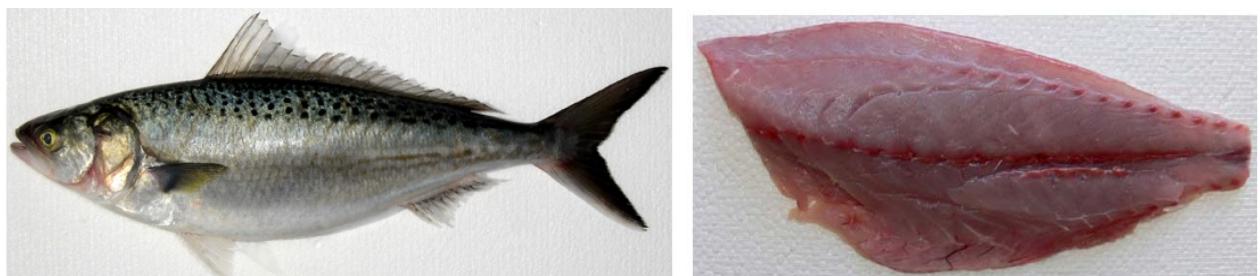


Figure 8. Kahawai and its fillet

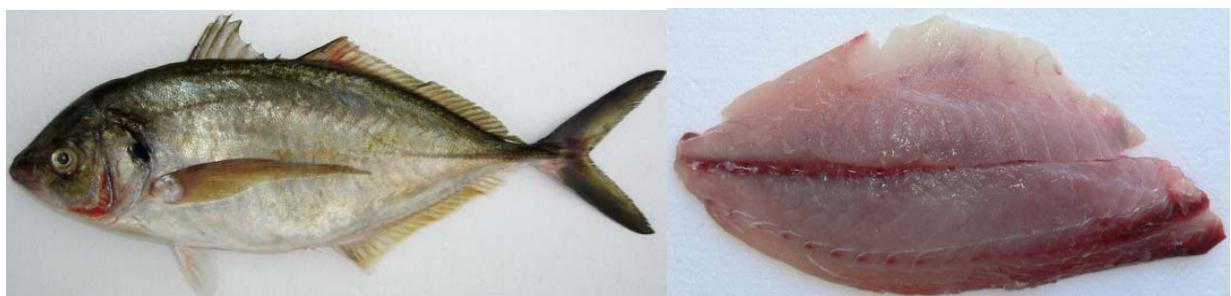


Figure 9. Trevally and its fillet

(All fish species images from Seafood Industry Council of New Zealand)

Chapter 2

Materials and Method

Chemicals

Trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were obtained from B.D.H Laboratory Chemicals. Man-Rogosa-Sharpe (MRS) medium was obtained from Difco, Becton Dickinson, France. Malonaldehyde was obtained from Fluka (Buchs, Switzerland). Dansyl chloride, histamine dihydrochloride, tryptamine hydrochloride, tyramine as free base, and 1,7-diaminoheptane were purchased from Sigma (St.Louis, MO, USA). Ammonium acetate, acetonitrile, sodium carbonate acid, perchloric acid, hydrochloric acid, sodium bicarbonate, sodium hydroxide, copper sulphate, sodium citrate, peptone water, ammonium hydroxide of analytical grade were purchased from B.D.H Laboratory Chemicals. Folin-Ciocalteau reagent was obtained from Scharlau Chemie S. A. (Barcelona, Spain). Bovine serum albumin was obtained from Serva Feinbiochimica (GmbH & Co, Germany). Folic acid was donated by Sanitarium, New Zealand.

Sausage casings

Sausage casing prepared from collagen were donated by Globus, Australia.

Novel equipment

Fifty millilitre syringes with a diameter of 25 mm were used as the casing for the FFM. After taking the plunger out, the needle end of the syringe was excised on a lathe (Figure 10).



Figure 10. The normal syringe and the syringe after the needle end was cut by the lathe



Figure 11. The syringe after extruding the FFM mixture and fitted with two layers, ParafilmTM and aluminium foil

The inside surface of the syringe barrel was lubricated with silicon and/or petroleum jelly. The plunger was then drawn to the 60-mL point, and the barrel was filled with fish mince (see next section). The open end of the filled syringe barrel was then tightly covered, with layer of Parafilm™ overlaid with aluminium foil. A rubber band secured the cover (Figure 11). The aim was to keep air out.

Fermented fish mince (FFM) preparation

Three commercial fish species commonly found in New Zealand waters were used for fermented fish production. These were trevally (*Pseudocaranx dentex*), kahawai (*Arripis trutta*) and hoki (*Macruronus novaezealandiae*) bought as unfrozen fillets from various Auckland retail outlets and maintained on ice. Using cold equipment in an ambient temperature laboratory, the fillets were minced through a 4-mm plate, and blended for 5 minutes in a domestic Kenwood mixer bowl with the carbohydrate source, minced (4-mm plate) peeled garlic cloves bought locally, and salted in the mass ratio of 1 (fish): 0.15: 0.05: 0.03, respectively (Saisithi et al., 1986). The mixtures were extruded into casings or more commonly into the 50-mL syringe barrels described in **Novel Equipment** above. The extruder was attached to the mincer so that the minced fish could be extruded into the syringe barrels and subsequently covered to keep air out. After sealing, the syringes were incubated horizontally at 30°C for 96 hours. Every 24 hours flat cylinders of sausage, with a range of heights depending on the test, were extruded and cut for analyses. Typically, three replicate barrels were prepared for each species at each trial.

The carbohydrate source was cooked white rice, which was later replaced with glucose because the rice failed to serve as a carbohydrate source for fermentation. A generic long grain white rice from Thailand (Foodtown, Quay Street, Auckland) was cooked in a rice cooker with the rice to water ratio of 1:1.5 cups respectively, to a normal tenderness.

Folic acid (food grade, donated by Sanitarium Limited, Auckland) was also added as a factor in some FFM experiments.

Physical analysis of FFM

Texture profile analysis

Texture profile analysis was performed only at 0 hours and 96 hours samples, and where the extruded cylinders were 30-mm high. The instrument used was a TAXT Plus Texture Analyser (Stable Microsystems, U.K.) fitted with a cylindrical aluminium probe whose 50-mm diameter flat surface contacted the sample. The extruded FFM samples were placed flat on the instrument's base (Riebroy et al., 2007). The tests were performed with two compression cycles

at room temperature. Textural parameters were measured at room temperature with the following test conditions (Riebroy et al., 2007): crosshead speed 5 mm s⁻¹; 50% strain; surface sensing force 0.971 N; threshold 0.294 N; time interval between first and second stroke was 1 second. Texture parameters were measured three times from three replicate barrels. The Texture Exponent 32 software (Stable Micro Systems, U.K.) was used to collect and process the data. Analyses were defined and calculated as described by Bourne (1978). Thus, hardness, adhesiveness, springiness and cohesiveness were calculated from the force-time curves generated for each sample.

Colour

The colour of the samples was measured in the L^* , a^* , b^* space using a Hunter meter (Model 45/0 Hunterlab ColorFlex, Reston, Virginia, U.S.A.). L^* , a^* and b^* indicate lightness, redness/greenness, and yellowness/blueness, respectively. The extruded cylinders of FFM were cut (10 mm high \times 25 mm diameter) and centrally placed on the base of a crystallising dish that sat beneath the black shroud (Figure 12). The measured values were corrected for the colour of the empty crystallising dish (mean of three readings). The colour of samples was measured three times from each of the three replicate barrels.



Figure 12. The extruded cylinder of FFM placed centrally on the base of a crystallising dish for colour measurement. The black shroud was removed to take this photograph

Microbiological analysis of fermented fish mince

The lactic acid bacteria counts (LAB) were determined using De Man Rogosa and Sharpe (MRS) agar according to the method of the Association of Official Analytical Chemists (AOAC, 2000). A 10-g sample was aseptically transferred to a sterile plastic pouch and stomached for 1 minute in a Lab-blender (Seward Medical, London, U.K.), to which 90 mL of 0.1% sterile

peptone water had been added. Decimal dilutions of the stomached liquid were made using the peptone water. Aliquots of each dilution (0.1 mL) were plated in triplicate on MRS agar and incubated at 30°C for two days. LAB counts were reported as log colony-forming units per g sample (cfu g^{-1}).

Chemical analysis of FFM

Determination of pH

The pH of the sample were determined according to the method of Benjakul, Seymour, Morrissey, & An (1997). A 4-g sample was homogenised with 40 mL of deionised water and the pH was measured using a portable pH meter (Meterlab, U.K.).

Determination of trichloroacetic acid-soluble peptides

The trichloroacetic acid-soluble peptides were determined by the method of Green and Babbitt (1990). The FFM sample (1 g) was homogenised at 9,500 rpm min^{-1} using an Ultra-Turrax homogeniser (IKA-Werke, Germany) with 9 mL of 15% (w/v) trichloroacetic acid for 2 minutes. The homogenate was kept on ice for 1 hour then centrifuged at 12,000 gravities for 5 minutes. The soluble peptides in the supernatant were measured by the method of Lowry, Rosebrough, Farr, & Randall (1951) and expressed as mg of bovine serum albumin equivalents kg^{-1} sample.

Determination of thiobarbituric acid reactive substances (TBARS)

The TBARS value was measured according to the method of Buege & Aust (1978). A 2.5-g sample was homogenised at 9,500 rpm min^{-1} using an Ultra-Turrax homogeniser (IKA-Werke, Germany) with 12.5 mL TBARS solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl). The mixture was heated for 10 minutes in a boiling water bath to develop the pink colour. Then, the mixture was cooled with running cold water and centrifuged at 5,500 gravities for 25 minutes. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Ultraspec 2100 pro, U.K.). The TBARS value was calculated from a standard curve of malonaldehyde and expressed as milligram malonaldehyde kg^{-1} of FFM.

Determination of biogenic amines

Sample were extracted according to Mah, Han, Oh, & Kim (2002) with a slight modification. A 2-g sample was added to 10 mL of 0.4 M perchloric acid and the mixture was homogenised at 9,500 rpm using an Ultraturrax homogeniser (IKA Werke, Germany) and

centrifuged at 3,000 gravities for 15 minutes. The supernatant was collected and the residue was extracted again with an equal volume of 0.4 M perchloric acid solution. The supernatants were combined and the final volume was adjusted to 25 mL with the same solution. The extract was filtered through Whatman paper No.1. Derivatisation of the sample extract was performed according to the method of Senoz, Isikli, & Coksoyler (2000). A 1 mL volume of each sample extract was mixed with 0.2 mL of 2 M NaOH and 0.3 mL of saturated sodium bicarbonate, 0.1 mL 1,7-diaminoheptane as internal standard (0.5 mg mL^{-1}), 2 mL dansyl chloride (10 mg mL^{-1} acetone), and then incubated at 40°C for 1 hour. Residual dansyl chloride was removed by the addition of 0.1 mL ammonia solution (25% v/v). After 30 minutes of incubation at room temperature, the extract was filtered through a $0.45 \mu\text{L}$ filter. Quantitation of biogenic amines was determined from the standard biogenic amines. The separation of biogenic amines was performed by High Performance Liquid Chromatography (LC-10AD Shimadzu, Japan) equipped with a Diode array detector (SPD-M10AV) using Nova-Pak C18 column $3.9 \times 300 \text{ mm}$ (Waters, Ireland). An isocratic elution program was used with 50:50 acetonitrile and ammonium acetate as mobile phase. The flow rate was 1mL min^{-1} .

Data analysis

Data were first marshalled by routines within Microsoft Excel. Data were often analysed for variance by the One Way routine in Minitab 15 (Minitab Inc., State College, Pennsylvania). Comparisons between individual means were done with the Tukey test in that routine.

Chapter 3

Process Development

Initial trial fermentations

In the earliest trials, cooked white rice was used as carbohydrate source with hoki, and the final mixtures were extruded into sausage casing and held at 30°C in an incubator (Figure 13). After 48 hours the room smelt strongly of spoilt fish. At 0 hours the pH was 6.33 and 6.19 for the folate and non-folate treatments, respectively. After 72 hours, the respective pHs were 6.35 and 6.50, confirming that a lactic fermentation had not taken place. At this time, the sausage was very hard and dry, clearly because the casing was permeable to water (Figure 14). At least some of the spoilt odour was presumably due to biogenic amine formation on the basis that in the absence of carbohydrate metabolism, the developing microflora extracted energy from decarboxylation of amino acids (Santos, 1996).



Figure 13. Incubation of FFM in collagen casing at 30°C

One obvious explanation for the failure to ferment was because the initial endogenous microflora was incapable to hydrolysing the rice starch to glucose, the fermentable form. Another reason may have been related to water activity in the dry incubator environment, which contrasts with the humid conditions of traditional banana or plastic bag methods employed in Cambodia (Chapter 1, Figure 2, Figure 3). Therefore another trial was performed, this time with

kahawai and trevally, using water-impermeable polypropylene plastic specimen vials as the packaging material. The 32 -mL vials (27 mm diameter) were lubricated with either silicon or petroleum jelly before filling and incubation. Cooked rice was again used as the carbohydrate source for fermentation.



Figure 14. The dried sausage in the collagen casing after incubation for 72 hours

The initial pH was not recorded, but subsequent experiments with these two species suggest that the starting pH would have been around 5.5. Over three days of incubation, the pH decreased slightly to about 5.2 from the 24 hour value of about 5.7, and the LAB count increased slightly as shown (Figure 15, Figure 16). The final pH was above 5, and final LAB count was between log 7 and 8 cfu g⁻¹. Folate was included as a third treatment (in kahawai only). It had no effect on pH or LAB kinetics. There were to be no obvious differences in outcomes between the two species. Thus a lactic fermentation did not develop, as with hoki. A successful lactic fermentation in FFM should result in a final pH below 5 and the final LAB count should be above 10⁸ cfu g⁻¹ (Riebroy et al., 2007).

