Fish Sauce Fermentation Technology using New Zealand Raw Materials

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Abstract

Despite abundant raw material, industrial fish sauce production is absent in New Zealand, partly due to a paucity of prior research on how well cold-climate South Pacific fish ferment under temperate conditions with local solar salt. The present study investigates the effect of different fish sauce fermentation conditions using New Zealand raw materials (Hoki and snapper heads and frames as well as green-shell mussel), New Zealand solar salts (stoved and unstoved salts) and different temperatures on volatile fatty acid (VFA) production as well as bacterial composition and diversity. Four different fermentation set-ups were conducted to investigate different aims:

1. Laboratory scale fermentation to investigate the influence of different variables (including seafood material, type of salt and temperature) on fermentation.
2. Pilot scale fermentation to investigate the influence of sampling depth on fermentation.
3. Shell-on mussel fermentation to investigate the influence of mussel shell on mussel fermentation
4. Snapper fermentation with mussel shell added to investigate the influence of mussel shell on snapper fermentation.

Four VFA were quantified in all fermentation in this study: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Butanoic acid was detected only in fermentations with unstoved solar salt regardless of seafood material and fermentation temperature. The presence of butanoic acid exclusively in unstoved fermentations may be associated with the presence or abundance of bacterial groups associated with butanoic acid production in these fermentations.

The current study found that VFA concentrations generally increased as the fermentation time increased. Salt type had more influence on VFA production than any other single variable including seafood material used, fermentation temperature and sampling depth.

It was also revealed that seafood material had more influence on bacterial compositions than any other variable and seafood material appeared to be the primary determinant of the predominant bacterial family groups for the fermentations (Hoki paste – *Micrococcaceae* and *Moraxellaceae*; snapper paste – *Carnobacteriaceae, Moraxellaceae* and *Pseudomonadaceae*; and mussel paste – *Flavobacteriaceae, Moraxellaceae, Paracoccaceae, Pseudomonadaceae* and *Roseobacteriaceae*). *Halobacteriales* were found only in unstoved salt fermentations suggesting that salt type may be responsible for these results. However, this postulate could not be validated since insufficient intact nucleic acid could be recovered from the salts used to generate adequate bioinformatics information.

Shell-on mussel and snapper plus shell fermentation revealed that the presence of mussel shell was associated with high pH in fish sauce fermentation, an observation not previously reported. However, no significant difference in pH was found in fermentations with different shell contents. In pilot scale snapper fermentation, sampling depth did not have a major influence on VFA production. However, sampling depth may influence the growth of some bacterial families to become the predominant groups by the end of the fermentation.

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To my friends, you are the family I chose, and I am so grateful for each one of you. Your support, laughter, and companionship have made this journey possible.

May this thesis bring benefit to all who seek its knowledge.

"Read, in the Name of your Lord Who created" – (96:1)

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List of Abbreviations

AEBAR	Aquatic Environment and Biodiversity Annual Review
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
DMA	Dimethylamine
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
FID	Flame ionization detector
GC	Gas-chromatography
GCMS	Gas-chromatography Mass-spectrometry
GDP	Gross Domestic Product
LB	Lysogeny broth
MPI	Ministry for Primary Industry
MS	Mass spectrometer
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling

OTU	Operational taxonomy unit
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PTFE	Polytetrafluoroethylene
QMS	Quota Management System
RDP	Ribosomal database project
RNA	Ribonucleic acid
RRF	Relative response factor
rRNA	ribosomal RNA
SDS	Sodium dodecyl-sulfate
SOLiD	Sequencing by Oligo Ligation Detection
TACC	Total allowable commercial catch
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TVB-N	Total volatile basic nitrogen
UV	Ultra-violet
VFA	Volatile fatty acid

Glossary

Ferment	Used to describe sample taken from actively fermenting fish sauce.
Fermentation	Used to describe a) the process and b) the entire fermentation bottle/barrel consisting of solid and liquid parts.
Ferment liquor	Used to describe the liquid sample from fish fermentation.
Chao1 index	Measures species (or OTU) richness present in a community where a high value indicates a high diversity.
Shannon-Wiener's Index	Measures the abundance homogeneity of different species in a community where the closer the index number to 1, the similar the abundances of different species in the community.
Simpson's index	Measures the richness and evenness of a species in a community where a higher number indicates higher diversity.
Solar salt	Types of solar salt used in the current study were: a) Stoved salt – refers to the crude solar salt that has undergone a high temperature dry heat treatment to kill halophilic bacteria b) Unstoved salt – refers to the untreated crude solar salt collected directly from the salt ponds, then washed with saturated brine to wash off mud before naturally dried out c) Iodised salt – refers to iodine fortified salt.

Chapter 1: General Introduction

Fish sauce is a clear brown liquid with a salty taste and pungent and fishy aroma (Lopetcharat et al., 2001; Saisithi, 1994). Fish sauce is a common cooking condiment in many Southeast Asian countries (Lopetcharat et al., 2001). Traditional processes of making fish sauce generally involve layering fish (normally whole small fish such as anchovies and mackerel) with salt in large barrels at a ratio of 1:3 salt: fish and then allowing the mix to ferment naturally (Saisithi, 1994). The fermentation process takes place between 6 – 18 months at ambient temperature between 30 – 40 °C (Lopetcharat et al., 2001). Once complete, the liquid is extracted, filtered, and sometimes aged further for enhanced flavour (Saisithi, 1994). The factors affecting fish sauce fermentation include the type of fish used, the type and concentration of salt, temperature, and oxygen concentration (Beddows, 1998; Salampessy et al., 2010). These factors consequently affect the abundance, diversity, and dynamic growth of the microflora as well as the chemical composition including volatile compounds and amino acid contents (Salampessy et al., 2010; Lee et al., 2015).

The fish industry is a major economic source for many countries worldwide including New Zealand (Chalamaiah et al., 2012). New Zealand waters provide diverse fisheries from deep water sea fish to inshore finfish and shellfish (Ministry of Primary Industries (MPI), 2017). In 2020, commercial fishing contributed $2.2 billion, representing 0.7 % of New Zealand Gross Domestic Product (GDP) (Dixon & McIndoe, 2022). It was reported that in 2020, Hoki (*Macruronus novaezelandiae)* accounted for 44 % of value for New Zealand's deepwater fisheries and 29 % of the total commercial fishing value (Dixon & McIndoe, 2022). On the other hand, the aquaculture industry dominated by green-shell mussel is projected to grow from NZ$ 600 million in 2019 to NZ$ 3 billion by 2035, making it a highly prospective industry for economic growth (MPI, 2023).

Chapter 1: General Introduction

A consequence of the growing fishing industry is the generation of fish waste and by-products (MPI, 2016). Current industrial practices often further process this waste into low value products such as fish meal and natural fertilizer (Mohtar, 2013). Thus, in recent years, researchers and fishing industry have taking active roles to increase seafood sustainability, reduce waste from fish by-product and to increase the income returns by utilizing seafood by-product more profitably. In New Zealand, despite abundant raw material, fish sauce manufacture is still absent, partly due to a lack of knowledge as to how well Southern Ocean fish will ferment with New Zealand solar salt under temperate conditions. The present research will explore fish sauce fermentation technology using New Zealand's raw materials as main ingredients under temperate condition with a final aim of producing high quality fish sauce. The current study also explores the chemical and microbiological properties during the fermentation time course.

This thesis will be presented in twelve chapters. Chapter 2 presents a general review of fish sauce, its manufacturing process and factors affecting the quality of a fish sauce. The chapter includes general review of analytical methods for analysing fish sauce quality. Research gap, hypotheses and objectives of the current study are included at the end of this chapter. Chapter 3 outlines the general methods and experimental plans of the current study. Chapter 4 presents the workflow of the development and validation of molecular methods used in this study. Chapter 5, 6 and 7 presents the results from laboratory scale fermentations using Hoki (frames and head), snapper (heads and frames) and green-shell mussel (whole), respectively. Chapter 8 presents the results from pilot scale fermentation using snapper head and frames and unstoved solar salt. The chapter also investigate the effect of sampling depth on fermentation. Chapter 9 investigate the effect of mussel shell content on mussel fermentation process. Chapter 10 explores the effect of mussel shell on snapper fermentation process. Chapter 11 provides general discussions based on the findings made from the different fermentation set-ups. Chapter 12 concludes the thesis by highlighting the overall conclusions and future direction of work based on the results obtained from this study.

Chapter 2: Literature Review

2.0 Introduction

This literature review provides comprehensive review of the following aspects of fish sauce: the manufacturing process, factors affecting fermentation, factors affecting the quality of the fish sauce and appropriate analytical analyses to assess the quality of the fermented fish product.

2.1 New Zealand Fishing Industry

The world's total marine catch reached 171 million tonnes in 2016, with approximately 70% contributed by the Southeast Asian countries followed by the European Union (9%), Latin America and Caribbean (8%), Africa (6%), North America (4%) and the Oceana (Australia and New Zealand) (4%) (Food and Agriculture Organization of the United Nations [FAO], 2018).

New Zealand is a maritime nation where the contribution of commercial fishing is substantial at $2.2 billion in 2020, representing 0.7 % of the country's Gross Domestic Product (GDP) (Dixon and McIndoe, 2022). New Zealand waters provide diverse fisheries from deep water sea fish to inshore finfish and shellfish (MPI, 2015). It was reported that in 2020, Hoki (*Macruronus novaezelandiae)* accounted for 44 % of value for New Zealand's deepwater fisheries and 29 % of the total commercial fishing value (Dixon and McIndoe, 2022). The inshore finfish like snapper (*Pagrus auratus*) accounted for 18 % of total fishing value meanwhile shellfish sectors that include green mussels (*Perna canaliculus*) provide about 9 % (Dixon and McIndoe, 2022). In 2019 it was reported that Hoki was New Zealand's most valuable deepwater fishery with an export value of NZ$ 232 million meanwhile in 2017 it was reported that snapper has an export value of NZ$ 33 million (Lundquist et al., 2023).

A consequence of the growing fishing industry is the generation of fish waste and by-products (Ministry of Primary Industries [MPI], 2016). Fish waste or by-products consist of heads, bones, skin, scale, fins, viscera, frames and trimmings (Ozogul et al., 2021). For a developed country, these large quantities of fish by-product waste could cause a severe pollution and disposal problem if the waste was concentrated in a few places and left untreated (Ozogul et al., 2021). Current industrial practices often further process this waste into low value products such as fish meal and natural fertilizer (Ozogul et al., 2021). Fish waste by-products contain useful nutrients such as omega-3 fatty acids and nitrogenous compounds including amino acids that are essential for human consumption (Shirai and Ramirez-Ramirez, 2011; Ozogul et al., 2021). Thus, one of the few ways left for fishing industry to increase seafood sustainability, reduce waste from fish by-product and to increase the returns (income on their current catch) is by utilizing seafood by-product more profitably.

In 1986, the Quota Management System (QMS) was introduced in New Zealand as an ongoing effort of the country for a sustainable fishery (MPI, 2022). There are 98 marine species listed in the QMS including Hoki (*Macruronus novaezelandiae),* snapper (*Pagrus auratus*) and green-lipped mussel (*Perna canaliculus*) with the total allowable commercial catch (TACC) of 110,000,000 kg, 6,907,300 kg, and 1,675,000 kg, respectively, each year (MPI, 2023). With a quota limit implemented, it has come into realization that fully utilizing the commercial allowance catch is important in generating a profitable and sustainable income for the country's economy (MPI, 2023).

In recent years, climate change has also shown significant impacts on New Zealand's fishing industry affecting both the distribution and abundance of fish stocks including wild-caught fisheries and aquaculture sector, as well as the overall health of marine ecosystems (Lundquist et al., 2023). Sea surface temperatures have warmed around New Zealand between 1981 and 2018 leading to shifts in the distribution of fish species (Lundquist et al., 2023).

Some species are moving southward in search of cooler waters, while others are becoming more prevalent in certain regions (Lundquist et al., 2023). This has disrupted traditional fishing patterns and affect the availability of specific species (Lundquist et al., 2023). Changes in water temperatures and environmental conditions has also influenced the timing and seasonality of fish migrations and spawning which consequently making it more challenging in predicting and planning the fishing seasons causing mismatches between fishing efforts and fish availability (Lundquist et al., 2023). It was reported that some of the marine species that are affected by the rises in water temperature in New Zealand's oceanic and coastal waters includes Hoki, snapper, blue cod, red cod, barracouta and albacore (Lundquist et al., 2023). To address the impacts of climate changes on fishing industry, a number of strategies have been outlined by the MPI including modifying stock assessments, reassessing stock boundaries and quota management areas and limiting catch size to protect sensitive size classes (e.g., immature or small fish) (Lundquist et al., 2023). Although these strategies are seen as sustainable approaches for the future of the fishing industry, in hindsight, the limited supply of wild-caught fisheries may impact any future on-shore fish sauce business and more interests may shift towards aquaculture sector for the sustainability of the highly potential fish sauce business.

2.2 Preserving Fish

A portion of 150 grams of fish contributes between 50 to 60 percent of an adult's total daily protein intake (FAO, 2018). In many developing countries, particularly in the Southeast Asia region, fish provides high-quality, easily digested animal proteins and is especially significant in reducing micronutrient deficiencies (FAO, 2018; Saisithi, 1994). Salting has been one of the most common techniques employed to preserve fresh fish along with smoking and sun-drying (Saisithi, 1994).

In tropical countries, salting and drying may be prolonged due to frequent rainfall and high humidity environments (Saisithi, 1994; Thongthai, 2005). For tropical countries, the warm and high humidity climate increases the rate of spoilage (Beddows, 1998).