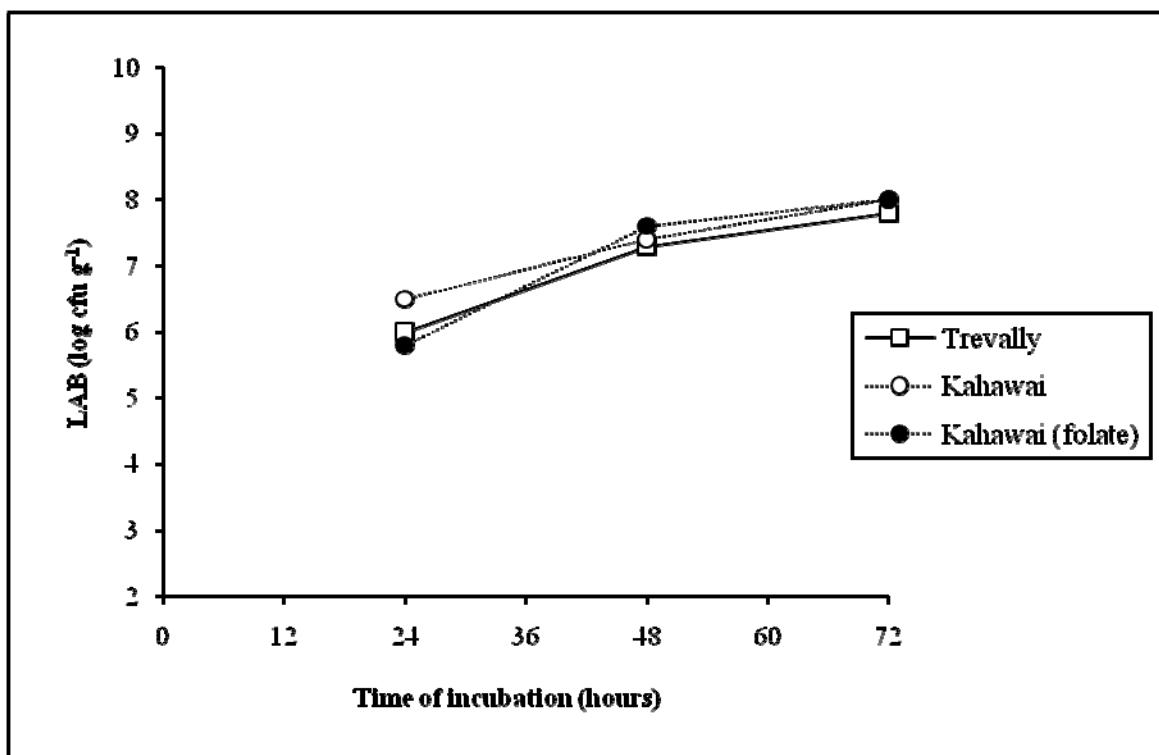


Figure 15. Changes in LAB of FFM produced from trevally and kahawai, using cooked rice as carbohydrate source. The FFM was packed in water-impermeable vials

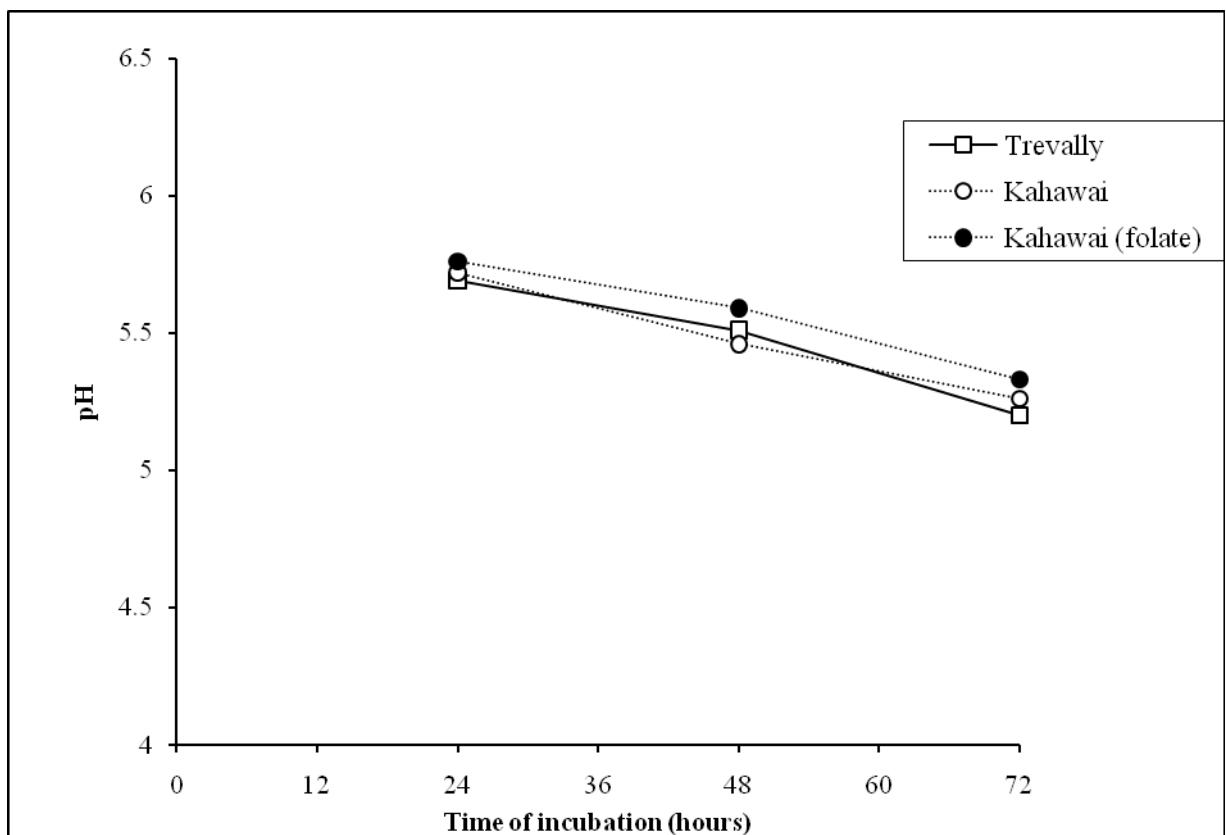


Figure 16. Changes in pH in FFM produced from trevally and kahawai using cooked rice as carbohydrate source. The FFM was packed in water-impermeable vials

Because cooked rice failed to ferment the fish mince mixture, it was decided to use glucose as the carbohydrate source in place of cooked rice. The simplest way of modifying the formulation was to simply substitute an equal weight of glucose. It was later realised that an equal mass of glucose and steamed rice would have a very different final glucose concentration in the event that the wet rice were totally hydrolysed. However, the high concentration of glucose used throughout subsequent work did not result in any problems and it was always certain that glucose would never be limiting, as steamed rice clearly was.

When vial incubations with trevally (\pm folate) were done with glucose, fermentation clearly occurred up to 96 hours (Figure 17, Figure 18). LAB counts increased from around 10^3 cfu g $^{-1}$ at 0 hours to over 10^8 cfu g $^{-1}$ after 72 hours of fermentation, and folate had no effect (Figure 17). There was a sharp drop in pH from 5.8 at 0 hours to around 4.5 after 96 hours of fermentation. Folate had no effect (Figure 18).

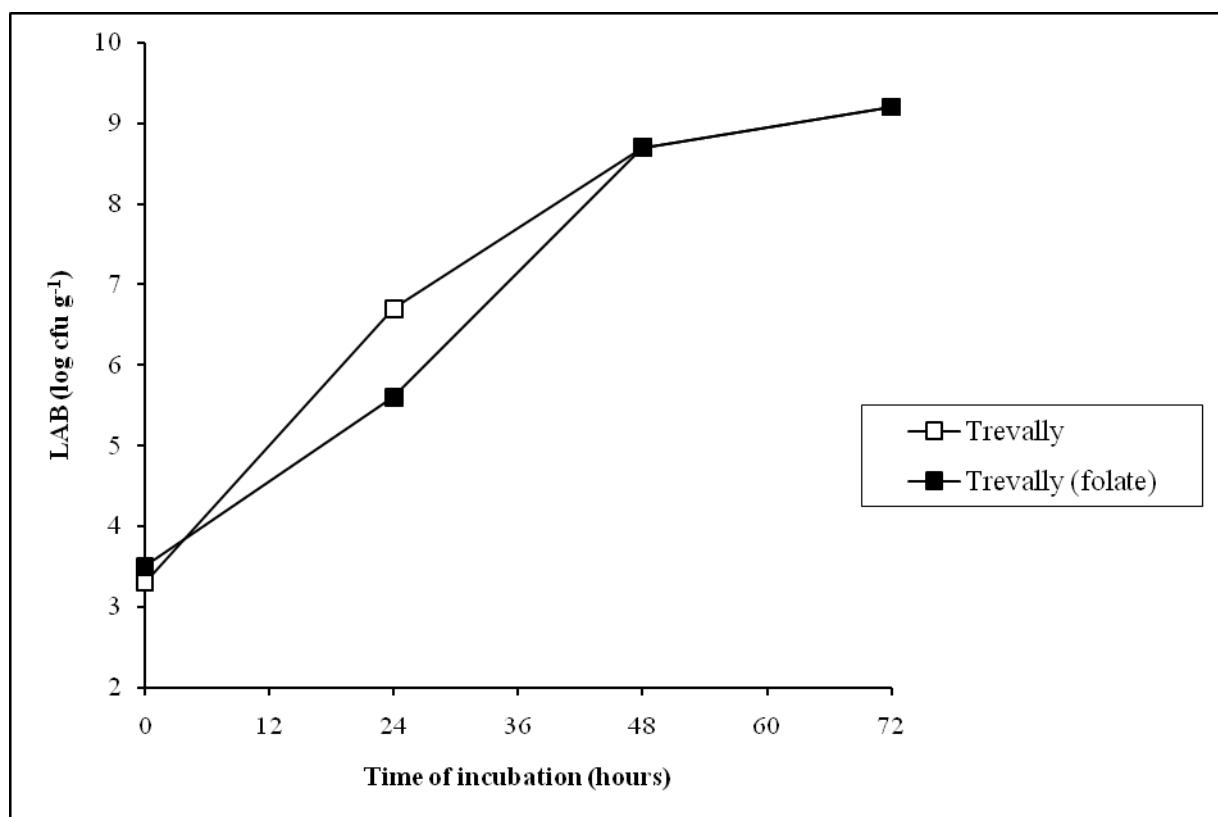


Figure 17. Changes in LAB of FFM produced from trevally with and without folate using glucose as carbohydrate source. The FFM was packed in water-impermeable vials

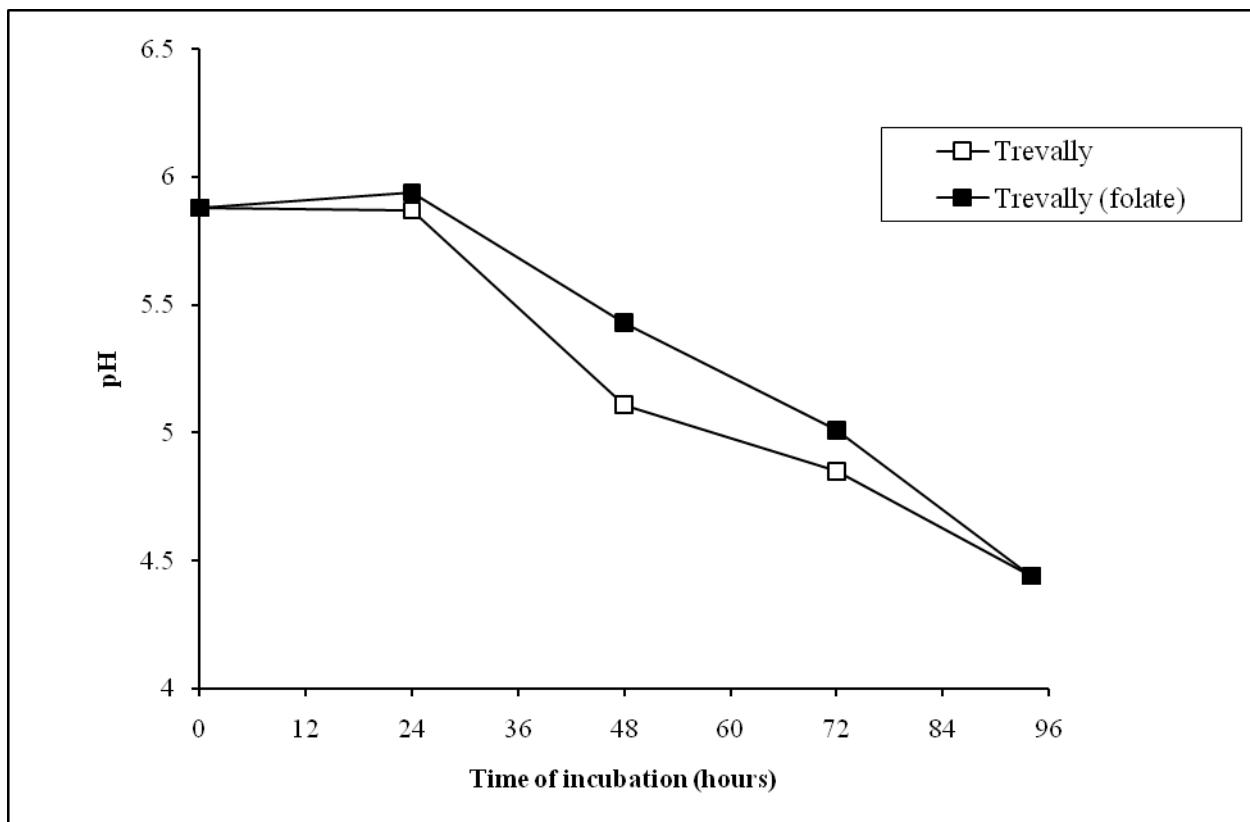


Figure 18. pH development of FFM produced from trevally with and without folate, using glucose as carbohydrate source. The FFM was packed in water-impermeable vials

The formal measurements of LAB counts and pH that indicated fermentation was successful and this was confirmed by the characteristic lactic acid smell of the samples accessed later in the incubation.

Subsequent work was based on the glucose design, but it was realised that the vial incubation method had limited utility in FFM recovery. The sample of each FFM treatment had to be scooped out in some way before testing could be done. Therefore a novel casing system was developed. This used lathe-cut 50 mL syringe barrels described in Chapter 2 (Figure 10, Figure 11). These were routinely and successfully used in all subsequent experiments.

Trial fermentations using syringe barrels

The syringe barrels proved useful in maintaining an anaerobic environment and in providing uniformly-sized samples of treatments at will. The times chosen were 0 hours (not packed in the syringe barrel), 24, 48 and 72 hours. With this timing, experiments could be comfortably done in a working week.

Figure 19 shows the kinetics of LAB counts for the six basic treatments (three species, \pm folate), but with no replication to allow statistical analysis.

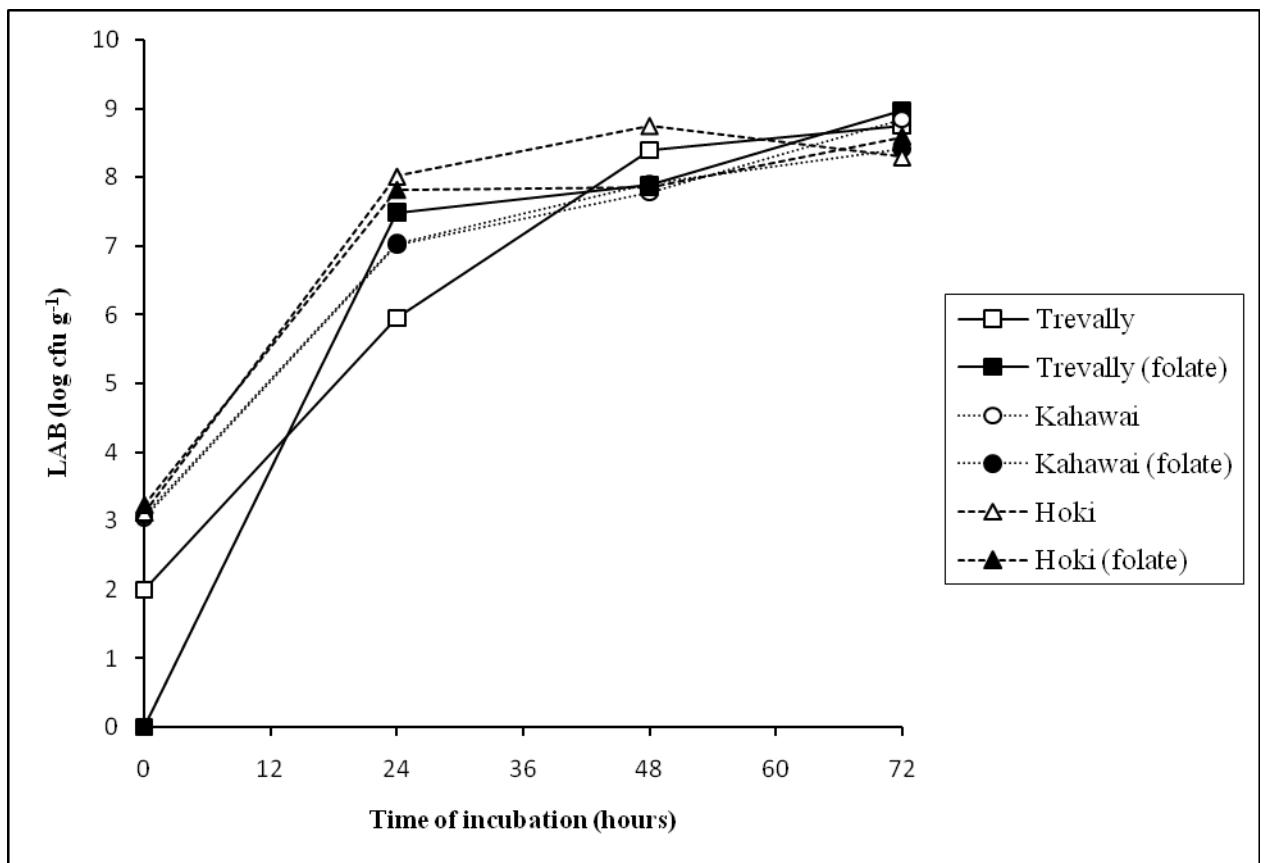


Figure 19. Changes in LAB of FFM produced from trevally, kahawai and hoki with and without folate, using glucose as carbohydrate source

During the first 48 hours of fermentation, the LAB count increased sharply up to 10^8 cfu g^{-1} . However, from 48 hours to 72 hours, the LAB count only increased slightly to over 10^8 cfu g^{-1} for all FFM produced from the three species. Interestingly, within the limits of a single replicate for each species, there was no difference in the growth of LAB due to folate. Figure 20 illustrates the pH development of FFM produced from the three fish species, with and without folate. pH drop fastest and the most with hoki. After 96 hours the pH of all treatments was below 5.0. The effects of folate were negligible within the single replicate limit.