These conditions have naturally allowed fermentation to take place and throughout the years, people have started to desire the unique aroma and taste of fermented fish products (Saisithi, 1994). The fermentation process provides a way to preserve foods, enhances their nutritional value and utilizes raw materials that otherwise are not usable for human consumption (Salampessy et al., 2010).

Fermentation process is defined as chemical transformation of organic matter via microbial metabolism facilitated by enzymes (Chai et al., 2022). According to Kaur et al. (2019), there are mainly four types of industrial fermentation process:

1. Alcohol fermentation – a process that produces ethanol as the final product via yeast fermentation, commonly observed in wine and beer production.
2. Lactic acid fermentation – a process mediated mainly by lactic acid bacteria (LAB) which commonly occur in milk products and cereals.
3. Acetic acid fermentation – a process occuring due to the work of *Acetobacter* species which converts ethanol to acetic acid in the presence of excess oxygen.
4. Alkali fermentation – a process occurs during the fermentation of either fish, eggs, seeds or any protein rich materials, commonly observed in food products used as condiments (e.g: fish sauce).

In the current study the term "fermentation" covers the evolving complex of enzymatic, chemical and biological transformations occurring in the liquid and solid phases within the fermentation vessel employed, small or large.

During the fish sauce fermentation, fish muscle proteins degrade into smaller peptides and amino acids which in turn provide nutrients for microorganisms (Salampessy et al., 2010). Due to this, fermentation is usually combined with a salting or drying method to reduce water activity and kill putrefying microorganisms (Salampessy et al., 2010; Saisithi, 1994). In addition, during salting,

proteolytic enzymes in the fish are liberated allowing the protein in cells and muscles of the fish to solubilize (Beddows, 1998). This solubilized protein is then hydrolyzed by both the liberated enzymes and microorganisms to produce a fish sauce (Beddows, 1998; Salampessy et al., 2010).

The transformation of organic substances into simpler compounds by both enzymes and microorganisms assist in the development of flavour and aroma unique to fish sauce (Beddows, 1998).Unlike fresh foods which can be too expensive for most people in developing countries, traditional fermented fish products offer cheaper and more affordable alternative (Saisithi, 1994). Fermented fish products may not be as good as fresh fish, but fresh fish is highly perishable (Saisithi, 1994). The food value of fermented fish will differ than the fresh fish, but nonetheless, fermented fish products provide many advantages including high consumers' acceptability due the acquired and desired taste and aroma, low production cost due to simple preparation procedures, improved food safety as high salt fermentation eliminates putrefying microorganisms and improved digestibility and absorbability compared to fresh fish (Saisithi, 1994).

Fermented fish products are consumed as a condiment in many Asian countries such as Thailand, Vietnam, Indonesia, Philippines, Malaysia, Laos, and Myanmar, Korea and Japan (Beddows, 1998; Lopetcharat and Park, 2002; Saisithi, 1994; Xu et al., 2008). There are many varieties of fermented fish products as fermentation can lead to a series of well-defined products (Lopetcharat et al., 2002). In Thailand alone, it is reported that there are 16 different types of traditional fermented fish products (Lopetcharat et al., 2002). Over the years, many attempts have been made to categorise fermented fish products according to presumed common characteristics (Saisithi, 1994). According to Beddow(1998), fermented fish products can be categorized into three groups according to the appearance of the final products which are: 1) fermented fish that retains its original form, 2) fermented fish that is degraded into paste-like form and 3) fermented fish that is completely hydrolysed into liquid form.

Nowsad (2007) categorized fermented fish products according to the processing methods which are: 1) fermented fish products that are fermented by the enzymes endogenous in the fish flesh and entrails and to which salt is added, 2) fermented fish products that are fermented by the combined effects of endogenous enzymes in the fish and microbial enzymes supplied in the form of starter cultures also to which salt is added and 3) fermented fish products in which the fermentation process is accelerated by chemical hydrolysis.

2.3 Fish Sauce

Fish sauce is a clear brown liquid hydrolysate which is obtained through protein hydrolysis that occurs during a fermentation process (Lopetcharat et al., 2001). Fish sauce is marketed with different names in different countries: *nouc-nam* in Vietnam, *budu* in Malaysia, *patis* in the Philippines, *aek-jeot* in Korea, *shottsuru* in Japan and *nampla* in Thailand which at present, is the largest producer of fish sauce Asia (Lopetcharat and Park, 2002; Nowsad, 2007).

In Thailand, fish sauce is categorized according to the production methods which are: 1) pure fish sauce is obtained from fresh fish or fish by-products fermented with salt or brine, 2) hydrolyzed fish sauce is obtained from chemical or enzymatic hydrolysis processes approved by the Thai Public Health Ministry and 3) diluted fish sauce is obtained by diluting the pure or hydrolysed fish sauce using approved additives or flavouring agents (Lopetcharat et al., 2001).

2.4 The Manufacturing Process for Fish Sauce

In general, the traditional production of fish sauce starts with heavily salted fresh whole pelagic fish with or without the guts, tightly packed and sealed in earthen jars where natural fermentation takes place at ambient temperature for 12 to 18 months (Beddows, 1998; Lopetcharat et al., 2001; Saisithi, 1994; Steinkraus, 2004). The ratio of fish and salt varies from 2:1 to 6:1 depending on the producing countries in order to satisfy specific consumer preferences (Lopetcharat et al., 2001).

The manufacture of *bakasang* in Indonesia uses small fish like skipjack tuna (*Katsuwonus pelanis),* a major fish caught in Indonesian waters (Beddows, 1998; Kailasapathy, Salampessy, and Thapa, 2010). The production of *bakasang* provides a way to reduce waste of the raw seafood materials in which the fish and the guts are both used by cutting them into smaller pieces before mixing with salt in the ratio of 1.5 to 3 parts and 5 parts of fish (Ijong and Ohta, 1994; Salampessy et al., 2010). The fish and salt mixture are then bottled and left to ferment for 3-6 weeks at temperatures varying between 30 °C to 50 °C (Ijong and Ohta, 1994; Salampessy et al., 2010). The production of *bakasang* remains as small local businesses and household food ingredients in the eastern Indonesia regions especially in the North Sulawesi and Maluku provinces (Salampessy et al., 2010). This particular fermented fish sauce is less popular in other parts of the country due to its strong flavor which is less palatable to most consumers disallowing commercial manufacturing of the fermented product (Salampessy et al., 2010).

In the Philippines, *patis* is a fish sauce obtained from the production of a partially or fully hydrolysed fish or shrimp paste called *bagoong* (Salampessy et al., 2010; Olympia, 1992). Fish such as anchovies and sardine and small shrimps are cleaned and mixed with salt in a ratio of 3:1 and placed in vats to ferment (Salampessy et al., 2010). *Patis* is the supernatant collected from *bagoong* production by decanting and/or pressing or centrifuging (Salampessy et al., 2010).

The supernatant is then left to ferment for another 6 to 12 months at a temperature varying between 37°C to 50 °C , developing its unique flavour and aroma which is described as salty with a slightly cheese-like odour (Olympia, 1992).

Another fish sauce from the Southeast Asian region is *nouc-mam* from Vietnam (Beddows, 1998). Traditionally, *nouc-mam* is produced by grinding fish, pressing them by hand, and then placed in earthen jars in a manner of alternate layers with salt in an approximate ratio of 3:1 fish to salt (Lopetcharat et al., 2001; Salampessy et al., 2010).

The tightly sealed jars are buried halfway in the ground and are allowed to ferment for several months depending on the size of fish used (Lopetcharat et al., 2001).

Figure 1: Several types of fish sauces from different countries. From left: Budu (Malaysia), nampla (Thailand), nouc-mam (Vietnam) and patis (Philippines).

Small fish takes about 6 months while bigger size fish extends the fermentation to approximately 18 months (Lopetcharat et al., 2001). After 3 days into the fermentation, turbid and bloody in appearance liquid called *nuoc-bai* is collected and drained off (Saisithi, 1994). A part of the *nuoc-bai* is returned to the jars to further ferment (Saisithi, 1994). The first supernatant is collected from the first fermentation cycle (a process that takes about 6-8 months) and is referred to as the primary and high quality *nuoc-mam* (Lopetcharat el al., 2001). Hot brine is then added into the fermentation jars to extract more nuoc-mam, this time this is referred to as the secondary or low quality *nuoc-mam* (Salampessy et al., 2010; Lopetcharat et al., 2001). The secondary nuoc-mam has a shorter shelf-life due to its lower salt content compared to the primary *nuoc-mam* (Lopetcharat et al., 2001). Additives such as caramel, molasses or roasted barley and maize can be added to improve the overall quality of the secondary product (Beddows, 1985; Salampessy et al., 2010; Lopetcharat et. al., 2001). In Malaysia, *budu* is traditionally produced by placing layers of fish, commonly *Stolephoris sp.* into salt in the ratio of fish to salt of 3:2 and allowing the mixture to ferment for 6 to 12 months (Beddows et al., 1980).

Tamarind and palm sugar are often added to the fish and salt mixture and then boiled prior to fermentation, giving *budu* its significant dark brown colour (Salampessy et al., 2010; Tanasupawat and Visessanguan, 2014). *Budu* also contains ground fish residue as the mature fermented fish products but are not traditionally filtered (Tanasupawat and Visessanguan, 2014). *Budu* is popular in the northeastern region and sometimes is produced using leftovers from fish drying or whole fish when the weather is not suitable for drying fish (Salampessy et al., 2010).Traditional production processes of *nampla, nouc-mam* and *bakasang* are presented in Figure 2.

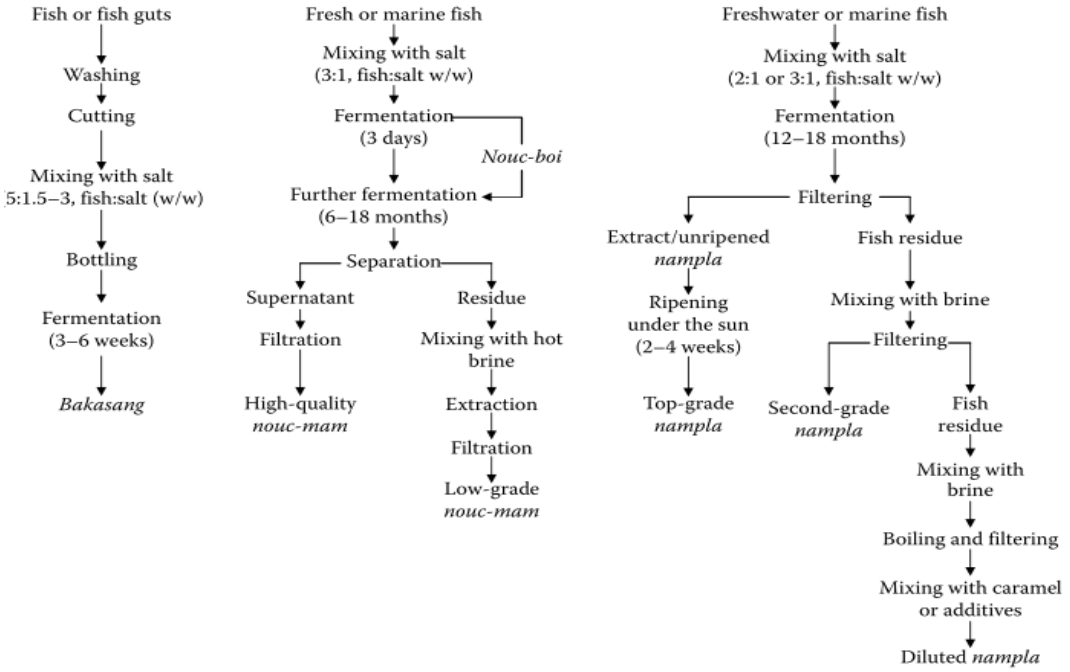


Figure 2: Flow chart of a traditional production of bakasang, nouc-mam and nampla (Kailasapathy, Salampessy, and Thapa, 2010).

Fish sauce from Thailand called *nampla* is more popular and well-known in many countries across the globe (Salampessy et al., 2010). To date, Thailand is the largest producer and exporter of fish sauce in the Southeast Asia region, and *nampla* is recognized in the international market, not only in Asia but also in the Western regions including the United States and the European Union (Steinkraus, 2004).

The *nampla* production starts by cleaning the un-eviscerated fish to reduce the number of microorganisms in the raw materials (Lopetcharat et al., 2001). The fish is mixed with salt in a ratio of 2:1 or 3:1 before transferring them into fermentation tanks such as earthen jars or wooden barrels (Salampessy et al., 2010). As fermentation progresses, liquid brine is produced from the osmotic dehydration of the fish, hence, bamboo mats are placed on top of the mixture and loaded with heavy weight to ensure that the fish is fully submersed in the brine (Saisithi, 1994).

The fermentation time varies from 6 to 18 months depending on the size of the fish, the amount of salt added and other environmental conditions (Steinkraus, 2004). The completion of the fermentation of process is determined based on the colour, flavour, aroma and the clarity of the *nampla* which is often subjected to the judgement and experience of the producers (Steinkraus, 2004).

The fish and salt mixture is allowed to ferment at varying temperatures between 30°C to 40°C in the open area and after the fermentation process is completed, the first batch of supernatant is collected and transferred to the ripening tank where the ripening process can take a further 2-12 weeks (Lopetcharat et al., 2001; Saisithi et al., 1966). The ripened liquid is then filtered and distributed as the first grade of *nampla* (Steinkraus, 2004).

Second grade and low-quality *nampla* can be produced using the same procedures except that Mikei water, nitrogen-rich by-products from the production of monosodium glutamate (MSG), can be added to improve the quality of the *nampla* (Beddows, 1998; Salampessy et al., 2010; Lopetcharat et al., 2001). The Mikei water is a rich source of glutamic acid that improves the nitrogen content of low quality *nampla* in order to meet the requirements of the Thai Industrial Standard Institute (Lopetcharat et al., 2001). Caramel colour is sometimes added to improve the colour of the second grade *nampla* (Lopetcharat et al., 2001).

Different recipes of fish sauce used in different countries are summarised in Table 1. In the majority of countries producing fish sauces, the production and manufacturing processes of the fermented fish products still remains a traditional process (Steinkraus, 2004). The traditional production of fish sauce typically does not employ control measures during the fermentation process which sometimes may result in inconsistent product qualities (Yongsawatdigul et al., 2004). In addition, the long fermentation process also requires a high capital land investment, limiting the growth of fish sauce industry (Akolkar et al., 2010). Scientific studies have been conducted to investigate modern approaches in the production of fish sauce without compromising the quality of the final product (Zaman et al. 2011).

Generally, natural fermentation methods are difficult to control with an accompanying risk in the growth of putrefying microflora, hence, the use of starter cultures have now become more important in the production of safe fermented fish products (Takada et al., 2023; Tanasupawat and Visessanguan, 2014). Starter cultures can accelerate the fermentation process hence shorten production times while ensuring consistent product quality (Li et al., 2024; Zaman et al., 2011). In addition, specific microbial strains can be selected to produce and/or improve desirable aroma and flavours (Yongsawatdigul et al., 2007). Although certain microbial strains have been shown to effectively accelerate fish fermentation, their performance can vary depending on the type of fish substrate, native microbiota, fermentation conditions, and desired product characteristics (Li et al., 2024). Relying on selected strains may lead to the loss of unique native microflora that define a traditional fish sauce, while also posing the risk of less optimal fermentation when used with new or less-studied raw materials (Li et al., 2024; Nguyen et al., 2021). The use of starter cultures may also disrupt microbial and biochemical dynamics of fermentation which consequently altering the production of beneficial or harmful compounds such as flavor precursors, biogenic amines, or Maillard reaction products (Kim et al., 2024; Li et al., 2024).

In addition, consumers may perceive fish sauce made with starter cultures as less natural which may potentially affect market acceptance (Li et al., 2024).

Table 1: Summary of different types of fish sauce and the traditional fermentation recipes.

Country	Name	Fish Species Commercially Used	Fish/Salt Ratio	Fermentation Time	References
Thailand	*Nampla*	*Stolephrous spp. Ristrelliger spp. Cirrhinus spp.*	2-3/1	6 - 18 months	Salampessy et al., 2010
Vietnam	*Nouc-mam*	*Stolephorus spp. Ristrelliger spp. Engraulis spp. Decapterus spp. Clarius spp.*	3/1	6-18 months	Beddows (1998)
Indonesia	*Bakasang*	*Stolephorus spp. Clupea spp. Leiagnathus Osteochilus spp.*	6/1	6 months	Lopetcharat et al. (2001)
Malaysia	*Budu*	*Stolephorus spp.*	3-5/1	3-12 months	Beddows (1998)
Philippines	*Patis*	*Stolephorus spp. Clupea spp. Decapterus spp. Leionathus spp.*	3-4/1 to 7/2	3-12 months	Beddows (1998); Steinkraus (2011)
Korea	*Aekjot*	*Astroscopus japonicus Engraulis japonica*	3-4/1	6-12 months	Tanasupawat and Visessanguan (2014)
Japan	*Shottsuru*	*Astroscopus japonicus Clupea pilchardus*	5/1	6 months	Lopetcharat et al. (2001)

2.5 Factors Affecting the Fermentation of a Fish Sauce

The factors affecting the fermentation of a fish sauce include the type of fish used, the type and concentration of the salt, temperature, and oxygen concentration (Beddows, 1998; Salampessy et al., 2010; Lopetcharat et al., 2001). All of these factors consequently affect the abundancy, diversity, and dynamic growth of the microflora (Salampessy et al., 2010). These factors are further discussed in turn in the following sections.

2.5.1 Fish species

Fish species is one factor that affects the final fish sauce (Lopetcharat et al., 2001). The fish species contributes to the type of proteins to provide nutrients for the microorganisms and the substrates available for enzymatic hydrolysis during the fermentation process (Lopetcharat et al., 2001). Traditionally, lean freshwater fish is preferable in fish sauce fermentation as fatty fish is more susceptible to lipid oxidation which may result in rancid flavour notes in of the final product (Saisithi, 1994). Different countries use different fish species in their fish sauce recipes (Lopetcharat et al., 2001). In Thailand, *nampla* is mainly produced from Indian anchovies (*Stolephorus spp*.) (Lopetcharat and Park, 2002). Other species such as bleeker smooth belly sardines (*Clupeoides sp.),* sardine *(Sardinella sp.),* Indian mackerel (*Rastrellinger spp.)* and threadfin bream (*Nemipterus sp.)* are also used in the production of *nampla* (Steinkraus, 2004). In Vietnam, *nouc - mam* is commonly produced from anchovies (*Engraulis spp*.), *budu* in Malaysia is often produced from Indian anchovies (*Stolephorus spp.*) and herring (*Sardinella spp.*) while *patis* in the Philippines uses mainly Indian anchovies (*Stolephorus spp.*) and flat oyster (*Ostrea spp*.) (Thongthai, 2005).

The extensive proteolysis and liquefaction of the fish protein that takes place during fermentation of a fish sauce is mainly the result if autolytic breakdown of fish muscles and tissues (Adam, 2009). The autolytic breakdown is found to be more rapid when whole fish is used because the head and viscera contain higher concentrations of proteolytic enzymes than other tissue (Adam, 2009). Generally, the fish viscera have the highest concentration of proteolytic enzymes during the feeding season (Saisithi, 1994). It was also found that cathepsin, an enzyme that is responsible for the degradation of myofibrillar proteins in fish muscle resulting in the softening of muscle texture, is activated in high salinity environments (Nonthaput et al., 2017). In many practices to produce semi-solid fish paste, eviscerated fish are used to decrease the rate of autolysis (Nonthaput et al., 2017).

It was also found that the muscle protein of active swimming fish is hydrolysed at a faster rate than that of slow-moving fish (Salampessy et al., 2010). Microorganisms play an important role in enhancing the degradation of fish proteins and the development of the unique flavour and aroma in the production of fish sauces (Salampessy et al., 2010). Microorganisms in fish vary depending on season, species, transportation, storage, and catching method (Lopetcharat et al., 2001). Seasonal changes influence water temperature, which in turn affects fish diet and alters the gut microbiome (Egerton et al., 2018). Additionally, wild-caught and farmed fish differ in microbial profiles, with wild fish showing greater diversity (Bereded et al., 2021). These factors can influence spoilage, fermentation behaviour, and overall product quality (Lopetcharat et al., 2001). Bacteria that are frequently associated with fish and seafood are *Acinetobacter, Bacillus, Alcaligenes, Aeromonas, Enterococcus, Enterobacter,* and *Corynebacterium* (Lopetcharat et al., 2001). Putrefying bacteria such as *Escherichia sp., Serratia sp., Pseudomonas sp.* and *Clostridium sp.* can grow effectively in raw fish; as fish muscles serve as available amino acids and additional nutrients (Salampessy et al., 2010).

2.5.2 Type of Salt and Salt Concentration

The amount of salt used in the production of fish sauce needs to be sufficient to reduce water activity (a_w) to below optimum levels at which bacteria on the fish can grow (Fernandes, 2009). In many conditions, the amount of salt added is sufficient to saturate the water phase and keeping an a_w of 0.74 or below (Fernandes, 2009). The osmotic effect of salt kills or retards bacteria due to plasmolysis, a process where the microbial cell loses water in a hypertonic environment (Majumdar and Basu, 2009). It is recommended the salt concentration between 18 to 20% is used to inhibit the growth of putrefying bacteria (Saisithi, 1994). The fish can easily spoil when the amount of salt used is below 7% (Steinkraus, 2004). The fish to salt ratio used to produce fish sauce differs in each country (Beddows, 1998) as summarized in Table 1. In current industrial practice, salting by hand is replaced by machine mixers allowing the process to be completed while the fish is still fresh (Saitsithi, 2011).

The autolysis of fish protein takes place immediately after the fish is dead and bacterial spoilage follows soon after (Nonthaput et al., 2017; Saisithi, 1994). For large fish, salt and fish are often put in alternate layers with salt at the bottom and top layer, speeding up the salting process (Beddows, 1998; Saisithi, 1994).

Meanwhile, bulk salting is often employed for small fish such as anchovies and sardines because small fish will have a larger surface area to volume ratio, allowing the salt to come into contact with the fish more easily (Saisithi, 1994). The readily salted fish are transferred to the fermentation vats where the topmost layer is then covered with approximately 1.5 to 2 cm thick layer of salt (Saisithi, 1994). It is also important that the fish are salted thoroughly for a better quality product (Steinkraus, 2004). Bacterial and enzymatic activity are affected by the salt concentration in a fish sauce mixture, resulting in different flavours and aroma (Tanasupawat and Visessanguan, 2014). Salt also controls the type of microorganisms and eliminates pathogenic bacteria during fermentation (Lopetcharat et al., 2001).

Often, sea salt is used in the production of fish sauce because it is easily available (Lopetcharat et al., 2001). Sea salt and rock salt mainly contain sodium chloride (Lopetcharat et al., 2001). Thai sea salt has approximately 88% sodium chloride which is slightly less compared to sea salt from other countries which are mostly composed of approximately 97% sodium chloride (Lopetcharat et al., 2001). Other elements in sea salt are calcium sulphate ($CaSO_4$) at 0.24%, calcium chloride ($CaCl_2$) at 0.24%, magnesium sulfate ($MgSO_4$) at 0.17%, magnesium chloride ($MgCl_2$) at 0.3%, water 2.4% and water insoluble substances at 0.4% (Lopetcharat et al., 2001). The Mg^{2+}, Ca^{2+}, SO_4^{2-}, and other impurities may retard the diffusion of NaCl into the fish muscle during salting and this may cause spoilage (Lopetcharat et al., 2001).

In addition, heavy metal ions contained in salt can also accelerate the lipid oxidation resulting in rancidity, thus reducing the quality of the final product (Lopetcharat et al. 2001). Sodium (Na^+) and chloride (Cl^-) in salt interrupt the acyl group in bacteria (Lopetcharat et al., 2001).

In high salt concentration, oxygen is less soluble creating an environment suitable for the growth of anaerobic bacteria (Lopetcharat et al., 2001). The high salt concentration used in the production of fish sauce also aids in the osmotic extraction of amino acids from the fish tissue particularly glutamic acid, volatile fatty acids and nucleotides which are responsible for the overall flavour and aroma of the final product (Hakimi et al., 2022). Halophilic archaebacterial are extreme halophiles that grow optimally in high salinity environments of about 20 to 30% salt concentration (Akolkar et al., 2010). These bacteria are common in unstoved solar salt and have been found to grow on salted fish (Akolkar et al., 2010).

Solar salt is produced by fractional crystallization method in solar pans where the insoluble salt (calcium carbonate) will first crystallises, then other salts crystallize at different pans based on their solubility and brine density (Bipin et al, 2022). In New Zealand, salt is sourced and purified from the sea at Lake Grassmere, Marlborough, some of which is further refined at Mount Maunganui, Tauranga (Reid, 1976). Sea water is pumped from ocean to large ponds where evaporation process relies on solar and wind energy (Reid, 1976). By the end of the evaporation process, the water volume is reduced to 27 % of the original, causing calcium sulfate to precipitate out (Reid, 1967). Then, the brine (without calcium sulfate) passes through series of ponds causing the sodium chloride content to rise from 2 % to 25 % (Reid, 1967). The increasing salinity gives the ponds a distinctive pink colour, as algae in high salinity environment can produce red pigments called haematochrome (Reid, 1967). Once the volume of salt brine has reduced to 10 % of the original, any further concentration will result in the deposition of sodium chloride (Reid, 1976). From September to February, more brine is added until at least 25 mm of salt has settled (Reid, 1976) Harvest season takes place in early March and lasts for four to six weeks (Reid, 1976). The crystallised solar salt harvested will be washed in clean saturated brine in which other salts that present as impurities are dissolved, thus producing crude solar salt (Reid, 1976).

The crude solar salt that does not undergo further processing is called unstoved solar salt (Reid, 1967). Unstoved solar salt may contain higher magnesium and calcium contents than processed salts, and it has coarser texture (Reid, 1976). Meanwhile, the crude solar salt that has undergone a high temperature dry heat treatment to kill halophilic bacteria is called stoved solar salt (Reid, 1967). Stoved solar salt tends to be whiter and more uniform in size (Reid, 1967).

Addition of koji (fermented soybean or wheat) and plant enzymes such as bromelain, ficin and papain can be included in the fermentation process of fish sauce to shorten the fermentation period to approximately 150 days and 21 days shorter, respectively (Akolkar et al., 2009). *Halobacterium salinarum strain ORE* was determined to be one of the protease-producing halophilic bacteria isolated from different fish sauces (Akolkar et al., 2009). The proteases produced by halophile are active at high salt concentration whereas the other enzymes from koji and plants are denatured ((Akolkar et al., 2009). In a study on the Vietnamese fish sauce, *nuoc-mam,* it was found that there was approximately 2.7 x 10^4 colony-forming units (cfu)/g of bacteria predominantly *Bacillus sp.* in the solar salt tested (Saisithi et al., 1966). In a study on Thai fish sauce, *nampla,* bacteria including *Bacillus sp., Micrococcus sp., Sarcina sp., and Halobacterium sp.* were isolated from sea salt used, indicating that salt is also the source of microflora that contribute to the flavour and aroma of fish sauce (Saisithi et al., 1966). However, the study on the microflora on salt were less explored as many current studies are primarily focused on the microflora present in the fish viscera due to their abundancy and high diversity which are thought to play more of a role in the fish sauce fermentation (Akolkar et al., 2009).