The pilot experiments above showed that FFM could be produced from the three species, relying on exogenous glucose as the carbohydrate source and the endogenous microflora of the fish fillets as bought at retail. This then was the working model for all subsequent work, for which treatments were triplicated so that the observed results could be analysed statistically.

The next chapter reports the physical, microbiological and chemical properties of FFM from the three New Zealand marine species.

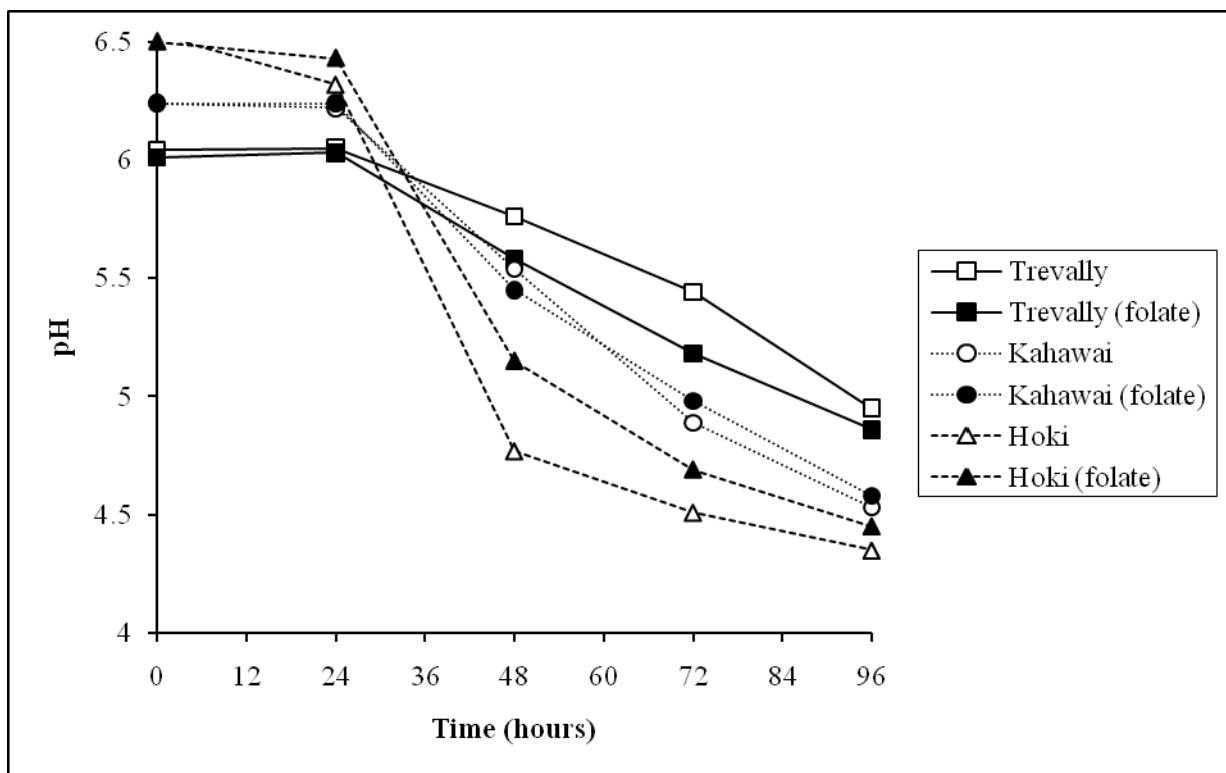


Figure 20. pH development of FFM produced from trevally, kahawai and hoki with and without folate, using glucose as carbohydrate source

Chapter 4

Results and Discussion

Textural properties of FFM during fermentation

The textural parameters of FFM at 0 hours and at 96 hours are shown in Table 4 with an example of the graph of the compression test shown in Figure 21. For trevally and kahawai, the hardness, adhesiveness, springiness and cohesiveness of the FFM increased numerically during fermentation, at various levels of statistical significance. Whereas the adhesiveness of hoki FFM increased numerically over 96 hours, the three other parameters decreased. At 96 hours, the FFM from trevally was clearly the hardest, with little difference between kahawai and hoki. Kahawai was the most adhesive at 96 hours ($-1,421 \pm 353$ mN s) compared with -676 ± 314 (trevally) and -872 ± 235 mN s (hoki) ($P < 0.05$ for both comparisons). At 96 hours, trevally FFM was the springiest and most cohesive of the three species ($P < 0.05$ for both comparisons). Saisithi et al. (1986) reported that the gel-forming ability of FFM depends on the kind of fish used, and that was certainly the case here.

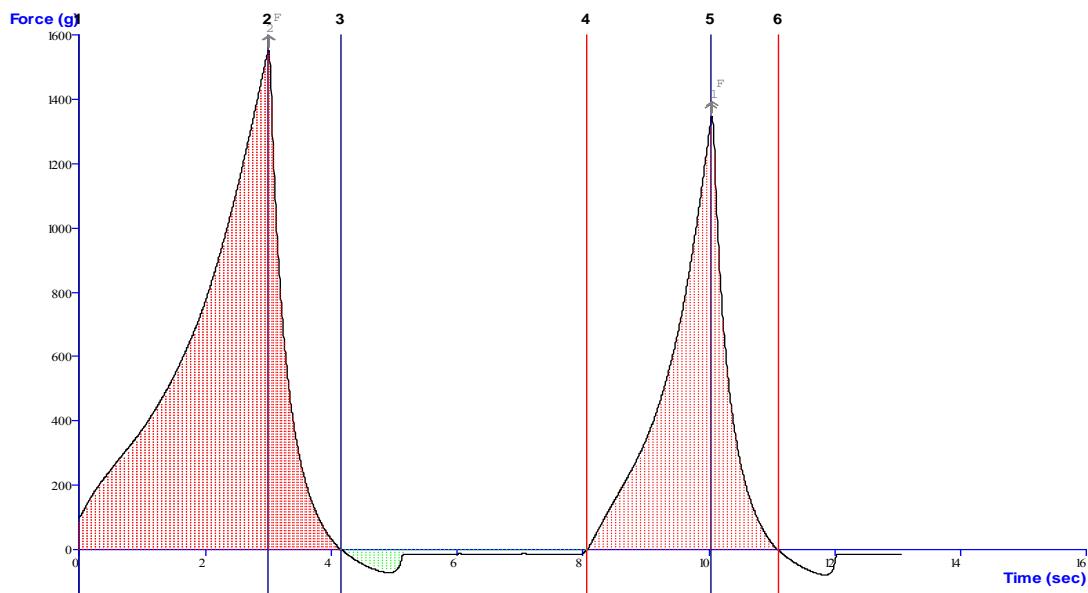


Figure 21. An example of compression test graph to measure textural properties of FFM from hoki FFM. Force is shown in grams in the graph, but the results are presented in SI units in Table 4. Shading represents data analysis in progress

Hardness, springiness and cohesiveness are primary mechanical parameters that can be used to characterise the textural properties of any sausage (Li, Carpenter, & Cheney, 1998). The hardness of sausage is a measure of the degree of maturation, resulting from gelation of denatured meat proteins, and the loss of water (Gimeno, Astiasaran, & Bello, 1999). However,

loss of water did not occur with the present experimental system indicating that hardness changes were due only to gelation.

Table 4. Changes in texture of FFM at 0 and 96 hours of fermentation

Fish species	Fermentation time (hours)	Hardness (N)	Adhesiveness (mN s)	Springiness	Cohesiveness
Trevally	0	¹ 7.71 ± 0.28	-1,666 ± 118	0.54 ± 0.13	0.34 ± 0.06
	96	28.01 ± 0.34	-676 ± 314	0.65 ± 0.03	0.46 ± 0.01
Effect of time		***	**	NS	*
Kahawai	0	5.82 ± 0.18	-1,921 ± 196	0.31 ± 0.01	0.24 ± 0.01
	96	9.19 ± 0.15	-1,421 ± 353	0.44 ± 0.04	0.26 ± 0.02
Effect of time		***	NS	**	NS
Hoki	0	11.13 ± 0.47	-1,352 ± 872	0.91 ± 0.48	0.51 ± 0.08
	96	8.99 ± 0.72	-872 ± 235	0.50 ± 0.04	0.28 ± 0.02
Effect of time		*	NS	NS	**

¹ Data are means ± standard deviation from three determinations

*, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant

In a study to compare FFM from seven commercial brands made from undescribed tropical freshwater fish, and randomly bought in Thai supermarkets, Riebroy, Benjakul, Visessanguan, & Tanaka (2005) found that the FFMs had different textural properties. At least some of these differences could arise from species effects (Saisithi et al., 1986). In the compression to 60% of the original high of cubed sample (2 cm × 2 cm × 2 cm), Riebroy et al. (2005) found a range from 28.86 N to 45.94 N, -81 mN s to -23 mN s, 0.84 to 1.81 and 0.52 to 0.69 for hardness, adhesiveness, springiness and cohesiveness respectively. In addition, an acceptability test showed that brands with the highest hardness correlated well with high acceptability of the product.

In a study to develop FFM from different tropical marine fish species, Riebroy, Benjakul, Visessanguan, & Tanaka (2007) found the hardness, adhesiveness, springiness and cohesiveness were respectively ranged from 9.92 N to 33.30 N, -38 mN s to -11 mN s, 0.70 to 0.87 and 0.60 to 0.78 after the completion of fermentation without the addition of starter culture. FFM from big-eye snapper had the highest mean hardness (33.30 N), and was the most preferred by a sensory evaluation panel. In a development of FFM produced from big-eyed snapper with a starter culture, Riebroy, Benjakul, & Visessanguan (2008) reported the hardness of FFM in the range of

19.60 N to 29.40 N during the time course of fermentation. Therefore, the FFM produced from fresh water species had better textural properties than those produced from marine species.

In comparing the present data (Table 4) with the Southeast Asian data of Riebroy and others, two factors are important. First, some textural parameters of FFM can obviously be affected by the size and shape of the FFM test sample. Intuitively, the greater the surface area in contact with the probe and base, the higher the values of hardness and adhesiveness. (However, springiness and cohesiveness are ratios and may not be affected.) With this limitation in mind, the developed FFM from New Zealand marine species, particularly trevally, showed comparable hardness values to those produced from marine species in Southeast Asia. Adhesiveness values were however, very different for the present study and the Southeast Asian data. Adhesiveness values of fully fermented FFM were of the order of -1000 mN s and -40 mN s, respectively. Why this is so is not clear, but the difference may relate to the nature of the test surfaces of probe and base, and to the nature of the FFM. Adhesiveness is effectively stickiness, which could be affected by test surfaces, and by the presence of wet rice as opposed to wet glucose. Whatever the reason for the difference, adhesiveness is probably less important than hardness as a quality attribute (Riebroy et al., 2005).

It is noteworthy that hoki had the highest hardness of the three species at the beginning of the fermentation, but after fermentation, hoki textural properties were poorer. This might be related to the integrity of the muscle protein polymers, especially those between myosin and actin. Typically fish muscle proteins undergo hydrolysis by endogenous proteolytic enzymes at a rate 10 times higher than those of mammalian muscle (Park, 2005). In a study on hoki muscle fibres by scanning electron microscopy, Bremner and Hallett (1985) concluded that the fibrils that connect the muscle fibres are destroyed by endogenous collagenases and/or other proteinases during chilled storage. The storage conditions in the present study were definitely not chilled, and presumably increased the rate of these texturally unfavourable reactions. In surimi production, minced fish flesh is washed to remove the proteolytic enzymes that reduce gel strength after cooking, the so-called *modori* effect (Alvarez, Couso, & Tejada, 1999). In a study on the gel strength of surimi made from hoki, Macdonald, Lelievre, & Wilson (1990) reported that the gel strength from washed mince was greater than from unwashed mince. They also found that surimi made from unwashed hoki mince underwent textural deterioration. Similarly, Guenneugues & Morrissey (2005) reported that surimi produced from hoki can form an excellent quality and high gel strength. Thus the inferior textural properties of hoki FFM might be due to proteolysis, although nothing is known of the proteolytic status of the other two species. There is also another factor that may be important for hoki as discussed next.

MacDonald, Hall, & Vlieg (2002) reported that the average protein content of hoki white flesh varied markedly with season, from 11.4 to 18.5% and the protein content was lowest after the spawning in October. The chilled hoki fillets used for the study were bought in November and December when the protein content of the fillet would relatively low. This has two implications. First, from a biological perspective, it is speculated that the potential for proteolysis would be low at that time of the year, a point that is further discussed in Chapter 5. Second, the FFM formulations all contained 81% fish flesh. If the moisture content for hoki FFM was the highest, this would go some way to explaining differences in textural properties. It is restated that the FFM texture is due to myosin and actin, the two proteins that dominate muscle.

On the basis of textural properties trevally is the best and has comparable textural properties to FFM produced from marine species in Southeast Asia, except for the adhesiveness.

Changes in colour of FFM

The L* values are shown in Figure 22. Summed over all times, it is clear that hoki had the highest L* values (light reflectance), followed by trevally and kahawai ($P < 0.001$). While the L* values of hoki FFM were essentially unchanged with time, those of trevally and kahawai increased significantly with time ($P < 0.001$ for each). If there were any significant differences in L* values due to folate, they were clearly unimportant.

Hoki flesh is notably white which explains the highest reflectance among the three species. It is well known that fish flesh becomes more reflective when denatured. This is obvious from cooking and from acidification due to lime juice for example. The increase in L* values of trevally and kahawai presumably results from myosin denaturation as acidification proceeds due to fermentation (see later). This poses the question as to why there was no obvious change with hoki? The answer may lie in the likely proteolysis that hoki undergoes during storage (Bremner & Hallett, 1985). It is proposed that any tendency to create cavities in structure would necessarily create light traps that would counter increased reflectance as denaturation proceeds with time.