2.5.3 Temperature

In a study on the effect of temperature in the production of *patis,* the fermentation time was reduced when the temperature is increased to 37 °C (Lopetcharat and Park, 2002). In another study on a Thai fish sauce, *nampla,* it was determined that a fermentation temperature of 50 °C had succeeded in producing a final product containing total nitrogen equivalent to that found in commercial fish sauce

within 15 days of fermentation (Lopetcharat and Park, 2002). This was due to the increase in the proteolytic activity of endogenous cathepsins at the optimal condition, 55 °C (Lopetcharat and Park, 2002).

In a study to understand the effect of fermentation temperature on the bacterial communities and metabolites in traditional Korean fermented anchovy sauce (*Myeolchi-Aekjot*), it was found that bacterial diversity and abundances differed depending on fermentation temperatures in which more bacterial growth were observed in samples fermented at higher temperatures, particularly at 20 °C, 25 °C, and 30 °C compared to sample fermented at 15 °C (Jung et al., 2016). In the same study, it was also found that at high temperatures (25 °C and 30 °C), *Photobacterium* and *Mycoplasma* were not detected after 30 days of fermentation, while at low temperatures (15 °C and 20 °C), the same bacteria genus were detected throughout the fermentation period suggesting that low temperatures may not be recommended for safety consumption of *Myeolchi-Aekjot* products as *Mycoplasma* is known to be pathogenic (Jung et al., 2016). However, it was reported that the optimum fermentation temperature for a safe and better-quality traditional Korean shrimp sauce (*Saeu-jeout*) is 15 °C in which the bacterial genus such as *Vibrio, Photobacterium, Aliivibrio,* and *Enterovibrio*, which may contain potentially pathogenic strains disappeared after 105 days of fermentation and maximum concentration of amino acids associated with the umami and sweet flavours were detected at the same fermentation day (Lee et al., 2014). These findings suggest that the effect of temperatures on fish fermentation can differ depending on the raw material used. Further discussion on the bacteria commonly found in fermented fish sauce is discussed in Section 2.6.3.

2.5.4 Oxygen Levels in Fermentation Tank

Low oxygen levels in fermentation tank are responsible for the dynamic and diversity of the microorganisms (Lopetcharat et al., 2001).

In the fermentation tank, the oxygen concentration decreases when moving further below the liquid surface, thus limiting the aerobic fermentation (Lopetcharat et al., 2001). Lopetcharat et al. (2001) reported that fish sauce fermentation was completed under partial aerobic and anaerobic conditions. Further discussion on the bacteria commonly found in fermented fish sauce is discussed in Section 2.6.3.

2.6 Factors Affecting the Quality of a Fish Sauce

The factors affecting the quality of a fish sauce is divided into three categories which are biochemical and chemical properties, sensory properties, and microbiological properties (Beddows, 1998; Lopetcharat et al., 2001). Based on Codex Alimentarius Commission (2011), the standard for fish sauce from the Food and Agriculture Organization of the United Nations (FAO), it is stated that the essential quality factors for fish sauce include; 1) chemical and biochemical properties such as salt, nitrogen, amino acid and histamine concentrations and 2) sensory properties such as odour and taste. Table 2 presents the standard parameters for fish sauce according to FAO.

Table 2: Standards for fish sauce according to Codex Alimentarius Commission (FAO, 2011)

Chemical properties	Standard for Fish Sauce
Total nitrogen content (w/v %)	≥1
Total amino acid content (w/v %)	≥40
Salt (w/v %)	≥20
pH	For traditional product: 5.0 – 6.5 For assisted fermentation: 4.5 ≥ pH ≤ 6.5
Histamine (mg/100 g fish sauce)	40

2.6.1 Biochemical and Chemical Properties of Fish Sauce

During fermentation of fish sauce, proteins and fats undergo degradation producing amino acids, peptides, fatty acids, organic acids and non-protein nitrogen compounds (Salampessy et al., 2010).

Amino acids can further degrade to produce amines, volatile acids and biogenic amines (Salampessy et al., 2010; Sanceda et al., 2003). This section will further discuss the biochemical and chemical changes during the fermentation process of fish sauce.

2.6.1.1 Chemical Properties

During fish fermentation, proteins, fat, and glucose are converted into peptides, amino acids, fatty acids and lactic acids by the action of enzymatic and proteolytic hydrolysis (Salampessy et al., 2010). Protein is the major constituent in fish, thus protein degradation represents the major change during the fish sauce fermentation process (Salampessy et al., 2010). The production of free amino acids, polypeptides and lactic acid by lactic acid bacteria (LAB) decreases the pH of fish sauce (Salampessy et al., 2010). Generally, fish sauce has a slight acidic pH value varying between 5 to 6.5 (Salampessy et al., 2010). The chemical composition such as total nitrogen content, pH and salt content varies between different types of fish sauces (Beddows, 1998). The chemical compositions of different types of fish sauce are summarized in Table 3.

Table 3: Chemical compositions of different types of sauce.

Reference	Bakasang (Ijong and Ohta, 1995)	Nouc-mam (Park et al., 2001)	Nampla (Ahmad Puat et al., 1994)	Budu (Beddows, 1979)	Ngapi (Beddows, 1998)	Japanese Shiokara (Saisithi, 1994)
Total nitrogen (w/v %)	NA	1.7	NA	1.8	NA	NA
pH	5.5 – 5.9	5.63	5.3	5.6	NA	NA
Salt (w/v %)	9.0 – 17.0	21.4	26.7	26.3	NA	7.8
Protein (w/v %)	14.0 – 17.4	NA	10.9	NA	45	11.6
Lipid (w/v %)	1.4 – 1.6	NA	NA	0.1	18.5	NA
Ash	NA	NA	NA	NA	11	8.68
Moisture content (w/w %)	66.3 – 71.9	61.4	71.9	NA	18	74.2

NA indicates that the value of the chemical property of the fish sauce was not included in the literature reference.

According to FAO in Codex Alimentarius Commission (2011), the total nitrogen content should not be less than 1 % (w/v), pH values are expected to be between 5.0 to 6.6 for a traditional fermented fish sauce but not lower than 4.5 if additional ingredients are used to assist fermentation and salt content should not be less than 20 % (w/v).

2.6.1.2 Biogenic Amines Formation

Biogenic amines are basic nitrogenous compounds commonly found in processed meat, fishery products and fermented foods and are described as organic bases with aliphatic, aromatic and heterocyclic structures (Naila et al., 2010; Sanceda et al., 2008; Tanasupawat and Visessanguan, 2014). Formation of biogenic amines are common in fish species such as anchovy (*Engraulis encrasicholus*), albacore tuna (*Thunnus alalunga*), Skipjack tuna (Katsuwonus pelamis), sardines and herrings (*Clupidae*) which are often used in the production of fish sauce (Lopetcharat et al., 2001; Sanceda et al., 2008; Yongsawatdigul and Udomporn, 2004). Biogenic amines are formed during amino acid decarboxylation activities of certain bacteria in foods (Naila et al., 2010; Zaman et al. 2011). Biogenic amines can cause health problems when consumed at a particular concentration or when the natural mechanisms for their catabolism are inhibited or genetically deficient in the human body (Zaman et al., 2011). Some of the health effects from biogenic amines consumption are severe headache, hypertension, renal intoxication or in other severe cases, intracerebral hemorrhage and eventually death (Sanceda et al., 2008). Fish sauce was reported to contain considerable levels of biogenic amines mainly histamine, cadaverine, putrescine and tyramine (Tanasupawat and Visessanguan, 2014). In addition, octopamine and dopamine were reported in meat, meat products and fish (Naila et al., 2010). Histamine concentration in fish sauce in particular, is used to indicate the safety level for its consumption (Tanasupawat and Visessanguan, 2014). According to the standard for fish sauce by FAO in Codex Alimentarius Commission (2011), the histamine concentration should not exceed 40 mg/100g of fish sauce.

Histamine toxicosis or better known as Scombroid poisoning is caused by consuming high concentrations of histamine in fish sauce which is formed by microbial decarboxylation of histidine (Figure 3) (Lopetcharat et al., 2001; Sanceda et al., 2008). Many halophiles including *Photobacterium phosphoreum, Photobacterium histaminum sp. nov., Enterobacteriaceae, Proteus morganii (Morganella morganii), Klebsiella pneumoniae, Citrobacter freundii, Enterobacter cloacae, Hafnia alvei, and Escherichia coli* are associated with the production of histidine decarboxylase (Lopetcharat et al., 2001).

In a study on a traditional Japanese fermented fish sauce made from squid liver, it was found that *Tetragenococcus muriaticus*, a halophilic lactic acid bacterium was responsible for the histamine formation regardless of the different fermentation conditions investigated (Kimura et a., 2001). The toxicity of histamine was also found to be enhanced by cadaverine and putrescine due to the inhibition of histamine-detoxifying enzymes including diamine oxidase and histamine N-methyltransferase produced by bacteria (Veciana-Nougues et al., 1996; Yongsawatdigul and Udomporn, 2004). According to Veciana-Nougues et al. (1996), the high level of accumulation of histamine, cadaverine and putrescine in fermented fish sauce was highly likely because the raw materials used were not fresh.

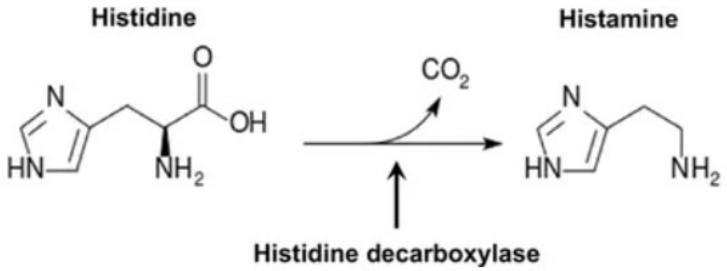


Figure 3: The conversion of histidine to histamine by histidine decarboxylase (Huang et al., 2018)

Histamine is heat stable and cannot be detected easily by organoleptic analysis (Lee et al., 2015). Many food processing approaches have been tested to eliminate or reduce histamine accumulation in food products including modified atmosphere packaging, high hydrostatic pressure, addition of food additives and preservatives and irradiation (Lee et al., 2015).

Histamine formation can be controlled by inhibiting microbial growth or inhibiting the decarboxylase activity of microbes (Naila et al., 2010). The prevention methods include temperature control, using high quality raw material, good manufacturing practice, the use of non-amine forming (amine-negative) or amine oxidizing starter cultures for fermentation, the use of enzymes to oxidize amines, packaging techniques and irradiation (Naila et al., 2010).

During irradiation, food products that contain water are likely to undergo both oxidation and reduction reactions due to the formation of an oxidizing agent like hydroxyl radicals and the formation of reducing agents such as aqueous electrons and hydrogen atoms (Kim et al., 2004). All of these oxidizing and reducing agents are water radiolytic products (Kim et al., 2004). Although irradiation is effective in reducing histamine formation, it also can potentially generate free radical compounds that are hazardous to health (Lee et al, 2015). Thus, the employment of starter cultures that produce negative amines that catalyze histamine degradation is a preferable approach (Lee et al., 2015). Histamine can further degrade via an oxidative deamination step to produce products that can be utilized as a carbon and/or energy source and as a nitrogen source (Tanasupawat and Visessanguan, 2014). The oxidation deamination step in histamine degradation is normally catalyzed by either amine oxidase (AO) or amine dehydrogenase (ADH) (Tanasupawat and Visessanguan, 2014; Zaman et al., 2011). The catalytic oxidative deamination by ADH often produces acetaldehyde and ammonia while that of AO produces hydrogen peroxide (H_20_2) in addition to acetaldehyde and ammonia (Figure 4) (Tanasupawat and Visessanguan, 2014).Many studies were conducted to investigate the roles of AO and ADH, particularly, histamine oxidase and histamine dehydrogenase activities in food fermentation to prevent the production and accumulation of histamine in fermented food products (Martuscelli et al., 2000; Lopetcharat et al., 2001; Lee et al., 2015). Histamine oxidase was found in *Staphylococcus xylosus, Staphylococcus carnosus, Bacillus amyloliquefaciens, Arthrobacter crystallopoietes,* and *Brevibacterium linens* (Martuscelli et al., 2000; Zaman et al., 2010).

However, the applications of microorganisms which are not halophilic or halotolerant may be restricted by unfavorable psychological conditions for enzyme activities due to the high salinity environment during fish sauce fermentation (Tanasupawat and Visessanguan, 2014).

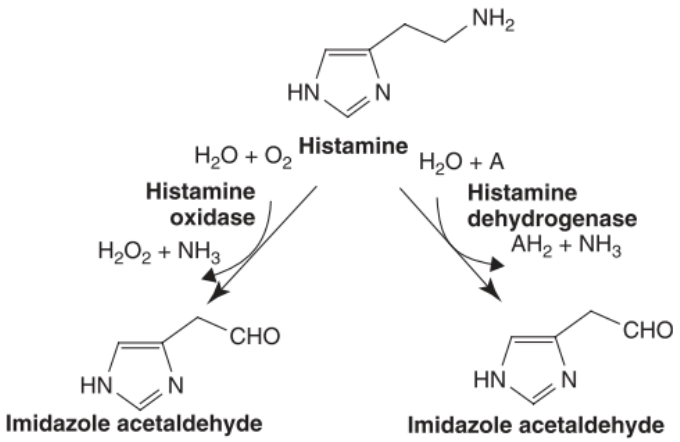


Figure 4: Histamine degradation pathways catalyzed by histamine oxidase and histamine dehydrogenase. The A and AH_2 are a two-electron acceptor and its reduced form, respectively (Tanasupawat and Visessanguan, 2014).

2.6.2 Sensory properties of Fish Sauce

Taste determines food selection, and which consequently contributes to the nutritional status and decision making regarding food purchasing (Zhao et al., 2016). This section will further discuss the factors affecting the sensory properties of fish sauce which include the production of peptides, amino acids and volatile compounds.

2.6.2.1 Peptides and Amino Acids

Food fermentation is one of the oldest methods used for food preservation and fermented food products are highly appreciated for their rich and complex taste and flavour (Day et al., 2022). The metabolic activity of microorganisms present during fermentations determines food quality, generates flavours and enhances palatability of the final products (Zhao et al., 2016).

Six basic tastes that can be detected by the taste receptors on the tongue are salt, sweet, sour, bitter, umami and oleogustus (kokumi) (Methven et al., 2023). In fermented food products, all six taste active peptides and amino acids can be generated through primary proteolysis of the raw material by proteases from either endogenous enzymes or microorganisms (Zhao et al, 2016).

Secondary proteolysis may take place by enzymatic or chemical hydrolysis which further converts amino acids or peptides into derivatives (Zhao et al, 2016). The overview of protein conversion from raw material into taste active peptides, amino acids and amino acid derivatives is presented in Figure 5. Taste active peptides, amino acids and amino acid derivatives are predominant tastants in many fermented food products and are often associated with bitter, umami or kokumi taste (Zhao et al, 2016). Umami taste is described as savory, meaty and broth-like (Yamaguchi and Ninomiya, 2000). Glutamic acid or its sodium compound, monosodium glutamate (MSG) is perceived as umami taste (Hartley et al., 2019; Thongthai, 2005). Glutamic acid is generated from the conversion of glutamine by glutaminase (Methven et al., 2023). Pyroglutamyl peptides which impart umami taste are produced by pyroglutamic acid cyclase from pyroglutamic acid (pGlu) in *Lactobacillus helveticus, L. delbrueckii subsp. bulgaricus,* and *Streptococcus thermophilus* (Figure 5) (Gazme et al., 2019). Maillard products such as alapyridaine, N-glycosides, pyroglutamyl peptides, and N-acetylglycine are also perceived to have umami taste (Zhao et al, 2016). Glutathione was the first taste active peptide identified that is associated with kokumi taste (Zhao et al, 2016). Kokumi is used to describe mouthfulness, thickness and increasing continuity of savory taste (Dunkel et al, 2007). The amino acid content in fish sauce is associated with the quality of the final product (Lopetcharat et al., 2001). According to the standard for fish sauce by FAO in Codex Alimentarius Commission (2011), the amino acid content should not be less than 40 % in a unit of fish sauce. The amino acid compositions differ slightly between different types of fish sauce (Park et al., 2001).

However, in many fish sauces the three most abundant amino acids are glutamic acid, aspartic acid and lysine which comprise about 40 % of the total amino acid content in fermented products (Thongthai, 2005). Meanwhile, valine, leucine and isoleucine make up at least 15 % of the total amino acids in a fish sauce (Thongthai, 2005). Lysine, in particular represents about 10 to 13 % of the total amino acids in Asian fish sauces (Thongthai, 2005).

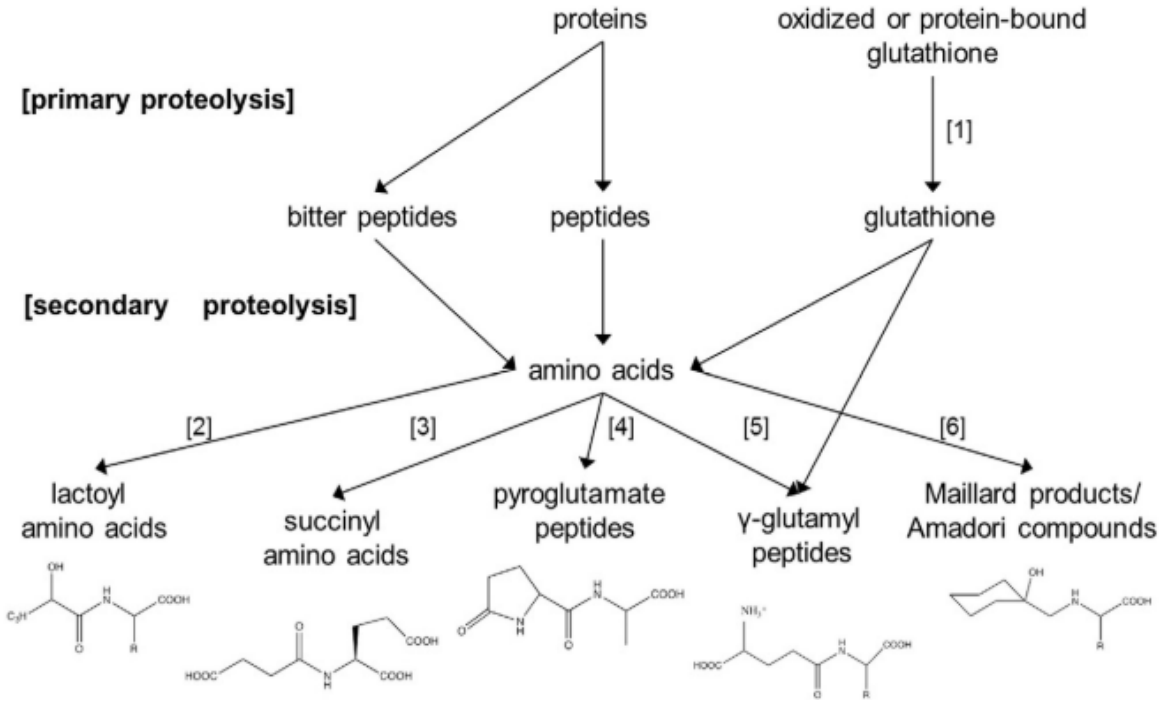


Figure 5: An overview on the generation of taste compounds in fermented foods. Primary proteolysis by endogenous proteases generate taste active peptides and amino acids. Glutathione reductase [1] generate glutathione which often associated with kokumi taste. Secondary proteolysis by enzymatic or chemical reactions further convert amino acids and peptides into amino acid derivatives. Enzymatic conversions are proposed to be catalyzed by lactoyl-transferase [2], succinyl transferase [3], pyroglutamyl cyclase [4] or γ-glutamyl-transferase [5]. Maillard products/Amadori compounds are formed by chemical conversion during heating [6] (Zhao, et al., 2016).

These amino acids were recognized to be responsible in the sensory profile of fish and shellfish (Park et al, 2005). In addition to contributing to the organoleptic improvement of fermented fish and shellfish sauces, taurine has also been known to have physiological benefits such as, antioxidant, detoxification and cell membrane stabilization (Park et al, 2005).

Taurine is found to be the most abundant in shellfish especially mussels, scallops and clams with concentration values of 655.4, 827.7 and 513.7 mg/100 g fresh raw material, respectively (Wójcik et al., 2010). In a Korean fermented blue mussel sauce, taurine was found to be a predominant amino acid that contributed to 10 % of the total free amino acids in the final product alongside glutamic acid, glycine, alanine and leucine (Park et al, 2005). Glutamic acid is naturally present in high concentration in foods such as seaweed, fish, mussels, crustaceans and egg yolks (Hartley et al., 2019). The curing, ageing and fermentation processes result in the increase in glutamic acid which consequently increases the umami flavor of the food products (Hartley et al., 2019). In a study on Vietnamese fish sauce, *nouc-mam*, omitting glutamic acid from the test sample eliminated the umami taste, thus indicated that the respective amino acid was a taste active compound in the fish sauce (Park et al., 2002). Alanine and valine were also found to impart umami taste (Park et al., 2002). The absence of alanine and valine decreased the umami taste of the fish sauce meanwhile bitter and sour tastes were found to be more prominent (Park et al., 2002). Thus, the generation of these taste active amino acids during the fermentation process was evidently responsible for the palatability of the fish product as a food condiment (Thongthai, 2005). In addition, the proteolytic degradation during fish sauce fermentation may release amino acids and peptides with biological activities that offer health benefits such as, antioxidants and antihypertensive (Cha and Yu, 2024; Nghia et al., 2017).

2.6.2.2 Volatile Compound Formation

The aroma of a fish sauce is used to assess the quality of the final product by consumers because the taste is very salty and often overpowering the other taste attributes (Beddows et al., 1980). The aroma of fish sauce is often described using three distinctive notes which are ammoniacal, cheesy and meaty (Beddows, 1998; Peralta et al., 1996; Steinkraus, 2004). The distinctive aromas of fish sauce are derived from protein hydrolyse and lipid oxidation products generated by either autolysis, microbial activity or endogenous enzymes (Fukami et al., 2002).

The ammoniacal note, often associated with fishy odour is produced by ammonia, volatile amines and other basic nitrogenous compounds (Beddows, 1998; Peralta et al., 1996). Some proteolytic enzymes can hydrolyse polypeptides into volatile amines such as ammonia dimethylamine (DMA) and trimethylamine (TMA) (Beddows, 1998). Total base volatile nitrogen (TVB-N) is used to represents these volatile amines and other basic nitrogenous compounds in marine fish (Etienne and Nantes, 2005).

Ammonia is formed by deamination of proteins, peptides and amino acids by bacteria and can also be produced by autolytic breakdown of adenosine monophosphate (AMP), a nucleotide used as a monomer in RNA (Etienne and Nantes, 2005). Fish muscle contains the non-protein-nitrogen (NPN) fraction including free amino acids and nucleotides which serve as a readily available bacterial growth substrate (Gram and Huss, 1996). Trimethylamine (TMAO) is part of the NPN fraction and is often found in marine fish (Gram and Huss, 1996). The TMAO is readily reduced to TMA, a volatile amine that has a pungent, strong fishy ammonia-like odour (Boraphech and Thiravetyan, 2015). In addition, DMA is also produced from TMAO found in fish muscle and is associated with a fish-like odour (Gill and Paulson, 1982; Gram and Huss, 1996). The reduction of TMAO to DMA can be catalyzed enzymatically by TMAO-ase found in the viscera and red muscle of fish (Gill and Pauloson, 1982).

The cheesy note is produced by low molecular weight volatile fatty acids (VFA) that are produced by microorganisms that use amino acids as substrates (Lopetcharat et al., 2001).

Microorganisms that are responsible for the production of VFA are *Bacillus* and *Staphylococcus* where the latter microorganism was found to produce twice as much of the VFA than that of the former (Lopetcharat et al., 2001). The VFA contributing to the cheesy note in fish sauce is also generated from lipid degradation during fermentation (Beddows, 1998; Peralta et al., 1996; Steinkraus, 2004).

Many studies have been conducted to determine the volatile fatty acids in fish sauces produced in different countries (Beddows, Ardeshir and Daud, 1980; Cha, 1994; Peralta et al., 1996; Sanceda et al., 1990). The VFA that are associated with the cheesy note in fish sauce include acetic acid, butanoic acid, pentanoic acids, propanoic acid, 3-methylbutanoic acid, benzoic acid, 2-methylpropanoic acid (Beddows, 1998; Peralta et al., 1996). The amount of total VFA increases as the fermentation time increases and reaches a maximum at approximately 9 months of fermentation (Saisithi, 1994). In addition to VFA, ketones such as *n*-pentan-2-one, iso-butan-2-one and 2-ethyl-butan-2-one are also responsible for the cheesy note in fish sauce (Beddows, 1998; Peralta et al., 1996).

The formation of the meaty note is more complex that the other two notes (Lopetcharat et al., 2001). To date, the meaty note is not attributable to a particular group of compounds but is associated with a combination of several volatiles (Beddows, 1998; Peralta et al., 1996). One of the earliest studies on VFA development in fish sauce suggested that the meaty note was produced by the oxidation of a substance that can be extracted entirely from fish sauce with isopropanol (Dougan and Howard, 1975). In a study on the sensory attributes of a Thai fish sauce, it was found that a combination of 2-methylpropanal, 2-methylbutanal and 2-ethylpyridine contributed to meaty odour in the sample studied (Lapsongphon et al., 2015; Dougan and Howard, 1975). Nitrogen-containing compounds such as pyrazines, pyridines, pyrimidines, amines and nitrile have a burnt or amine-like aroma and together when combined with aldehydes, they produce meaty notes in the fish sauce (Lopetcharat et al., 2001).

Volatile flavour compounds can be divided into acidic, basic and neutral fractions using a series of extraction and distillation processes using chemical solutions such as 10% sodium bicarbonate solution, hydrochloric (HCl) solution and ethyl ether (Min et al., 1979). Three lactones in the neutral fraction including γ-butyrolactone, γ-caprolactone, and 4-hydroxyvaleric acid lactone extracted from *nampla* were associated with the meaty note in that particular fish sauce (Dougan and Howard, 1975).