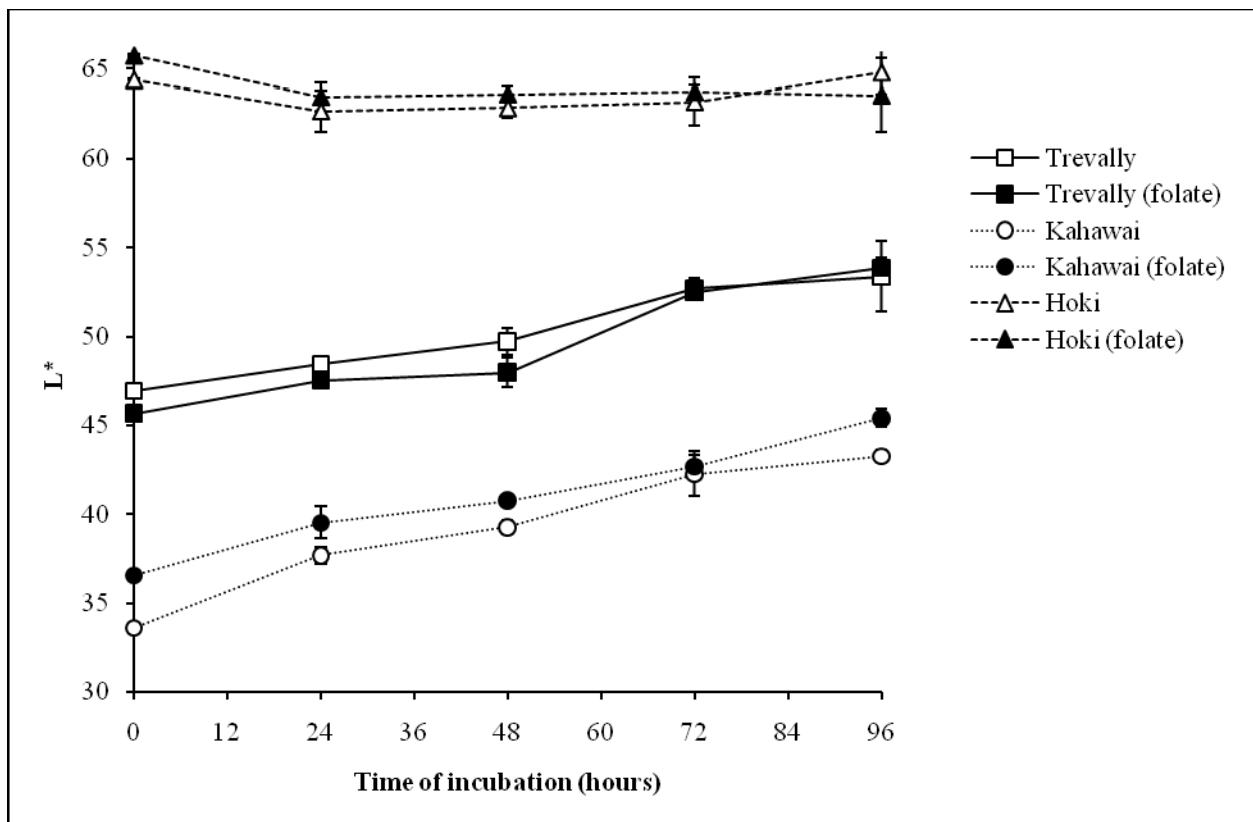


Figure 22. Development of the reflectance (L^*) of the FFM during fermentation. Bars indicate standard deviation from three determinations

The whiter colour of FFM was reported to contribute to high consumer acceptability (Riebroy et al., 2004). On the face of it, hoki FFM would be the most acceptable due to its high L^* value.

The kinetics of a^* values of FFM are depicted in Figure 23. At all times for kahawai, the a^* values were the highest ($P < 0.001$) compared to the other two species because the kahawai flesh is relatively red, as discussed in the Chapter 1.

Over 96 hours of fermentation, the kahawai a^* values decreased significantly ($P < 0.001$). By contrast, the a^* values of trevally fluctuated until the completion of fermentation, where the final a^* values were slightly lower than at 0 hours ($P < 0.001$). With hoki, a^* values appeared to increase slightly during fermentation, but the change was not significant ($P > 0.05$). If there were any significant differences in a^* values due to folate, they were clearly unimportant.

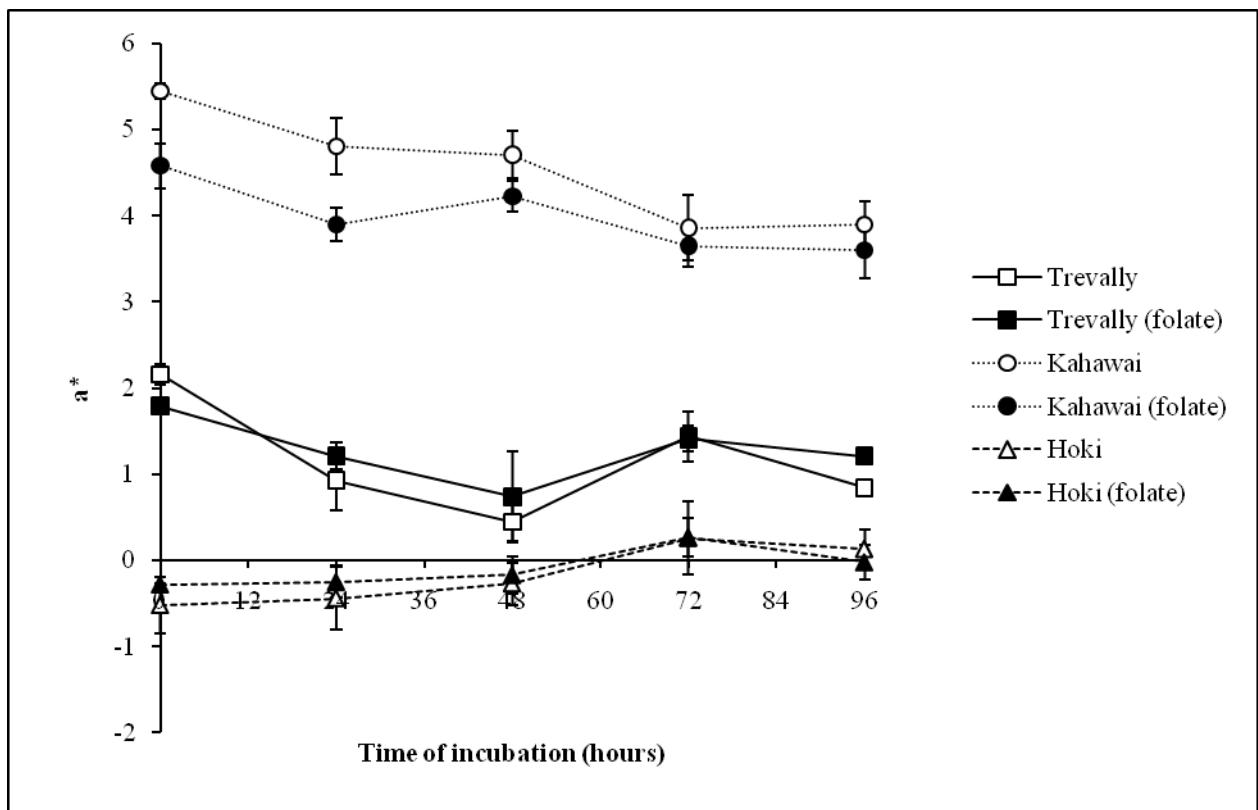
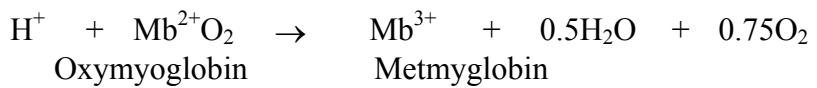


Figure 23. Development of redness of the FFM during fermentation. Bars indicate standard deviation from three determinations

The kinetics of b* values are shown in Figure 24. The b* value of hoki increased sharply until the completion of fermentation ($P < 0.001$) while the other two species showed few changes. There was a slight decreased in b* values of trevally FFM during the first 48 hours of fermentation, but that decrease was not maintained to the completion of fermentation. As noted for L* and a* differences due to folate treatment, if any, were unimportant.

The reason for the increase in yellowness in hoki FFM is unclear. As was described in Chapter 1, kahawai muscle (flesh) is notably red pigmented due to myoglobin – whatever its exact form at any time – and is responsible for the typical iron content of 2.1 mg 100 g⁻¹ (Nelofar, 2006) in that species. The equivalent values for trevally and hoki are lower (especially for hoki) as is described in **Fat oxidation in FFM during fermentation** in this chapter. Under deteriorative conditions such as occur during fermentation at 30°C, the original myoglobin and oxymyoglobin in the fish muscle tends to oxidise to the brown pigment metmyoglobin (Young & West, 2001). This is accompanied by an increase in b* values compared with a* values. Thus of the three species, kahawai might be expected to be the treatment most likely to exhibit colour changes due to metmyoglobin formation. However, metmyoglobin formation is favoured by acidic conditions as is clear from the following equation as reported by Brown & Mebine (1969).



Of the three species, hoki showed the greatest fall in pH which may go some way to explain the differences. But at the same time pH is not the only factor affecting metmyoglobin formation. The oxidoreductive status of muscle is also important (Greaser, 2001); the general status of these species and the specific status of the fillets chosen is completely unknown.

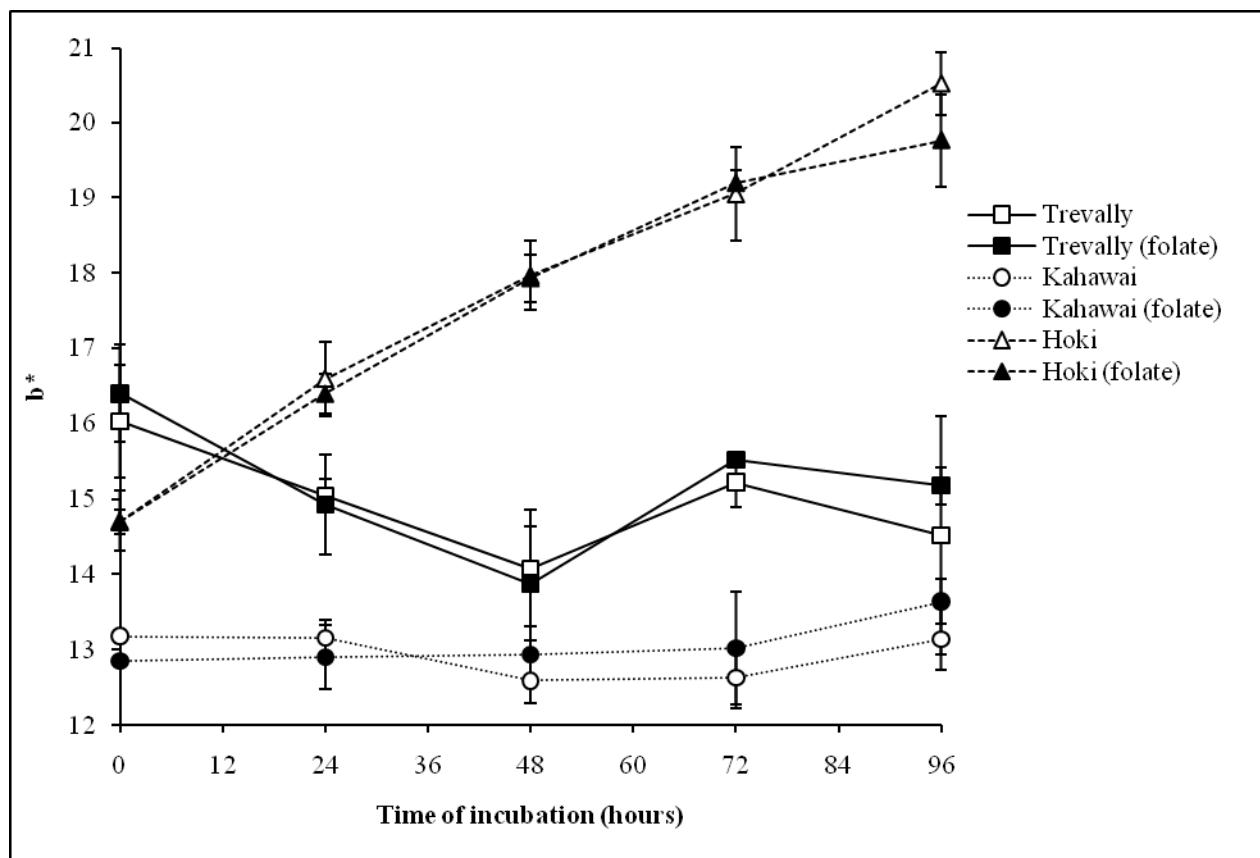


Figure 24. Development of yellowness of FFM during fermentation. Bars indicate standard deviation from three determinations

Changes in LAB of FFM during fermentation

The changes in lactic acid bacteria (LAB) are depicted in Figure 25. The initial microbial load for each species was just above 10^3 cfu g⁻¹ and increased sharply for all species reaching the similar count of over 10^8 cfu g⁻¹ ($P < 0.001$). There is a sharp increase in LAB during the first 48 hours of fermentation. The counts then continued to increase slightly until the completion of fermentation for all species. Hoki FFM had the lowest LAB count during the first 48 hours of fermentation ($P < 0.001$); however, the LAB count for all species was similar at the completion

of fermentation. Though there were differences between the control and folate treatments, they were inconsistent, unimportant and irrelevant by the time fermentation was complete.

In tropical marine species Riebroy et al. (2007) reported that the final LAB counts of FFM from a number of species was approximately 10^8 cfu g⁻¹ from an initial microfloral load ranging from 4.7×10^4 cfu g⁻¹ to 5.2×10^6 cfu g⁻¹. These initial loads indicate that the hygiene of fish processing is inferior to that in New Zealand.

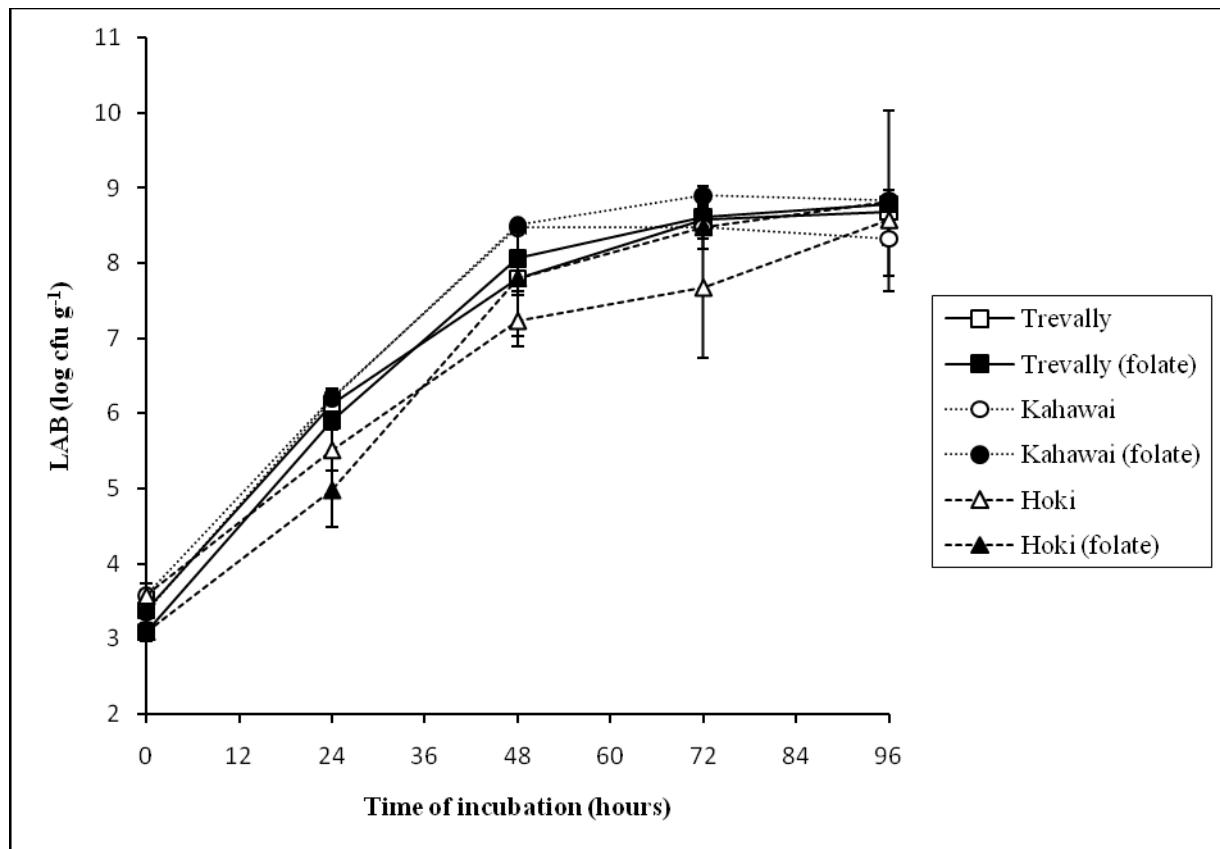


Figure 25. Changes in LAB of FFM produced from trevally, kahawai and hoki with and without folate during fermentation. Bars indicate standard deviation from three determinations

In the case of fermented pork, the initial microflora derives mainly from the raw materials and the processing equipment (Wonnop et al., 2006), and there seems no reason why this should not be true for fermented fish. However, it must be pointed out that hygiene maintenance is always likely to be more difficult in a tropical environment. Whatever the initial load, the outcome in the present study is comparable to that reported by Riebroy et al. (2007) and according to Saisithi et al. (1986), the LAB count in FFM should be above 10^8 cfu g⁻¹ after the completion of fermentation to ensure the pH is lowered to a desirable of 4.6. In short, FFMs produced from New Zealand marine species have desirable final LAB counts.