In addition, short-chain fatty acids (SCFA) including acetic acid, propionic acid and butanoic acid play important roles in immunity by regulating the activities of innate immune cells such as T and B cells (types of white blood cells that play vital roles in the adaptive immune response in the body) (Yao et al., 2022). The SCFA also promotes fatty acid oxidation in liver and muscular tissues by promoting the activation of adenosine monophosphate-activated protein kinase (AMPK) (Campos-Perez and Martinez-Lopez, 2021). Acetic acid ingestion in particular is associated with the reduction in lipid accumulation in adipose tissue, reduced hepatic adiposity and improved glucose tolerance (Coven, 2024). A study by Yamashita et al. (2007) suggested that acetic acid in the form of acetate was responsible for modulating insulin secretion from pancreas and regulating hunger and satiety by encouraging the release of hormones peptide YY and glucagon-like peptide-1.

2.6.2.3 Microbiological properties of Fish Sauce

Microorganisms play an important role in enhancing the degradation of fish proteins and the development of unique flavour and aroma in the production of fish sauces (Lee et al., 2015; Salampessy et al., 2010). The microflora during fish sauce fermentation can be divided into two categories which are proteolytic enzyme-producing bacteria and flavour- and aroma-producing bacteria (Salampessy et al., 2010). Enzyme-producing bacteria include *Bacillus sp., Pseudomonas sp., Halococcus sp., Halobacterium salinarium, Micrococcus sp., Staphylococcus sp. and H. cutirubrum* (Thongthai et al., 1992). Meanwhile, *Staphylococcus* strain 109 and *Bacillus spp.* are bacteria that are involved in the development of flavour and aroma in the fish sauce (Saisithi et al., 1966).

The microflora in the fish sauce mixture change during fermentation (Salampessy et al., 2010). To date, many studies have been conducted to investigate the microbial dynamics and diversity that take place during the fermentation process in different types of fish sauces (Majumdar and Basu, 2010; Gram et al., 1999; Lee et a., 2015; Lopetcharat et al., 2001; Steinkraus, 2004).

The microflora diversity differs between the different types of fish sauce (Salampessy et al., 2010). During the fermentation of *bakasang,* a fish sauce from Indonesia, the number of microorganisms increased rapidly during the first 10 days during which there was a concomitant decrease in pH of the mixture (Ijong and Ohta, 1996). The decrease of pH was associated with the production of lactic acid by lactic acid bacteria (LAB), which are abundant during the early stages of the fermentation (Beddows, 1998; Ijong and Ohta, 1996). The number of the microorganisms then dropped after 20 days of fermentation with *Lactobacillus sp., Streptococcus sp., and Pediococcus sp.* being the predominant microorganisms (Ijong and Ohta, 1996). This was because, salting killed the spoilage bacteria present on the fish leaving only the halophilic and halotolerant bacteria to slowly grow in the high salinity medium (Saisithi, 1994). In *bagoong*, a fermented fish paste in the Philippines, bacteria such as *Bacillus sp., Micrococcus sp.,* and *Moraxella sp.* were found to be predominant in the fermented product (Beddows, 1985). It was determined that LAB and *Bacillus sp.* were the predominant bacteria in *hentak* and *tungtap,* fermented fish pastes from India (Beddows, 1985). The presence of *Bacillus sp.* in the LAB-dominant environment was due to their ability to produce endospores to survive under the prevailing conditions (Beddows, 1985). Natural fermentation of fish sauce leads to the growth of numerous halophilic and halotolerant microorganisms due to the high salinity environment (Lee et al., 2015). Due to the high salt concentration in fish sauce, most microflora found are halophilic or halotolerant bacteria (Steinkraus, 2004). Halotolerant bacteria including *Clostridium spp., Micrococci, and Staphylococci* do not require salt for growth but can grow in the presence of up to 10% or more salt (Kushner, 1968).

Halophilic bacteria can be divided into two groups which are moderate halophilic and extremely halophilic (Kushner, 1968). Moderate halophilic bacteria such as *Achromobacter, Pediococcus, Pseudomonas, Micrococcus, Vibrio, Coryneform, and Bacillus* require 3-25% salt for growth while extremely halophilic bacteria such as *Halobacterium* and *Halococcus* can only grow in the presence of 2.5 M or higher salt content (Steinkraus, 2004).

Eleven microorganisms isolated during the fermentation of *aekjeot,* a Korean fermented anchovy sauce were all either aerobic or anaerobic halophiles (Lopetcharat et al., 2001). During the early stages of the fermentation, when the bacterial population is not yet established, it is claimed that the endogenous enzymes from the fish guts are mainly responsible for the protein hydrolysis that takes place (Le et al., 2015). In *saeu-jeot,* a Korean fermented shrimp sauce, the dominant genera including *Pseudoalteromonas, Photobacterium, Enterovibrio, Aliivibrio, Vibrio,* and *Psychrobacter* that belong to the phylum Proteobacteria, were initially the predominant species but were rapidly replaced by *Staphylococcus, Salimicrobium,* and *Alkalibacillus*, genera belonging to *Firmicutes,* as the fermentation progressed (Jung et al., 2013). After 49 days of fermentation, *Halanaerobium* appeared and was predominantly the main species present after 66 days of *saeu-jeot* fermentation (Jung et al., 2013).

A similar pattern was found in the fermentation of *myeolchi-aekjeot,* another fermented fish product from Korea, where *Proteobacteria* was initially the predominant species but was rapidly replaced by *Firmicutes* as the fermentation progressed (Lee et al., 2015). The identification of the bacterial population of the *myeolchi-aekjot* showed that *Salinivibrio, Staphylococcus,* and *Tetragenococcus* or *Halanaerobium* appeared in a subsequent pattern throughout the fermentation process (Lee et al., 2015). The *Halanaerobium* are responsible for the production of acetate, butyrate and biogenic amines through the fermentation of monosaccharides, amino acids and glycerol thus, indicating that the bacterium may potentially be an indicator of putrefaction or over-fermentation (Brown et al., 2011; Jung et al., 2013). A *Halanaerobium* species, *Halanaerobium hydrogenifirmans*, have an ornithine decarboxylase gene that produces putrescine (Lee et al., 2015). The bacterial growth during fermentation affects the production of metabolites and amino acids in fermented fish sauce (Lee et al., 2015). The rapidly increasing concentration of amino acids despite the low prevalence and minimal growth of bacteria during the early stages of the fermentation process of *myeolchi-aekjot* indicated that endogenous enzymes were responsible for the protein hydrolysis during this fermentation period (Lee et al., 2015).

There are still contradictory findings about the role of specific bacteria responsible for the fermentation of fish sauce despite many organisms having been isolated, identified and investigated (Steinkraus, 2004). Isolating specific bacteria responsible for fish sauce fermentation is a continuous study as the diversity and abundancy of microflora present during the process are affected by various factors including type of raw material used, salt concentration, the type of carbohydrate and sugar added and the fermentation stage (Steinkraus, 2004).

2.7 Analytical Methods for Analysing the Fish Sauce Quality

Analytical methods are employed to provide qualitative and quantitative analyses of the fish sauce samples during and after fermentation processes.

2.7.1 Gas Chromatography (GC) Analysis for Volatile Compounds

Gas chromatography is widely used in applications involving food analysis typically to obtain the quantitative and/or qualitative analysis of food composition, flavour and aroma compounds, and contaminants such as pesticides, environmental pollutants and natural toxins (Mohamed et a., 2012). Gas chromatography is typically used for analyzing non-polar and semi-polar, volatile and semi-volatiles chemical compounds (Mohamed et a., 2012). The mechanism of GC is based on the principle of differential partitioning between a mobile phase and a stationary liquid or solid phase which is typically coated on a column (Agilent Technologies Inc, 2020). A liquid sample is introduced to the GC using a micro syringe by the injecting sample through a septum and into a heated sample port set a temperature of at least 50 °C above the boiling point of the least volatile compound that may be present in the sample of interest (Hussain and Maqbool, 2014). The sample will be then carried to the capillary column by the mobile phase, commonly helium, where the different components of the sample are separated (Hussain and Maqbool, 2014).

The compounds are then detected by detectors such as flame ionization detectors (FID), thermal conductivity detectors (TCD), electron capture detectors (ECD), and mass spectrometers (MS) which is chosen depending on the specific requirements of the analysis (Hussain and Maqbool, 2014).

There are several factors that affect the optimization of GC methods including oven temperature, extraction time, type of capillary columns and sampling method (Hussain and Maqbool, 2014). Different conditions for GC analysis of volatile compounds are summarized in Table 4.

Table 4: Summary of the different conditions for Direct Injection Gas Chromatography Mass Spectrometer (GCMS) analysis of volatile compounds in fermented fish products.

Instrument	Oven temperature	Capillary Column	Sample	Volatile compounds detected	References
A 6890 GC (MSD; Agilent Technologies, Inc.) system. Each aroma extract (1 µL) was injected into a cooled injection system. Cold splitless-mode.	For cooled injection system: −50 °C initial temperature (0.10 min hold); ramp rate 12 °C/s; 260 °C final temperature (10 min hold); 1.10 min valve-delay, the 50 mL/min vent flow. For GC oven temperature: 35 to 225 °C at a rate of 4 °C/min with initial and final holding times of 5 and 20 min, respectively. Helium as carrier gas (constant rate of 1.0 ml/min)	Stabilwax (30 m × 0.25 mm i.d.; 0.25 µm film; Restek) and Sac-5 columns (30 m × 0.25 mm i.d.; 0.25 µm film; Supleco, Bellefonte, PA).	Two commercial Thai fish sauce: Fish sauce A (Rayong Fish Sauce Industry Co., Ltd., Rayong, Thailand) and fish sauce B (Marine Resources and Development Co., Ltd., Chantaburi, Thailand).	Volatile short-chain fatty acids and volatile amines	Lapsongphon et al. (2015)
Hitachi G-3900 model gas chromatograph equipped with a flame ionization detector (FID). The GC was connected to a thermal desorption cold trap injector CP4020 TCT (GL Sciences).	The oven temperature was programmed from 50 to 230°C at 3 °C/min. The injector and detector temperatures were set at 200 and 250 °C, respectively. The flow rate of helium as a carrier gas was 0.7 mL/ min.	A fused silica capillary column (60 m × 0.25 mm i.d.) coated with cross-linked polyethylene glycol (20M) at a film thickness of 0.25 µm (TC-Wax; GL Sciences).	*Nampla* (Thai fish sauce) from the Thai Fish Sauce Factory Co., Ltd.	Volatile short-chain fatty acids and volatile amines	Fukami et al. (2002)
A Hewlett Packard 5790 Model gas chromatograph.	The column temperature was programmed from 60 to 170°C at the rate of 4°C/min, the injection port at 180°C and the detector (FID) at 230°C. The carrier gas was helium with a flow rate of about 1.0 ml/min and a split ratio of 1: 28.	A fused silica column (0.25 mm i.d. x 50 m) coated with Carbowax 20M	*Shottsuru* (Japanese fish sauce), n*ampla* (Thai fish sauce) and *noucmam* (Vietnamese fish sauce).	Acids, alcohols, nitrogen containing compounds, sulfur containing compound, phenols, carbonyls, easters	Sanceda et al (1985)

Instrument	Oven temperature	Capillary Column	Sample	Volatile compounds detected	References
An Agilent Technologies 6890N with Agilent 5975 inert XL	The column was kept at 35 °C for 4 min, heated from 35 to l00 °C at a programmed rate of 30 °C/min and held at l00 °C for 2 min, and then heated from 100 to 300 °C at a rate of 15 °C/min and held at 300 °C for 10 min. The temperatures for the injection port, ion source, quadrupole, and interface were set at 250, 230, 150, 280 °C, respectively. Helium with a flow rate of 0.5 ml/min was used as carrier gas, and split ratio was 2:1.	A fused silica capillary column, HP-5MS flexible glass capillary gas chromatography column (30 m×0.25 mm×0.25 μm, Agilent Technologies Co., Ltd., Palo Alto, USA).	*Fuzhou Yulu* (chinese fish sauce)	Volatile short-chain fatty acids	Yang et al. (2008)
A Hewlett Packard 5890 Series II gas chromatograph	The oven temperature was programmed from 50 to 230 °C at 2 °C/min. The injection port and detector temperatures were 220 and 250 °C, respectively. The helium carrier gas flow rate was 22 cm/s, with a split ratio of 1:25.	A fused silica capillary column (60 m × 0.25 with a film thickness of 0.25 μm (DB-Wax; J&W Scientific, Folsom, CA).	Taiwanese fish sauce	Volatile compounds	Peralta et al. (1996)

2.7.2 Metagenomics Sequencing for Bacteria Identification and Quantitation

In traditional production of fish sauce, the food product undergoes natural fermentation without the use of starter cultures or sterilisation thus leading to the growth of various microorganisms (Saisithi, 1994). Bacteria involved in the fermentation of fish sauce have been actively studied as discussed in Section 2.6.3. Taxonomic studies of bacteria isolated from fish sauce have shown that *Bacillus sp., Lactobacillus sp., Pseudomonas sp., Halococcus sp., Halobacterium salinarium, Micrococcus sp., Staphylococcus sp. and H. cutirubrum* are some of the microbes responsible for the fermentation of the fish product (Beddows, 1998; Ijong and Ohta, 1996; Thongthai et al., 1992). However, culture-based approaches are limited to the reproducibility and culturability of some bacteria (Jung et al., 2011). It is estimated that less than 2% of bacteria in the environment can be cultured in the laboratory (Jung et al., 2011). Hence, the set cultured isolated bacteria may not be representative of the true composition of fermented fish sauce (Beddows, 1998).