Since folate treatment showed no important effects for all parameter up to now, folate has been excluded from subsequent experiments.

pH development of FFM during fermentation

Changes in pH in FFM samples during fermentation are shown in Figure 26. Generally, the pH of FFM decreased during fermentation ($P < 0.05$). While the pH values of FFM produced from kahawai was relatively unchanged, those of trevally and hoki decreased markedly with time ($P < 0.01$ for each). The pH of trevally and hoki FFM dropped to 4.6 and 4.4 respectively. Interestingly, hoki had the highest initial pH and a lowest final pH, while kahawai had the lowest initial pH and the highest final pH. During the first 24 hours of fermentation, pH of hoki did not fall; however, after 72 hours of fermentation pH dropped sharply and continue to do so until the completion of fermentation. This delay for hoki FFM may be a result of the slower development of a LAB microflora in that species (Figure 25).

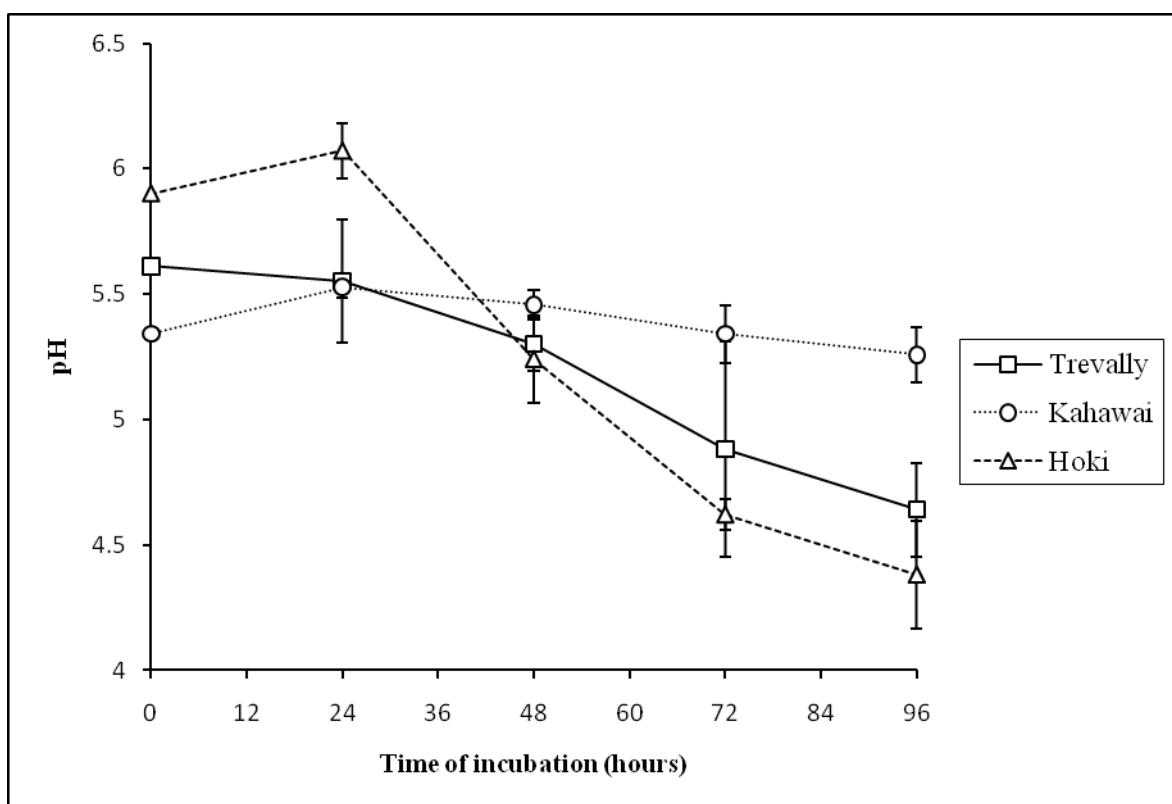


Figure 26. pH development of FFM produced from trevally, kahawai and hoki. Bars indicate standard deviation from three determinations

The pH of FFM is generally an important factor to ensure the safety with the desired value of 4.6 (Riebroy et al., 2004), which could inhibit pathogenic and spoilage bacteria (Owen & Mendoza, 1985). In this study, trevally and hoki exhibit the desired pH value, but kahawai did

not. On the face of it, kahawai FFM made as was done here would be commercially unacceptable.

The lowering of pH in FFM (and other fermented flesh foods) is associated with the production of organic acid, especially lactic acid from LAB with traces of acetic acid (Riebroy et al., 2004). The hydrogen ions associated in these acids are responsible for the lowering of pH, but the pH change achieved will be affected by the different buffering capacities of the muscle proteins of different fish species (Riebroy et al., 2004).

In the section **Textural properties of FFM during fermentation** it was pointed out that the protein content of hoki fillets was low at the time the work was done, a fact that could go some way to explaining the poor textural properties of hoki FFM. If the low protein content were the fact this would reduce the buffering capacity of hoki FFM, possibly accounting for the lower pH attained. The inverse of this argument might explain the high pH results obtained with kahawai.

The differences in final pH cannot be attributed to a lack of fermentation because the final LAB counts for all FFMs were much the same, and to achieve this growth required an energy source. Glucose is the obvious energy source that glycolytically yields lactic acid as the source of hydrogen ions. The concentration of these was therefore likely to have been similar in all three treatments. However, this analyte was not measured.

Proteolysis of FFM during fermentation

Proteolysis of meat products is conveniently determined as tricholoroacetic acid (TCA)-soluble peptides and amino acids. Figure 27 shows the calibration curved based on bovine serum albumin (BSA) used to quantify TCA-soluble peptides and amino acids. A quadratic equation was fitted to the data.

The kinetics of TCA-soluble peptides are shown in Figure 28. Generally, protein hydrolysis increased with increasing fermentation time ($P < 0.001$ within each species). FFM produced from trevally showed the highest proteolysis at all times, followed by kahawai and hoki FFM in that order ($P < 0.001$ between each species). Differences in TCA-soluble peptide and amino acid contents are due to differences in muscle components and post mortem proteolysis (Benjakul, Visessanguan, Riebroy, Ishizaki, & Tanaka, 2002) and proteolysis due to the action of microbial peptidases, which further degrade the protein fragments to small peptides and free amino acids (Molly et al., 1997).

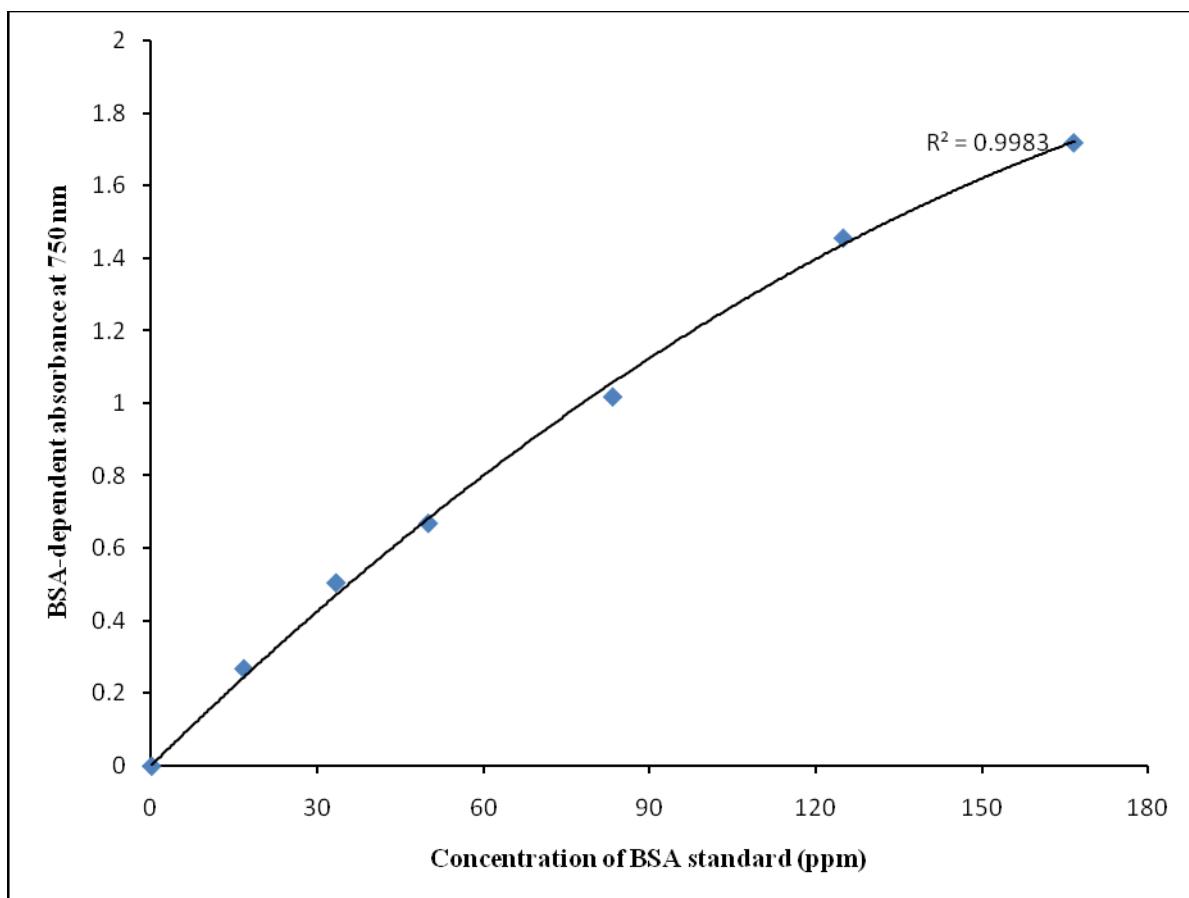


Figure 27. Calibration curve used to quantify TCA-soluble peptides and amino acids.

The absorbance at 750 nm was described by the equation:

$\text{Absorbance} = -0.000026 x^2 + 0.01455 x + 0.01908$, where x was the concentration of proteinaceous matter. The R^2 value was 0.998. After solving for x , TCA-soluble peptides and amino acids were quantified.

Hoki showed the lowest values for TCA-soluble peptides and amino acids. On the face of it this seems contrary to the earlier results and discussion (**Textural properties of FFM during fermentation**), where proteolysis in hoki was proposed to be responsible for the loss of texture during fermentation. The results may be reconciled as follows.

In a study on the protein degradation in fish by SDS-polyacrylamide gel electrophoresis, Benjakul, Seymour, Morrissey, & An (1997) showed that myosin heavy chain was most susceptible to proteolysis among all the proteins. This was confirmed by Riebroy et al. (2004). Microbial peptidases further degrade the protein fragments to small peptides and free amino acids. Thus, Valyasevi & Rolle (2002) reported strong proteinase activity from staphylococci and bacilli is responsible for breakdown of fish protein into peptides and free amino acids.

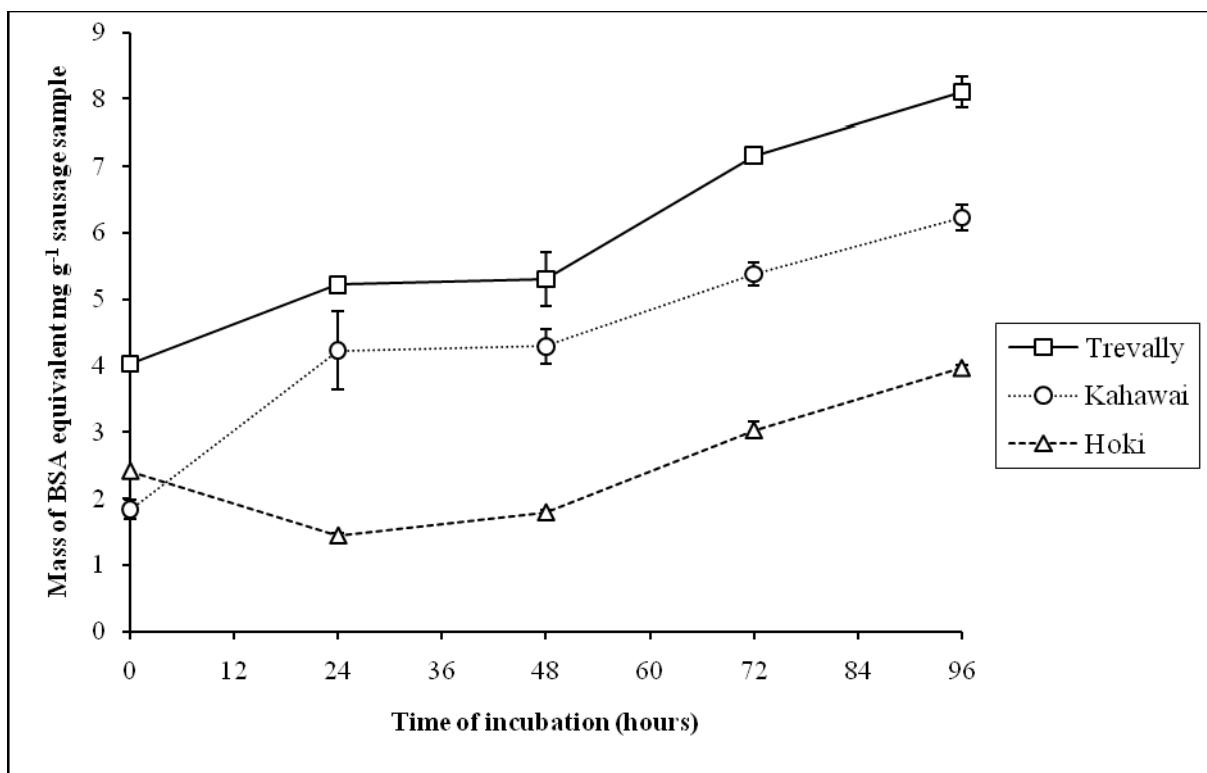


Figure 28. Protein hydrolysis of FFM produced from trevally, kahawai and hoki during fermentation. Bars indicate standard deviation from three determinations

When myosin is cleaved by trypsin, for example, it splits into two fragments that retain the α -helical character of the parent molecule. The molecular weight of these so-called light and heavy meromyosins is approximately 100 KDa and 360 KDa respectively. These have the physicochemical behaviour of the parent myosin and would be expected to precipitate (Neurath & Hill, 1979). Any damage to myosin is likely to contribute to loss of favourable texture (Table 4), because, myosin heavy chain is central to gel formation due to heat or acidification (Lanier, Carvajal, & Yongsawatdigul, 2005), and thus textural properties. Thus, proteolysis by staphylococci and bacilli to yield peptides and free amino acids may be relatively unimportant in gel formation. Trevally had the best textural properties and yet had the highest TCA-solubility values. (As will be discussed in **Changes in biogenic amines in FFM during fermentation**, trevally had the highest biogenic amine formation, deriving from free amino acids.)

Looking now at why the TCA-soluble peptides and amino acids were lowest in hoki, two points may be important. Recall that the protein contents of the hoki FFM may be lower than for the other two species (MacDonald et al., 2002) in **Textural properties of FFM during fermentation**. First, with putatively less protein to act upon fewer peptides and amino acids may be produced. Second, the growth of microorganisms in hoki was lower compared to the other two species between 0 hours and 72 hours (Figure 25). It is important to realise that the

scale in Figure 25 is logarithmic, so seemingly small differences in LAB log counts could translate to large linear differences in protein hydrolysis to peptides and amino acids (Figure 28).

Peptides and amino acids produced by proteolysis are flavour active (Belitz, Grosch, & Schieberle, 2004; Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1993). In addition, compounds such as peptides and amino acids may contribute to the taste in a complex manner, exceeding the taste properties of the pure compounds due to synergistic interactions (Riebroy et al., 2008). On the face of it, trevally FFM should be the most flavourful from an amino acid/peptide perspective. However, the amino acid/peptide profiles may differ between species, and until sensory trials are performed this proposal can only be conjecture.

Fat oxidation in FFM during fermentation

Based on the calibration curve in Figure 29, fat oxidation in FFM is shown in Figure 30.

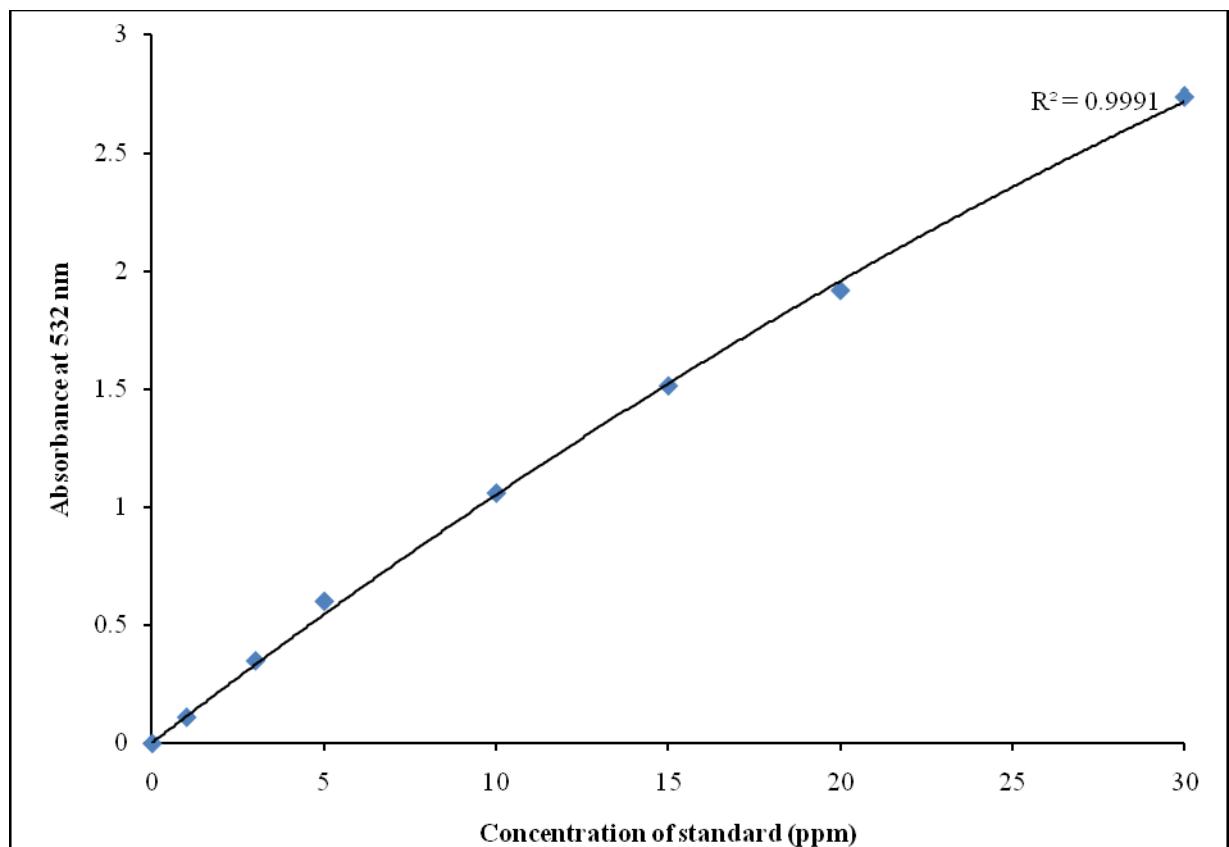


Figure 29. Calibration curve used to quantify TBARS values. The absorbance at 532 nm was described by the equation: $\text{Absorbance} = -0.000650x^2 + 0.1096x + 0.02081$, where x was the TBARS values. The R^2 value was 0.999. After solving for x, TBARS values were quantified.

FFM showed a lot of variation in fat oxidation during fermentation. Overall, hoki had the lowest TBARS ($P < 0.001$), with trevally and kahawai showing equal and highest TBARS at 96

hours. Compared with the other two species, the kinetics of TBARS for hoki were essentially static, with minimal variation between replicates. The pattern for hoki presumably reflects that hoki was the least fatty of the three species. MacDonald et al. (2002) reported that the mean fat content of hoki was $0.63 \pm 0.08\%$ (w/w) and was unaffected by season. Kahawai is a fatty fish (2.7% w/w) with iron content $2.1 \text{ mg } 100 \text{ g}^{-1}$ in the form of haem ion (Nelofar, 2006). When lost from the haem, as happens in degradative muscle foods, the free iron as Fe^{2+} is a strong prooxidant through the Fenton reaction (Belitz et al., 2004). Hoki by contrast contains typically 0.2 mg of iron 100 g^{-1} (Athar, McLaughlin, & Taylor, 2003), the least of the three. Like kahawai, trevally is a relatively fatty fish (typically 2.4%) with a typical iron content of $1.6 \text{ mg } 100 \text{ g}^{-1}$ (Food Standard Agency, 2002). Thus the TBARS values appear to reflect the iron and fat contents of the three species.

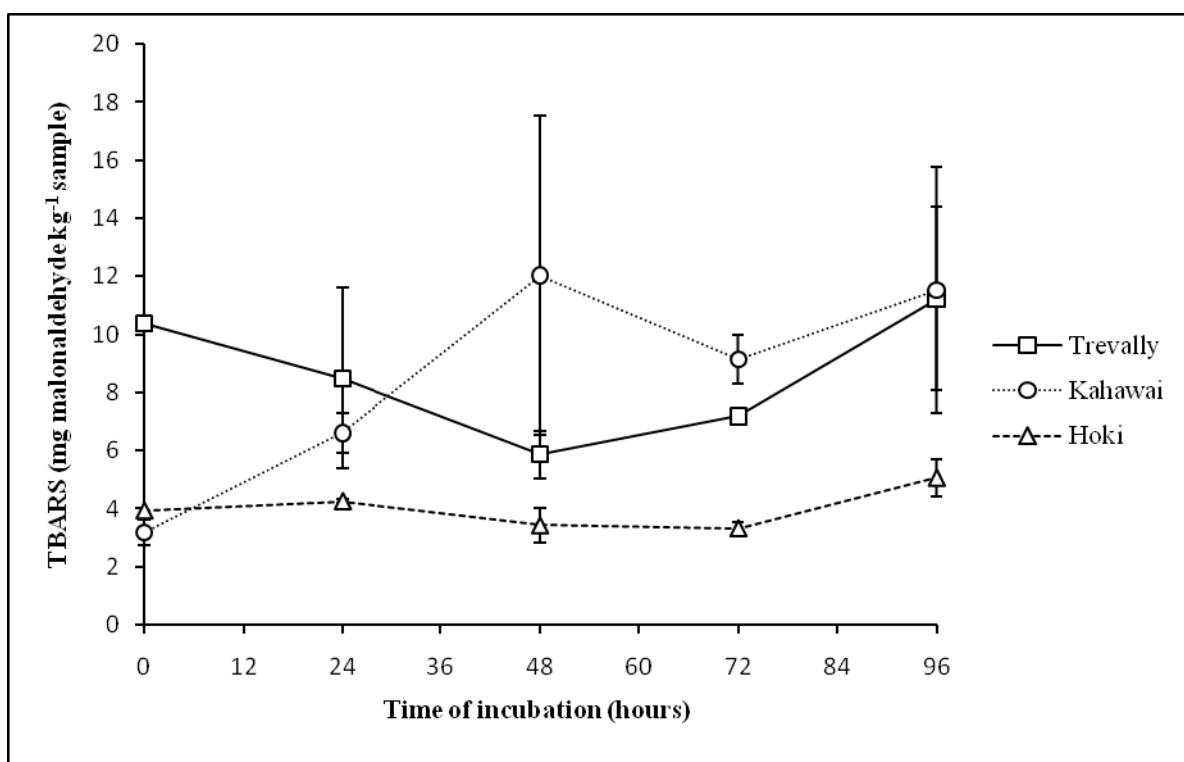


Figure 30. Fat oxidation of FFM produced from trevally, kahawai and hoki during fermentation. Bars indicate standard deviation from three determinations

Riebroy et al., (2007) reported different fat oxidation in FFM produced from six marine species having TBARS values from 10 to 40 mg kg^{-1} . Comparing to the commercial FFM produced from fresh water species, which have TBARS values from 5 to 14 mg kg^{-1} (Riebroy et al., 2004), FFM produced from marine species have much higher fat oxidation than those from the fresh water species.

Generally marine fish species contain more unsaturated fatty acid content, which is prone to fat oxidation, than fresh water fish. In addition, different fat contents and fatty acid compositions among fish species could also be a factor (Riebroy et al., 2004). Moreover, fat oxidation is also accelerated by processes like comminution that damage the muscle structure. These expose the fatty acids to oxygen and catalysing factors such as iron and haem (Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). As in the case of FFM from trevally, the decrease in TBARS values with fermentation time might be due to the loss of volatile secondary products from fat oxidation and those products, especially aldehydes, might bind with the muscle protein (Riebroy et al., 2007).

Lipid oxidation producing alcohol, aldehydes and ketones which are necessary to develop desirable flavour (Manley & Mankoo, 2005; Park, 2005). In short, fat oxidation in this study, particularly for trevally and kahawai, is comparable with the fat oxidation in commercial FFM produced from fresh water species in Southeast Asia. Some fat oxidation, which produces volatile alcohols, aldehydes and ketones, is necessary to develop desirable flavour (Manley & Mankoo, 2005; Park, 2005). It is expected, but untested, that the three FFMs produced here would not have a rancid flavour.

Changes in biogenic amines in FFM during fermentation

Examples chromatograms of biogenic amine standards, and of biogenic amines in FFM from trevally after the completion of fermentation are shown in Figure 31 and Figure 32. Changes in the biogenic amines, histamine, tryptamine and tyramine are shown in Table 5. No biogenic amines were detected before fermentation; however, after the completion of fermentation histamine had increased for all treatments. It increased from 0 to 209 mg kg⁻¹, 15.8 mg kg⁻¹ and 11.95 mg kg⁻¹ in FFM from trevally, kahawai and hoki respectively ($P < 0.001$ between species).

Figure 28 showed that TCA-soluble peptide and amino acids increased in all three species with fermentation time, and the concentration of these was in the sequence trevally > kahawai > hoki. Free amino acids are the substrates for amino acid decarboxylases (Silla-Santos, 2001), and it may be significant that the same sequences applies to the biogenic amines (but not in direct proportion). Several factors have been reported to contribute to the increase of biogenic amines in fermented food such as the availability of free amino acids, microorganisms with amino acid decarboxylase activities, low pH, and available fermentable carbohydrate (Adams & Nout, 2001).

The TCA-solubility test does not distinguish between soluble proteins (not all are precipitated by TCA), soluble peptides and amino acids, the latter being substrates for the decarboxylases. It may be that the trevally treatment yielded more free amino acids due and microbial hydrolytic activity, but this could only be determined by different analyses.

Histamine contents in FFM varied from 12 to 209 mg kg⁻¹. According to Nout (1994) good manufacturing practice demands histamine contents in fermented sausage in the range of 50 to 100 mg kg⁻¹. Senoz, Isikli, & Coksoyler (2000) found histamine content in Turkish style sausage between 6.72 and 362 mg kg⁻¹. Commercial FFM in Southeast Asia has histamine, tryptamine and tyramine contents respectively from 55.1 to 291 mg kg⁻¹, 19 to 71 mg kg⁻¹ and 19 to 225 mg kg⁻¹ (Riebroy et al., 2004). The allowable maximum level of tyramine in foods is 800 mg kg⁻¹ and tyramine with a level of 1080 mg kg⁻¹ is toxic (Shalaby, 1996). Food Standards Australia New Zealand has set upper limit of 200 mg kg⁻¹ for histamine in fish (Food standards Australia New Zealand, 2006)

Table 5. Changes in biogenic amines of FFM produced from trevally, kahawai and hoki

Species	Fermentation time (hours)	Histamine (mg kg ⁻¹)	Tryptamine (mg kg ⁻¹)	Tyramine (mg kg ⁻¹)
Trevally	0	n.d.	n.d.	n.d.
	96	¹ 209.1 ± 26.7	47.7 ± 47.7	168.9 ± 31.9
Kahawai	0	n.d.	n.d.	n.d.
	96	15.8 ± 0.00	n.d.	n.d.
Hoki	0	n.d.	n.d.	n.d.
	96	11.95 ± 1.68	n.d.	n.d.

¹ Data are means ± standard deviation from three determinations
n.d., not detected

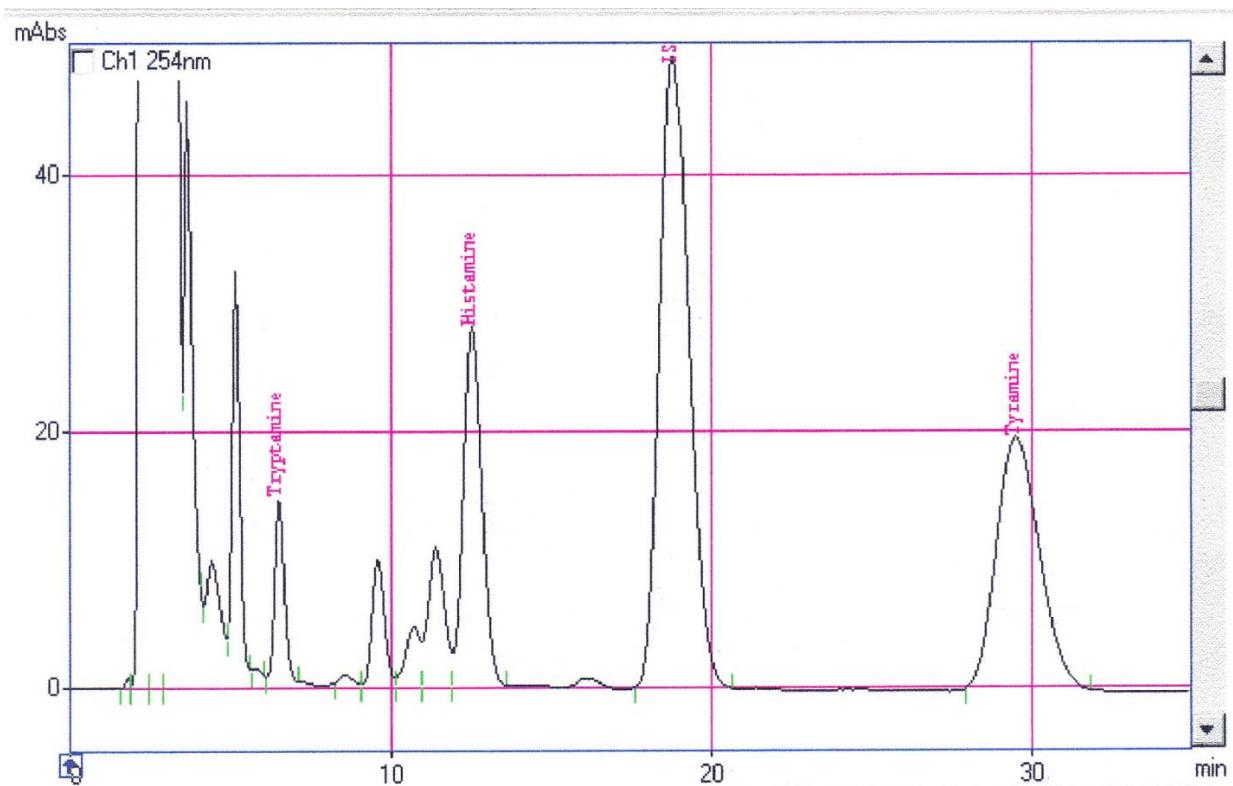


Figure 31. Chromatogram of biogenic amine standards. Identified amines from left to right are tryptamine, histamine, internal standard and tyramine