Metagenomics is a rapidly evolving discipline that allows the study of microbial genomic identity in their natural environmental settings (Tshikhudo et al., 2013). One of the main benefits of metagenomic approach is the ability to provide a comprehensive microbial profile, including both culturable and unculturable species, which are often overlooked in conventional culture-based methods (Pinto & Raskin, 2012). The metagenomic approach is widely used in food industry particularly for fermented products such as beers, wine, cured meat, fermented dairy products (cheese, yogurt) and fermented fish products (fish sauce, fish paste, Japanese dried bonito – *katsuobushi)* (Chanprasartsuk et al., 2013; Jung et al., 2011; Lestari et al., 2024; Ohwofasa et al., 2024; Ohwofasa et al., 2025; Srinivas et al., 2022). Beyond microbial profiling, metagenomic approaches are used in quality control and standardization, where metagenomics helps define microbial community signatures associated with high-quality fermentation leading to quality control and process standardization (Wang et al., 2022).

In addition, the metagenomics approach allows for the rapid identification of pathogenic bacteria thus enabling proactive interventions to prevent defects and ensure food safety in the final product (Billington et al., 2022).

Metagenomics can provide genetic information on genomic linkage between function and phylogeny of microbial communities, potential novel biocatalysts or enzymes and evolutionary profiles of community function and structure (Jung et al., 2011; Thomas et al., 2012; Tshikhudo et al., 2013). Generally, in a metagenomics study, nucleic acids are extracted from a sample collected from an environment of interest and are directly sequenced (Bowers et al., 2017). The sample that is suitable for metagenomic analysis is limited by the quality and quantity of DNA that can be extracted from it (Bowers et al., 2017). Various environments pose different challenges regarding the collection of samples before resolving for appropriate DNA extraction techniques (Bowers et al., 2017). The DNA extraction approach for the analysis of community composition from environmental DNA differs depending on the media from which the DNA is extracted and the organism of interest (Lear et al., 2018). The extraction method employed should also be effective for diverse microbial taxa to prevent the sequencing results from being dominated by DNA derived only from easy-to-lyse microbes (Quince et al., 2017). DNA extraction methods that include mechanical lysis (also called beads beating) are often considered more superior compared to methods that rely on just chemical lysis (Quince et al., 2017). However, vigorous extraction method using beads beating can result in shortened DNA fragments. Table 5 summarises DNA extraction methods that were used in the metagenomics studies of varying environments. Table 5 shows that different DNA extraction methods are employed depending on the type of environmental samples and targeted microorganisms to be identified in downstream applications.

Table 5: Summary of the DNA extraction kits for varying samples. The prices were determined in February 2025.

Name	Sample	Application	Price	Reference
DNeasy PowerSoil kit (Qiagen)	Extract DNA directly from soil or sediment material The kit uses both mechanical (beads beater) and chemical lysis to ensure maximum cell lysing. Available in a 96 well extraction format.	Commonly used in studies targeting DNA of both prokaryotic and eukaryotic microorganisms. Provides high DNA yield (from 0.25 g samples), superior microbial diversity, uses Inhibitor Removal Technology (IRT) that efficiently removes PCR inhibitors.	$510/50 isolations	DNeasy PowerSoil Pro Kit Handbook (2017); Lear et al. (2018)
DNeasy Blood and Tissue kits (Qiagen)	Extract DNA from freshwater and marine samples.	Commonly used when animal (such as fish and amphibians) or plant derived DNA is the main target for extraction.	Not listed	Lear et al. (2018); Thomas et al. (2014);
DNeasy PowerMax kit (Qiagen)	Extract DNA directly from soil or water.	Used for isolation of microbial DNA from large quantities (50ml) of soil. Commonly used in studies targeting DNA of both prokaryotic and eukaryotic microorganisms.	$ 402/10 isolations	DNeasy PowerMax Soil Kit Handbook (2017); Lear et al. (2018)
DNeasy PowerWater Kit (Qiagen)	Target DNA from fish, amphibians and aquatic reptile. Use filter to free sample from salts, metals, humic substances and other organic materials.	Commonly used in freshwater studies. Also used for extraction of DNA from microorganisms in water and ice.	$1432/100 isolations	DNeasy PowerWater Kit Handbook (2017); Lear et al. (2018)

DNeasy PowerFood Microbial kit (Qiagen)	Extract DNA from food samples. Efficient removal of PCR inhibitors including lipids and polysaccharides	Commonly used to isolate variety of food pathogens. Simplified methods for DNA extraction from a variety of food types, pathogens, yeasts and fungi.	$509/100 isolations	DNeasy PowerFood Microbial Kit Handbook
Wizard Genomic DNA Purification Kit (Promega)	Extract DNA from various type of samples.	Provides wide applications for DNA extraction from white blood cells, tissue culture cells, animal tissue, plant tissue, yeast and Gram-positive and Gram-negative bacteria.	$433/100 isolations	Wizard Genomic DNA Purfication Kit Handbook

The extracted DNA samples are then sequenced. The two most common approaches for downstream DNA sequencing are shotgun metagenomics sequencing and targeted metabarcoding sequencing (Bowers et al., 2017). The Shotgun metagenomics approach sequences the entire DNA sample, often including many members of a population, directly without a prior amplification step. This approach allows the recovery of potentially the whole microbial population contained in the target environment (De Filippis et al., 2017). Shotgun metagenomic approaches can also track and compare the abundance between bacteria and other organisms such as fungi and viruses at the same time providing a more comprehensive understanding of the community composition of the subject environment (De Filippis et al., 2017). The bioinformatics analysis of shotgun data however is more complex and computationally intensive compared to the metabarcoding sequencing (De Filippis et al., 2017). Particularly, the applications of shotgun metagenome in the studies of food fermentation is limited to only a few pioneer studies which are mainly focused on cheeses and Korean fermented cabbage (kimchi) (Jung et al., 2011).

Unlike the shotgun metagenome approach, metabarcode sequencing requires a polymerase chain reaction (PCR) step using primers to amplify a single or a few copies of a target piece of DNA prior to sequencing (Lear et al., 2018). Primers are short single-stranded DNA and are required to initiate a PCR amplification of a region of DNA (Lear et al., 2018). The choice of primers determines the DNA region that will be amplified (Abdulamir et al. 2010). In various metagenomics studies on prokaryotes, the 16S rRNA gene is the most commonly amplified region. 16S rRNA amplification can be achieved by using universal primers to investigate the phylogenetic information of bacteria contained in an environment (Barghouthi, 2011; Peng et al., 2002). Universal primers are primers that allow the detection of a large number of bacteria simultaneously (Barghouthi, 2011).

The use of 16S rRNA genes as phylogenetic marker eliminates the need for in vitro culture which in turns provide a wide information on the naturally occurring microbial communities in various habitats (Jung et al., 2011). Ribosomes act as protein synthesis machinery in all living cells (Tshikhudo et al., 2013). The ribosome consists of two subunits which are 30S subunit (small subunit) and 50S subunit (large subunit), each is composed of protein and ribosomal RNA (rRNA) (Kolbert and Persing, 1999). Usually in a prokaryotic ribosome, the 50S subunit is composed of two rRNA species which are 5S rRNA and 23S rRNA while the 30S subunit is composed of 16S rRNA and 21 polynucleotide chains (Kolbert and Persing, 1999; Sharpton, 2014). The rRNA genes are omnipresent making them suitable tools for bacterial species identification and taxonomic classification (Tshikhudo et al., 2013). In prokaryotes, there are generally three to four copies of rRNA genes in a single genome (Tshikhudo et al., 2013). The most commonly used rRNA gene in molecular techniques for bacterial identification and classification is the 16S rRNA gene because it is present in almost all bacteria, it has shown functional consistency over time and its length of approximately 1500 base pair (bp) is considered sufficient for bioinformatics analysis (Janda and Abbott, 2007; Tshikhudo et al., 2013). The 16S rRNA gene provides genus- or species-specific identification due to the presence of highly conserved nucleotide sequences, interspersed with hypervariable regions that are an indication of divergence over evolutionary time (Tshikhudo et al., 2013). There are nine hypervariable regions (V1- V9) in 16S rRNA genes that represent sequence diversity in different bacteria species (Chakravorty et al., 2007).

Table 6 summarises some of the universal primers that are commonly used in PCR targeting 16S rRNA genes. Specific primer set is often used when identification of specific bacteria is needed unlike universal primers that are more commonly used when a more diverse identification of bacteria communities contained in a particular sample is required (Barghouthi, 2011). Specific primers were proved to be highly sensitive in the identification and quantitation of specific bacteria (Barghouthi, 2011).

In a study on the efficiency and versatility of various primers for 16S rRNA gene amplification, primers pMyc14 bio (a forward primer: GRGRTACTCGAGTGGCGAAC) and pMyc7 (a reverse primer: GGCCGGCTACCCGTCGTC) were proved to be highly sensitive in the 16S rRNA amplification particularly for the identification and quantitation of Mycobacteria (Kox et al., 1995). The study found that the respective primer set allowed a rapid and accurate identification of mycobacteria in clinical samples from patients suspected from having mycobacterial disease (Kox et al., 1995).

Table 6: Summary of universal primers used in PCR amplification of 16S rRNA genes.

Primers Code (forward; reverse)	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**	**PCR product size**	**References**
8F; 907R	GGATCC AGACTTTGATYMTGGCTCAG	CCGTC AATTCMTTTGAGTTT	0.9 kbp	Ben-Dov et al. (2006)
CF319a-F; EubA	TGGTCCGTGTCTCAGTAC	AAGGAGGTGATCCANCCRCA	N/A	Kirchmen et al. (2003)
R1n; U2	GCTCAGATTGAACGCTGGCG	ACATTTCACAACACGAGCTG	1.0 – 1.1 kbp	Weidner and Arnold (1996)
FD1; 1540R	AGAGTTTGATCCTGGCTCAG	GGAGGTG WTCCARCCGC	0.6-07 kbp	Jiang et al. (2006)
Bac27F; Univ1492R	AGAGTTTGGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	0.6-07 kbp	Jiang et al. (2006)
340F; 758R	CCTAC GGGAGGCAGCAG	CTACCAGGGTATCTAATCC	0.7 kbp	Jung et al. (2011)
Bac27F; Bac541R	5'-adaptor B-AC- GAG TTT GAT CMT GGC TCA G-3'[1]	5'-adaptor A-X-AC- WTT ACC GCG GCT GCT GG-3'[1]	0.8kbp	Lee et al. (2014)

[1]Adaptor A and B are specific nucleotide sequences used in the primer sets used in this particular study. The X-AC denotes the unique barcode sequence inserted between the adaptor used in this study. The list of adaptor and barcode sequences can be retrieved from the cited reference (Lee, Jung and Jeon, 2014).

Even though many primers (some were as summarised in Table 7) are labelled as universal primers, however, their applications do not cover all bacteria especially the non-target bacterial species (Barghouthi, 2011). Thus, the major limitations concerning the application of metabarcoding sequencing based on 16S rRNA gene are PCR primer and enzyme biases which would impact the results of the study (Abellan-Schneyder et al., 2021).

Primer and enzyme biases could limit the sequence reads of different variations of the 16S rRNA genes thus affecting the identification of certain bacteria (Abellan-Schneyder et al., 2021). To overcome these limitations, primers can be designed specifically to the targeted microorganism (Peng et la., 2002). A combination between universal primer and specific primer were also employed depending on the target microorganisms (Jiang et al., 2006; Brandon-Mong et al., 2015). The selection of primers can be made based on the high coverage rate, the size of fragments based on the subsequent sequencing approach and the absence of single mismatches in the four nucleotides close to the 3' end of the primer to prevent biases (Abellan-Schneyder et al., 2021). Besides, the PCR products may also be purified prior to downstream application using a PCR purification kit (Kox et al., 1995). The PCR purification kit is employed to facilitates the removal of enzymes, nucleotides, primers and buffer components that may interfere during the downstream application (Lee et al., 2015). Despite the continuous studies attempting to determine the most suitable primers for the amplification of the genomic region of target bacteria, unfortunately this does not deny that most methods are still limited by primers biases to completely be claimed as universal primers.

Based from the literature findings, the selection of primer set for this study can be made based on:

1. Primers that can provide a large size of PCR product. Larger PCR product provides a longer sequence read when subjected to downstream application, consequently providing more information for the identification of bacteria communities contained in the fish sauce samples.
2. Primers that are used for the identification of bacteria commonly found in fermented food products (as discussed in Section 2.6.3, this includes, at genus level, *Proteobacteria, Firmicutes, Photobacterium, Vibrio, Phychrobacter* and *Halanaerobium*). According to literature, some of the primer sets that may be suitable for PCR amplification of DNA samples from fermented food products are (forward primer; reverse primer) 340F; 758R, and Bac27F; Bac541R (Table 6).

Further investigation is to be conducted to determine the suitability of these respected primer set for this study. In the case of either of the approaches described above, metagenomic shotgun sequencing or metagenomic barcoding, a next generation sequencing (NGS) technology will be used downstream. Over the years, the development of NGS technologies such as 454 pyrosequencing, Illumina sequencing and SOLiD (Sequencing by Oligo Ligation Detection) has led to the larger scale metagenomics sequencing of environmental communities such as soil, water and human gut (Jovel et al., 2016; Lear et al., 2018; Suzuki et al., 2011). The most significant feature of NGS is that it provides millions of short sequence reads varying between 30 base pairs (bp) to 50 Gbp depending on the type of analysis employed (Suzuki et al., 2011). The short sequence reads are then assembled to extract the sequence features of the DNA samples that will be used to identify and quantify target microbial communities in the selected samples (Suzuki et al., 2011). Table 7 summarises several sequencing platforms commonly used in studies concerning DNA sequencing.