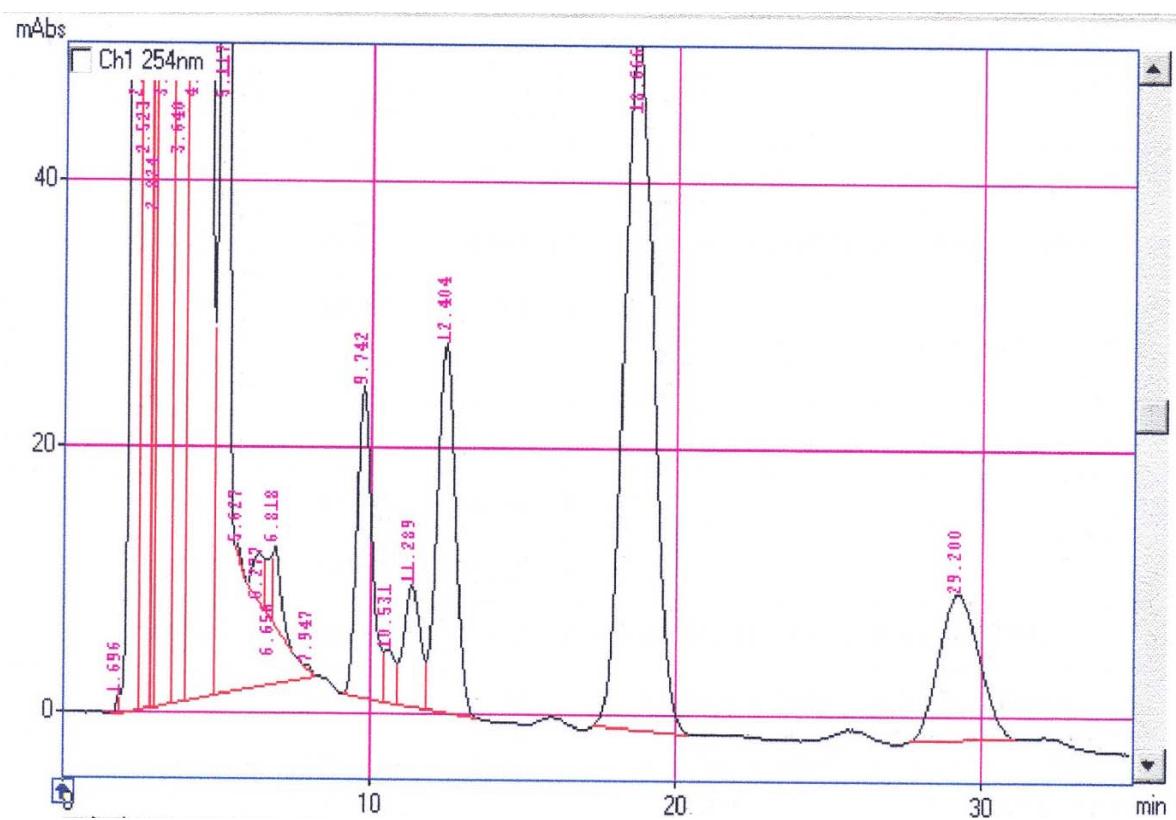


Figure 32. Chromatogram of biogenic amines from trevally FFM at 96 hours

Summary of the properties of the three FFMs

Physically, FFM produced from three marine fish species, trevally, kahawai and hoki showed variation in colour due to the different colour of fish muscle before processing, and changes in response to fermentation. Hoki FFM was the outstanding treatment in that light reflectance (L^*) did not increase with protein denaturation on fermentation and a possible reason for this was discussed. Nonetheless, hoki FFM had the highest reflectance at all times. It also showed a marked increase in yellowness during fermentation for which there was no obvious explanation.

Folate had no significant effect on any of the properties measured.

Texturally, trevally FFM had the highest hardness, adhesiveness, springiness and cohesiveness following the completion of fermentation.

In the early stages of fermentation hoki FFM had the lowest LAB count and kahawai FFM had the highest, but by the end of fermentation at 96 hours, the LAB count was similar for all species. However, this did not translate to a similar fall in pH. The final pH for kahawai was notably high and for hoki was notably low. Reasons for this were discussed.

At all times of fermentation, trevally FFM had the highest concentration of soluble peptides and free amino acids, which will have flavour implications. Likewise, flavour is strongly affected by fat oxidation products. Although TBARS data were often highly variable it was clear that trevally and kahawai FFM showed a similar higher level of fat oxidation at the end of fermentation compared with the least fatty species hoki.

Biogenic amines were not detected in unfermented FFM mixes, but were present in all species at the end of fermentation. Trevally FFM had the highest concentration of biogenic amines, and this could plausibly be linked to higher concentration of soluble peptides and free amino acids in that species.

The next chapter discusses the results obtained to date in the context of further product development and surrounding issues.

Chapter 5

Overall Discussion

Fish muscle properties and their effect on FFM

Trevally and kahawai are pelagic fish species that respectively inhabit the depth range between 0 and 100 m and 0 and 50 m of the continental shelf and coastal waters (Hirt-Chabbert, 2006). Though there is no specific information on the stability of the proteins of these two species, it is likely that the muscle proteins of the pelagic species are generally more stable than the deep water species. Unlike trevally and kahawai, hoki is a demersal species found in the depth range between 150 and 1100 meters over the upper continental slope (Hirt-Chabbert, 2006). The average water temperature in the demersal zone is significantly lower than in the pelagic zone (Jacob, McClatchie, Probert, & Hurst, 1998). Muscle proteins (and probably other proteins) are increasingly unstable to heat and acidity as the ambient temperature of the fish's environment decreases (Lanier et al., 2005). Thus, hoki muscle protein may form the FFM gel in a way that is different from that of the pelagic species, perhaps helping to explain why hoki FFM's texture properties were inferior. At the same time there is the possibility of protease activity being higher in hoki, as discussed in Chapter 4.

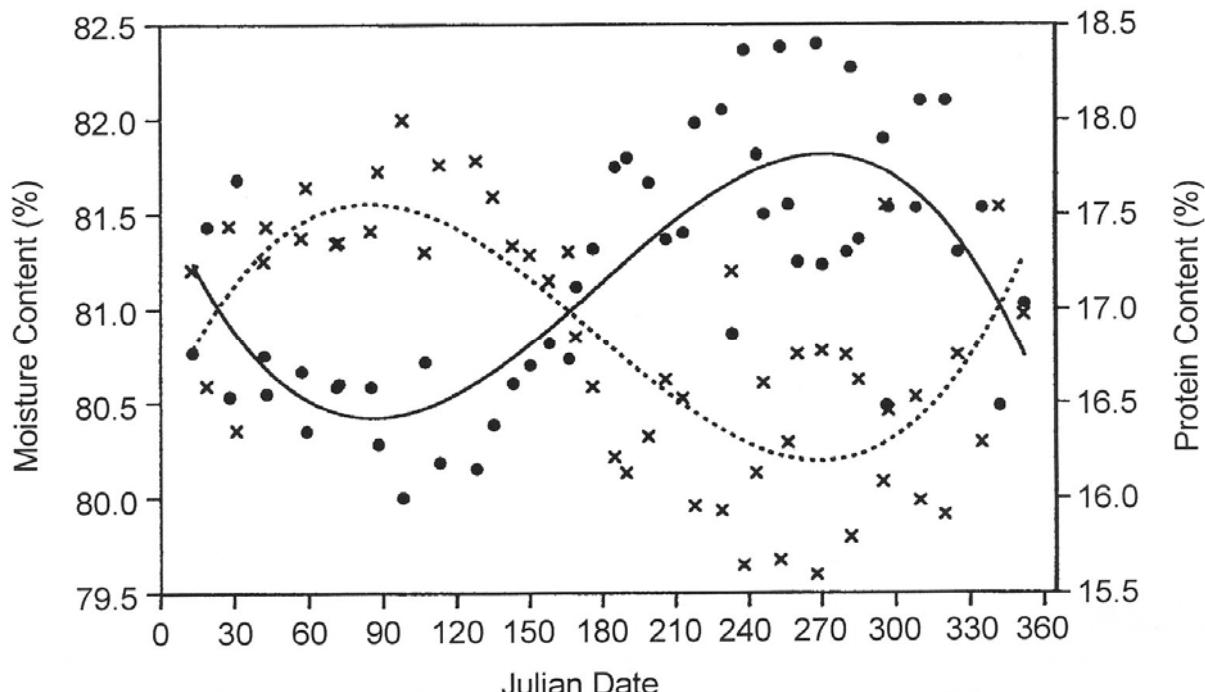


Figure 33. General seasonal variation in muscle moisture (●) and protein (x) of hoki.
From MacDonald et al. (2002)

There is also another likely reason why hoki FFM had inferior textural properties. The protein content of hoki varies widely with season as is shown in Figure 333 (MacDonald et al., 2002).

The protein content of hoki in spring was lowest which could explain the poorer textural properties (and the low pH achieved, perhaps from reduced buffering by protein). When this project was planned, the decision had to be made about what parameters to measure. On the basis that all 81% of the FFM mixture was fish mince, the decision was made not to do proximate analyses. In hindsight that was a mistake as the information of the protein content in the three FFMs might have helped interpretation of some of the results. However, it should be noted the time available for research is limited and the number analyses has to be limited. One other assay that ideally should be added to the basic list is the analyte lactate. Measuring its concentration would distinguish between the true extent of the glycolytic fermentation (lactate formation) and pH, the latter being affected by the buffering capacity. Lactate will best be determined by the lactate dehydrogenase (LDH) assay, which is specific for only lactate.

Cooked rice as a carbohydrate source

At the early trial experiments, ground steamed rice was used as a carbohydrate source for the endogenous LAB to ferment. However, fermentation did not take place. This was obvious because of the smell of spoilt fish and the fact that the pH was relatively unchanged. This failure to ferment has two implications. First, the initial microbial load of the FFM mixture prepared here was around 10^3 cfu g⁻¹, compared those of FFM in Southeast Asia where the initial microbial load was between 10^4 cfu g⁻¹ and 10^6 cfu g⁻¹ for products made from marine species (Riebroy et al., 2007), and between 10^5 cfu g⁻¹ and 10^7 cfu g⁻¹ for fresh water species (Saisithi et al., 1986). These high initial loads may be a consequence of the tropical environment of Southeast Asia where the hygienic conditions are difficult to maintain. Thus the tropical examples of FFM had a ‘head start’. Second, in the production of fermented fish product in Southeast Asia repeated for many years, it is likely that a stable LAB population capable of hydrolysing starch to glucose (amylolytic LABs) may have developed in the production chain environment. This contrasts with the New Zealand chilled fish production chain where hygiene standards are very high (witnessed by the low initial load) and carbohydrate is rigidly excluded from the production chain.

Amylolytic LAB can hydrolyse the starchy biomass and also ferment the glucose to lactic acid. Amylolytic LAB strains include *Lactobacillus amylophilus* GV6 (Altaf, JanakiramNaveena, & Reddy, 2005), *Lactobacillus amylophilus* B4437 (Mercier, Yerushalmi,

Rouleau, & Dochain, 1992), *Lactobacillus amylovorus* (Cheng, Mueller, Jaeger, Bajpai, & Iannotti, 1991), and *Lactococcus lactis* combined with *Aspergillus awamorii* (Hiroshi, Hidenori, & Hideo, 1988). *Lactobacillus plantarum* strains were reported to have potential for industrialising fermented cassava production (Huch et al., 2008). It is likely that none of these strains was endogenous to New Zealand fish fillets or were outgrown by other LAB strains.

In the present study the failure to ferment cooked rice was easily overcome by substituting glucose for rice. In hindsight, it was a mistake to substitute on a weight basis because about 80% of the mass of cooked rice is water, meaning that the dry mass of potentially fermentable starch was 2.4%. The 12% glucose added was excessive, but certainly did not impede fermentation.

The potential for starter LAB cultures in FFM production

The failure to ferment cooked rice and the knowledge that amylolytic LABs have been identified, suggests there is an opportunity to revisit the use of cooked rice in FFM production from New Zealand marine species. This concept is discussed later in the section **Further development of FFM from New Zealand marine species** where New Zealand-typical starch sources are proposed to be used in place of rice. But even if starch is not used and fermentation is supported by glucose addition, there are distinct advantages to using starter cultures.

A starter culture is a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by promoting and dominating the fermentation. A particular group of LABs occupies a central role in the inoculation of the raw material to produce fermented food, partly because this group has a long and safe history of application and consumption (Leroy & Vuyst, 2004). In addition, direct addition of selected starter cultures to raw materials has been a success in the production of fermented food from the high degree of control over the fermentation process and standardisation of the end products.

Different strains of LAB have been used to produce different fermented food. *Lactobacillus* or *Pediococcus* genus and coagulase-negative staphylococci and members of *Micrococcaceae* are normally used in the fermented sausage (Cocconcelli, 2007). *Lactobacillus alimentarius* and *Carnobacterium piscicola* have been used in the fermented fish products (Leroy & Vuyst, 2004). These or others might be employed in FFM production from New Zealand marine species.

The concentration of active bacterial cells to be inoculated in the raw material is another factor to be considered, and it depends on the growth potential of the organisms in the food

matrix. In most European-style sausage fermentations, about 10^6 cells of LAB are added per gram whereas 10^8 g^{-1} of *Lactococcus lactis* had to be added to produce the desired final effect in meat fermentation (Lucke, 2000). By contrast, inoculation at 10^4 and 10^6 of *Lactobacillus curvatus* was reported to successfully produce fermented pork sausage (Visessanguan et al., 2006).

In a study on FFM produced from the marine species big-eye snapper, Riebroy et al. (2008) reported that FFM inoculated with *Pediococcus acidilactici* at 10^4 cfu g^{-1} resulted in a reduction of fermentation time, better textural properties and acceptability and higher quality than the wild-type control without the starter. Similarly, Hu, Xia, & Ge (2008) found that FFM produced from freshwater species, silver carp (*Hypophthalmichthys molitrix*), inoculated with mixed starter cultures at the level of 10^6 to 10^7 cfu g^{-1} could reduce fermentation time, suppress TBARS values, total volatile base nitrogen, trimethylamine production, and the growth of spoilage bacteria and pathogens (Enterobacteriaceae, *Pseudomonas*, yeasts and moulds) compared with the wild-type control. Flavour, digestibility, and nutritional values were also improved by the use of these cultures. The groups of LAB used were drawn from *Lactobacillus plantarum*, *Lactobacillus casei*, *Staphylococcus xylosus* and *Pediococcus pentosaceus*. Similarly, in the production of FFM from marine species of mackerel (*Scomber australasicus*) inoculated with *Pediococcus pentosaceus* and *Pediococcus pentosaceus* at 10^5 to 10^6 cfu g^{-1} , Yin, Tong, & Jiang (2005) found a rapid growth of LAB, decline in pH and suppression in the growth of microflora such as Enterobacteriaceae, *Staphylococcus*, and *Pseudomonas* and a better quality FFM compared to the control.

In short, the production of FFM from New Zealand marine species with starter cultures is likely to provide benefits such as the reduction in fermentation time and a concomitant rapid decrease in pH, the suppression of pathogens and the improvement of the sensory quality. Therefore, the use of LAB starters such as *Lactobacillus* and *Pediococcus* at a level of 10^4 to 10^6 cfu g^{-1} in the present study could offer promising quality improvements to the products.

Further development of FFM from New Zealand marine species

As discussed in the previous section the use of starter cultures is probably a step forward in quality improvement and standardisation for marine species FFM. There are also other potential improvements that could be made. These include choice of carbohydrate source, use of phosphate, spicing, curing and smoking, and drying.

The most commonly used carbohydrates in meat fermentation are sugars such as sucrose, lactose, glucose, corn syrups, different starches and the sugar alcohol sorbitol (Ruiz, 2007). The available concentration and type of sugar are related to the lowest pH achieved and the rate of pH fall, respectively. Glucose can be utilised directly by lactic acid while other sugars such as sucrose, lactose or maltose are less easily fermented (Stahnke & Tjener, 2007), and as shown in Chapter 3, steamed rice was not fermentable by the endogenous LABs on the three marine species used. However, the use of a starchy ingredient is attractive for the main reason that less fish – the most expensive ingredient – is used in the FFM. But at the same time starches do not form the gels that give FFM their desirable textural properties such as were measured in Chapter 4. Thus there remains a case for using glucose rather than a starch product.

If a starch product were to be used, what would be a good choice? Steamed glutinous rice, steamed cassava root and cassava starch were used in comparison with rice in the production of fresh water FFM (Saisithi et al., 1986). Although cassava starch gave a smoother texture and a drier product, FFM made with it did not possess the characteristic odour and flavour of FFM. However, sensory scores for colour, odour, sourness, saltiness, texture and acceptability of FFM from steamed cassava root were only slightly lower than those of FFM from cooked rice. While steamed rice or similar could be used in the commercial development of marine species FFM (along with an amylolytic LAB), it may be better to use a carbohydrate source more closely identified with New Zealand. This could confer a degree of ‘geographical exclusivity’ in the parallel way to geographical exclusivity seen in products like Scotch whiskey (Scotland), tequila (Mexico), and champagne (Champagne, France).



Figure 34. Kumara flesh can be cream or orange

The most commonly used carbohydrate source from root vegetables in New Zealand is potato, and other notable sources are kumara, and in some areas taro. In addition to their abundance throughout the year, they are also cheap compared with fish, particularly potato. In terms geographical exclusivity, kumara seems to be the best choice and moreover, comes in a wider variety of colours than mainstream potatoes. Figure 34 shows cream and orange options. Kumara is obviously not a New Zealand-exclusive plant, but its Maori name has a strong New Zealand-only link.

In FFM production with rice, prior cooking (steaming) is required to make the amylose and amylopectin polymers available for enzyme-catalysed hydrolysis, and indeed prolonged cooking of rice for example yields sweet fragments (e.g. maltose) from the initially long polymers. Rice porridge and sticky rice are examples of this. By analogy, kumara, potato and taro would probably require extensive cooking to support fermentation.

In 100 g of taro, kumara and potato, there are about 0.45 mg, 0.27 mg and 1.34 mg of manganese respectively (U.S Department of Agriculture, 2007). Manganese has been identified as an active component in the enhancement of acid production by LAB in meat fermentation (Zaika & Kissinger, 1984). On these grounds alone kumara, potato, and taro could be choices for further product development.

Polyphosphates are multipurpose ingredients which are commonly included in sausage formulations. Of their several roles in foods, two roles are particularly important in sausage production. First, the polyphosphate, pyrophosphate ($\text{P}_2\text{O}_7^{4-}$) is a chemical analogue of adenosine triphosphate that serves to dissociate actomyosin into its component parts, actin and myosin. This reduces viscosity because the rigor complex is broken, and because myosin by itself is more useful than actomyosin for gelation in sausage products (Knipe, 2004). A reduction in viscosity is important in processing since the unfermented FFM can be forced into casings more readily. Second, phosphates bind metal ions like Fe^{2+} that can otherwise catalyse fat oxidation. Roncales (2007) has summarised the role of phosphates in sausage: a better binding of meat and fat particles, more equilibrated drying, a longer shelf life, a smoother texture, and greater juiciness.

Spices and herbs have been added to flesh foods for millennia (Chi & Wu, 2007). By analogy to the Cambodian FFM models, garlic was included as a basic ingredient at a concentration of 5% by weight. It is not proposed to change that concentration. Spices, which garlic is arguably not, are included in foods at substantially lower concentrations. This is common experience. Many spices have been used in the production of fermented sausage at

various concentrations depending on the sausage types. Spices that are in common use in fermented meat sausages include pepper, chilli, paprika, garlic, mace, pimento and cardamom depending on culture of producing countries (Chi & Wu, 2007; Verluyten, Leroy, & Vuyst, 2004). The main purpose of adding spices is to add flavour and colour. In addition, they are also a source of many other substances such as sugars, nitrates, and metal ions (Aguirrezabal, Dominguez, & Zumalacarregui, 1998), which may or may not be desirable. However, Zaika & Kissinger (1984) showed that spices added to fermented meat promote the growth of LABs particularly due to the manganese content in the spices.

Anecdotally, and from the author's experience in his native Cambodia, spices are commonly added to FFM. The most popular of these is chilli. Chilli is not only popular in Southeast Asia, but as with many spices serves a preservative role against pathogens, which as argued by Billing & Sherman (1998) is the ultimate reasons that spices are used. Also, spices such as pepper, dried and powdered lemon grass, galangal and ginger have all been tested as a means of reducing the fishy odour (Saisithi et al., 1986). All spices were reported to give favourable aroma to the product, except pepper.

In addition to the above properties, many if not all herbs and spices also exhibit antioxidant activities. Thus 1% whole oregano (10,000 ppm) was equivalent to 200 ppm butylhydroxyanisol (BHA) in controlling oxidation of mackerel oil (Tsimidou, Papavergou, & Boskou, 1995). This was in addition to its manganese content of 1.4 mg 100 g⁻¹ dried ground sample (Food Standard Agency, 2002). Rosemary which is a very commonly used spice in food processing worldwide (Yanishlieva, Marinova, & Pokorny, 2006). It is reportedly both a direct fat antioxidant and metal chelator. In addition, rosemary extracts were found to scavenge superoxide radicals (Basaga, Tekkaya, & Acikel, 1997).

Sage (*Salvia officinalis L.*) is used in food flavouring and seasoning. The mixture of sage and rosemary was found to be the best antioxidant activity among the numerous herbs and spices tested (Djarmati, Jankov, Schwirtlich, Djulinac, & Kjordjevic, 1991). In addition, sage has one of the highest manganese content 25 mg 100 g⁻¹ dried ground powder (Food Standard Agency, 2002). Thyme (*Thymus vulgaris L.*) has been commonly used as culinary herb spices for adding flavour and eliminating unpleasant smell (Yanishlieva et al., 2006). It also possess strong antioxidative effect which is associated with the high content of cavacon and thymol (Schwarz, Ernst, & Ternes, 1996) in addition to its high content of manganese, 7.6 mg 100 g⁻¹ dried ground powder (Food Standard Agency, 2002).

Returning to the theme of geographical exclusivity, the leaves of horopito (*Pseudowintera colorata*) have a peppery flavour (Figure 35). Horopito is also known as bush pepper. Horopito has traditionally been used by Maori of New Zealand and early European settler for medicinal purposes, notably antifungal. The active substance which have been found to exhibit fungicidal activity against yeast and filamentous fungi is polygodial (Berry-Kilgour, 2002). In the past decade or so, horopito has been revived as a condiment. It is not available in mainstream retail shops, but is available online targeted at the restaurant trade (Epicurean Supplies, 2007).



Figure 35. Horopito leaves, a New Zealand native herb

In short, spices and herb such as horopito, rosemary, sage and thyme – the latter three being well recognised in New Zealand – could be potential ingredients in FFM from marine species. They would help prevent fat oxidation and would probably accelerate the production of lactic acid. Whether horopito also has these properties is not known but is does seem likely.

To sum up, FFM products which could well suited with the New Zealand market might include the Zealand staple carbohydrate kumura, and horopito as a ‘signature style’, but subject to **Sensory analysis** to be described in the next section.

Of the three species tested here, trevally and kahawai , had the highest fat content, the highest iron content, and generated the most fat oxidation products as determined by TBARS values. Both species are good candidates for nitrite curing. According Honikel (2007) curing enhances colour and minimises fat oxidation because iron remains locked in haem, and therefore not significantly present as free Fe²⁺ or Fe³⁺ ions, both of which promote fat oxidation. Curing also minimises the proliferation of *Clostridium* spp., which is useful from a food safety perspective. In addition to the nitrite curing, smoking can also assist other preservation effects

by covering the surface of the FFM with bacteriostatic and mycostatic compounds (Toldra, 2007).

Finally there is the matter of casings. The present study used 50 mL syringe as the casing, a form totally unsuited for commercial purpose. Commercial FFM sold in Southeast Asia is normally packed with banana leaves or in small plastic bags. Since the product is fermented anaerobically, the packaging suitable for the product should be substantially airtight. In a study of different packaging materials such as low density and high density polyethylene, polypropylene, oriented polypropylene laminated with polyethylene and polypropylene, Saisithi et al. (1986) concluded that polypropylene casing is well suited with the product requirement and it is also cheap compared to the other packaging materials. In addition, it is also convenient for printing which add another benefit to the product presentation.

Whereas a polypropylene packaging could be easily applied to FFM, including cured FFM, it could not be applied where the FFM was to be smoked. Therefore, natural casing (intestinal) or a synthetic collagen casing would be more useful where smoking is required.

Sensory analysis

Each of the improvements listed in the previous section would require some research activity through comparison of various treatments. Many of these could be explored with the assays employed in the present study, along with the more mundane proximate analysis. But the end of all this experimentation, sensory analysis must be done.

The first step will be to ensure the product is safe for consumers. Food products to be on sale must meet a certain standard by regulatory authority. In New Zealand, the body responsible for administering legislation covering food for sale is the New Zealand Food Safety Authority (NZFSA). According to the draft guidelines for the production of uncooked comminuted fermented products, several pathogens need to be screened for as a verification procedure. These include coagulase positive staphylococci, *Escherichia coli* and *Salmonella* with the specification listed in the table below (New Zealand Food Safety Authority, 2008).

Table 6. Microbiological limits for uncooked comminuted fermented meat products

Organism	No. of samples from a lot of food	Max. number of sample allowed to have results > m but < M	Accepted microbiological level in a sample (m)	Max. level which when exceeded in one or more samples would cause the lot to be rejected (M)
Coagulase positive staphylococci g^{-1}	5	1	1,000	10,000
<i>Escherichia coli</i> g^{-1}	5	1	3.6	9.2
<i>Salmonella</i> 25 g^{-1}	5	0	0	0

When it is clear that the products are free from pathogens and safe for consumption, consumer sensory testing should be conducted (Lawless & Heymann, 1998). This will be hedonic focusing on liking of texture, colour and flavour, typically testing four treatments at a time. A key point to consider in this testing is context. Normally, in Southeast Asia, the product is sliced into pieces suitable for eating and served with lettuce, roasted peanut, and ginger as a side dish. How context should be controlled in New Zealand context is not clear at this time. There is further complication. As is discussed in **Route to market**, the intended consumer is much more likely to be a gourmet than a frequenter of Subway or McDonalds. Thus, organising a consumer trial would involve more than just accessing students on the AUT campus.

Putting context and intended consumer to one side for the moment, the sorts of comparisons that could be made are as follows. First, a basic Southeast Asian FFM formulation could be compared with FFM – probably from trevally – using kumura as the carbohydrate source (this will require an amylolytic starter culture). Further trials would compare different New Zealand formulations, of increasing complexity and diversity in spicing for example. From these techniques, a preferred model could be selected for a commercial launch.

Route to market

From the sensory tests, the most preferred samples of FFM from trevally made from different spices and carbohydrate sources could be selected for a commercial launch. The following assumptions are also made. The initial production will be done with little money in rented production facilities that meet food safety standards, and supermarket chains will not be interested in stocking a completely novel product.

It is proposed to develop a market through delicatessen retailers who target gourmets, and through full service restaurants at the top end of the market. There are several reasons for selecting this route. Gourmets are receptive to new flavours, and frequent these retailers and

restaurants. Consider the retailers, with Auckland's Sabato as an example (<http://www.sabato.co.nz>). Even casual inspection of Sabato's pricing structure shows that it is expensive, focusing as it does on geographically distinct products sourced from around the world. A New Zealand FFM would fit well in this marketing framework. Moreover, samples of new products are frequently offered instore, along with the opportunity to buy. The relative price inelasticity in the gourmet demographic would also be useful, because the small initial production of New Zealand FFM is likely to be expensive no matter how cheap the fish was. In restaurants, FFM would most likely be presented as a starter snack with breads etc, and or as an entrée. For both the delicatessen retailers and the restaurants, there will be a single way to approach placement: identification of the decision maker(s) within each entity, followed by direct contact and free samples to test consumer reception. This will cost money and time. How the market could progress from this starting point is unknown, but a likely way forward would be through publicity and photographs in gourmet magazines such as *Cuisine*.

Chapter 6

Conclusion

FFM has been produced from three New Zealand marine fish species, trevally, kahawai and hoki. The fish fillets selected were necessarily limited to a few examples of each species, whereas the flesh properties within each species (certainly for hoki) will probably change with age, gender, season and location. None of these factors could be realistically controlled in this pilot study. Thus the results are a ‘snapshot’ of the properties of FFM from each species. Within this limitation, each FFM showed different physical, microbiological and chemical properties. FFM produced from hoki had the highest reflectance due to its white flesh, while kahawai FFM showed the least reflectance due to its red flesh colour. However, the reflectance of both trevally and kahawai increased during fermentation, while that for hoki did not and the possible reason for this was discussed. Nonetheless, hoki FFM showed a marked increase in yellowness during fermentation for which there was no obvious explanation.

Folate had no important effect on any of the properties measured.

FFM from trevally showed the best textural properties, making it the most likely candidate for further product development. Lactic acid bacteria counts were different for each species at the beginning of the fermentation, but reached similar levels at the completion of fermentation. However, these counts did not translate to a uniform decrease in pH. The pH for FFM from hoki and trevally decreased significantly after the completion of fermentation while that for kahawai was relatively unchanged. At all times of fermentation, trevally FFM had the highest concentration of soluble peptides and free amino acids, which would have flavour implications. Likewise, flavour is strongly affected by fat oxidation products. Although TBARS data were often highly variable it was clear that trevally and kahawai FFM showed a similar higher level of fat oxidation at the end of fermentation compared with the least fatty species, hoki.

Biogenic amines were not detected in unfermented FFM mixes, but were present in all FFM samples at the end of fermentation. Trevally FFM had the highest concentration of biogenic amines, and this could plausibly be linked to higher concentration of soluble peptides and free amino acids in that species. Although the histamine content of trevally FFM was slightly higher (209 ppm) than the limited standard (200 ppm), this may be reduced by altering the production variables, typically by using a defined starter culture (see below). Notably, histamine poisoning is associated with concentrations in fish typically between 600 and 20,000 ppm, with three cases

in New Zealand where the concentration was 2,000 ppm (New Zealand Food Safety Authority, 2001).

The results from the analysis of FFM point to the commercial opportunities for FFM from trevally because it has comparable properties with the commercial FFM in Southeast Asia. On the face of this, further research could also be done in order to produce the products with ingredients commonly used in New Zealand.

In this respects, the substitution of cooked rice with staple New Zealand carbohydrate source such as kumara, taro and potato together with spices and herbs commonly used in New Zealand such as horopito, oregano, rosemary and thyme could be conducted.

In order to get uniform quality, a starter culture should also be used, in particular a starter culture which could reduce the production of biogenic amines.

Sensory studies should also be conducted to indentify the most preferred FFMs so that the risk in launching products in the market can be minimised. Finally, a route to market has been proposed, through delicatessen retailers who target gourmets, and through full service restaurants at the top end of the market. FFM could have bright future in New Zealand cuisine.

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