Table 7: Summary of different sequencing platforms

Sequencing platforms	**454 pyrosequencing**	**Illumina MiSeq**	**SOLiD**	**Ion Torrent**
Data output/run	0.7 GB	120 MB – 1.5 GB	120 GB	10 MB – 100 MB
Read length	700 bp	150 bp	50 bp	400 – 600 bp
Time/run	24 hours	3 hours	7 days	2 hours
Accuracy	99.9%	99%	99.94%	98%
Reference	Lear et al. (2018)	Liu et al. (2012)	Huang et al. (2012)	Rothberg et al. (2011)

The performance of sequencing platforms is generally assessed based on the read length, accuracy, applications, costs and informatics infrastructure (Liu et al., 2012). The pipelines that are commonly used to analyse sequencing data include RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/), BLAST, NGS QC Toolkit and IlluQC.pl (Jiang et al, 2019; Jung et al., 2011; Lee et al., 2015; Mengoni et al., 2015).

There are several aspects to note when choosing the suitable metagenomics approach for the bacterial identification and quantification. For example, metabarcode sequencing may be suitable when the nature of the study is mainly focusing on the bacterial taxonomy contained in the sample (Jovel et al., 2016). The cost for metabarcode sequencing is also less expensive compared to the shotgun sequencing thus it is more preferable when large number of samples are involved (Jovel et al., 2016). However, it is also important to note that there may be primer biases in the results obtained from the metabarcode sequencing approach due to the use of primers during the PCR amplification prior to downstream application (Jovel et al., 2016).

In comparison, the shotgun sequencing is usually more expensive but can provide a more comprehensive understanding on the microbial content in a sample including the bioinformatic information of bacteria, viruses and fungi (Jovel et al., 2016).

In this study, it was decided that the metabarcoding approach is more suitable as the current interest is the diversity of bacteria communities in the fish sauce sample during the fermentation period. It is believed that the knowledge gathered in this study could provide a stepping stone for further research on the fish sauce produced from New Zealand raw materials.

2.8 Research gap

Fish sauce production is a major industry in many Southeast Asia countries including Thailand, Vietnam, Indonesia and Malaysia (Beddows, 1998; Lopetcharat and Park, 2002; Xu et al., 2018). In these tropical countries, the high average temperature (between 21 to 30 °C) and humidity (relative humidity of approximately 70 %) play important roles in the fermentation process of fish sauce that affect the chemical, biochemical and microbiological aspects of the fish product (Lopetcharat et al., 2001).

The majority of the current scientific studies covering different types of fish sauce produced in the tropical countries, use local raw materials as main ingredients (Beddows, 1998; Salampessy et al., 2010; Lopetcharat et al., 2001; Ijong and Ohta, 1994; Steinkraus, 2011). In New Zealand, despite abundant availability of the required raw material, fish sauce manufacture is still absent, partly due to a lack of knowledge as to how well Southern Ocean fish will ferment with New Zealand solar salt under temperate conditions. This research will explore fish sauce fermentation technology using New Zealand's raw materials as main ingredients under temperate condition with a final aim of producing Thai-style fish sauce.

Based on literature findings, it was found that fish sauce made from fish by-products including heads, skins, trimmings and frames are not widely produced. Commonly, fish sauce is made by salting either uneviscerated or eviscerated, cut or whole fish which are then placed in earthen jars to allow fermentation to take place over a duration of 6 to 18 months at temperatures ranging from 25 °C to 30°C (Beddows, 1998; Saisithi, 1994). In 2011, New Zealand generated as much as 59,900 tonnes of fish waste, worth $173.7 million (MPI, 2015). Currently, the fishing industry is exploring new ways to increase seafood sustainability, reducing waste and increase the returns (income on their current catch) by utilizing seafood by-product more profitably. Hence, this research will also determine if New Zealand's fish waste by-products as the raw material can produce a good quality fish sauce.

To date, scientific studies are widely focused on the proteolytic microflora present in the fish viscera due to their abundancy and diversity which are thought to play a major role in the fish sauce fermentation (Avolkar et al., 2009). Current studies are also primarily focused on the dynamics and diversity of planktonic microflora, as the studies were mostly conducted by sampling the fish sauce supernatant.

Therefore, this research will also explore the role of microflora pre-existing on the salt surfaces as the key inoculum source in the fish sauce fermentation and will also investigate the viable microflora on biofilms particulates (bones, tails and scales) in the fish sauce as opposed to planktonic microflora.

The factors affecting the fermentation of a fish sauce include the type of fish and salt used, the ratio of fish and salt and the temperature at which the fermentation takes place. These factors will affect the chemical, biochemical and microbiological properties of the fermented fish product making each fish sauce unique and distinct. This research aims to investigate how these factors will affect the fermentation process when utilising New Zealand's fish waste by-product and New Zealand's sea salt to produce a fish sauce unique to New Zealand.

12.8.1 Research Hypotheses

In this particular study, hypotheses to be tested are:

- Halophilic and halotolerant bacteria pre-existing in the salt are the key inoculum source that will dominate the fish sauce fermentation as opposed to bacteria from the fish.
- Unstoved salt provides more diverse and/or abundant halophiles and/or halotolerant bacteria compared to the stoved salt as the key inoculum source for fish sauce fermentation.
- The concentration of the volatile fatty acids will increase as the fermentation time increases.
- The volatile fatty acids (VFA) found in fish sauce include acetic acid, propanoic acid, butanoic acid and 3-methylbutanoic acid.
- The microbiological dynamic and diversity are affected by the different fermentation conditions of the fish sauce.

2.8.2 Research Objectives

For this study, research objectives are identified and listed to address the hypotheses made, respectively. The research objectives are:

- To characterise the viable microflora pre-existing in both unstoved and stoved salts used in the fermentation.
- To characterise and follow the changes in the microbiological population and diversity in the fish sauce during the fermentation period of fish sauce using different combinations of New Zealand's raw seafood materials and salts at different temperatures.
- To determine the volatile fatty acids produced during the fermentation process as a quantitative assessments of the fish sauce.
- To investigate the influence of the sampling depth on the fermentation process.
- To investigate the influence of the mussel shell on the fermentation process.

Chapter 3: Fermentation Set-Up and General Materials and Methods

3.0 Introduction

This chapter describes the fermentation set-ups and general materials and methods of analysis used in this study.

A preliminary fermentation was conducted to determine and validate the suitable analytical methods used in this study, using 2 litre containers. This was followed by the design of four different fermentation set-ups to investigate different aims:

1. Laboratory scale fermentation to investigate the influence of different variables (including seafood material, type of salt and temperature) on fermentation.
2. Pilot scale fermentation to investigate the influence of sampling depth on fermentation
3. Shell-on mussel fermentation to investigate the influence of mussel shell on mussel fermentation
4. Snapper fermentation with added mussel shell to investigate the influence of mussel shell on snapper fermentation.

3.1 Raw Material for Fermentation

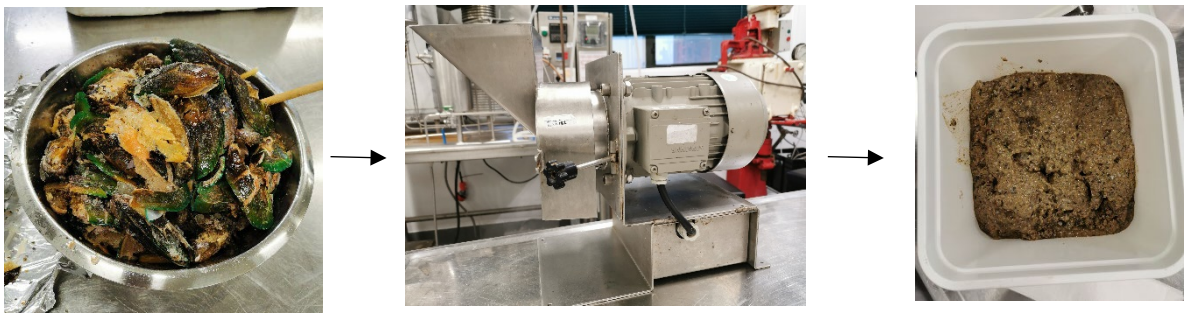
Hoki (*Macruronus novaezelandiae*) heads and frames, snapper (*Pagrus aurtus*) heads and frames and green-shell mussels (*Perna canaliculus)* were obtained as 20 kg frozen blocks from Sanford Ltd., Auckland, New Zealand in August, 2019. All seafood materials were stored at -20 ± 1 °C prior to their preparation for fermentation to minimize bacterial growth and reduce the potential impact of storage on the fermentation process.

The storage methods align with current fishing industrial practice in New Zealand where fish are processed and frozen at sea, then transferred to cold storage upon docking before export or distribution.

Unstoved solar salt (Pacific Grade 11) and stoved solar salt (Pacific Grade 22) were received in 25 kg bags from Dominion Salt Ltd., Mount Maunganui, New Zealand in November, 2019. The salts were stored as delivered in double layered polybags at room temperature (18 ± 5 °C) until used.

3.1.1 Preparation of Seafood Material

All seafood materials were prepared as shown in Figure 6. The frozen blocks of Hoki and snapper heads and frames, and green-shell mussels were thawed at 4 ± 1 °C for 72 hours. After thawing, the seafood materials were washed in tap water twice before they were ground into a paste using a hammer mill (Siemens, Germany). Hoki and snapper heads and frames were chopped into ~5cm^3 piece using bowl chopper (Scharfen, Germany) before ground using the hammer mill. The paste was stored at -20 ± 1°C until used.



(a) (b) (c)

Figure 6: The preparation of green-shell mussels. (a) Green shell mussels were thawed and washed twice under tap water. (b) Cleaned green-shell mussels were ground using a hammer mill (c) Green shell mussels were ground into a paste and stored at -20 ± 1°C .

3.2 Preliminary Studies

Preliminary studies were conducted to select and validate the analytical methods suitable for the qualitative and quantitative assessments of the fish sauce samples.

3.2.1 Preliminary Lab Scale Fermentation Set-up

Two 2-litre fermentation units were set up using snapper heads and frames and two different salts: Marlborough iodised sea-salt flakes (Cerebos, New Zealand) and iodised table salt (Pams, New Zealand). It is to be noted that the preliminary lab scale fermentation set ups were conducted in July, 2019 before the selected unstoved and stoved solar salts were delivered from Dominion Salt Ltd.

The snapper heads and frames paste and salt were placed in a Kilner glass fermentation bottle in alternating layers with a final ratio of 3/7 of fish/salt (Lopetcharat et al., 2021) and left to ferment at 30 ± 1 °C in a controlled temperature incubator (Figure 7). The fish sauce samples were used in preliminary tests to find suitable analytical methods to be employed for this research.

Figure 7: Preliminary laboratory scale fermentation set-up.

3.3 Fish Sauce Fermentation Set-Up

Four different fermentation sets were set-up to investigate the different aims in this study as outlined in Section 3.0. In this study, these terms will be used:

1. Ferment - to describe sample taken from actively fermenting fish sauce. The reason "fish sauce" is not used to describe the samples taken during the fermentation period is because "fish sauce" is commonly referred to the final product (has completed the fermentation process and solid residues are filtered out).
2. Fermentation – to describe a) the process and b) the entire fermentation bottle/barrel consisting of solid and liquid parts.
3. Ferment liquor – to describe the liquid portion of the sample drawn from a fish fermentation.

3.3.1 Laboratory Scale Fermentation

The laboratory scale fermentation was carried out in individual small 20 ml glass bottles (AliExpress, China) which were then incubated at the same temperature. At each sampling time single bottles were removed for analysis. Each type of pre-prepared seafood paste was mixed with two different types of salt in different ratios of seafood to salt, the combined mixture was then ground to a paste using the hammer mill to produce a homogenous mixture.

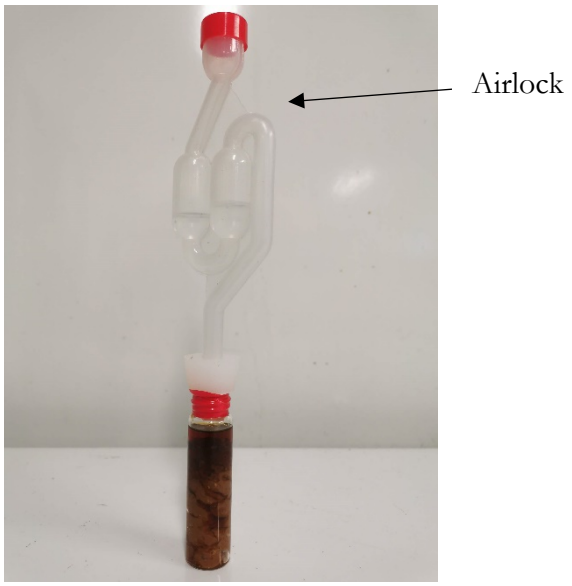


Figure 8: Laboratory scale fermentation set-up using 20 ml glass bottle. The bottle was sealed with a rubber stopper and attached with and airlock.

Approximately 20 g of the seafood and salt mixture was placed into a 20 ml glass bottle. Each glass bottle was sealed with rubber stopper attached with an airlock as shown in Figure 8.

The bottles were then incubated at 18, 25 or 30 ± 1 °C. The different combinations of raw materials, type of salt, fish/salt ratios and incubation temperatures resulted in a total of 18 different fermentation conditions as shown in Table 8.

Table 8: Fermentation sets of different combinations of raw material, type of salt, fish/salt ratio and incubation temperature.

Fermentation Set	Temperature	Type of Solar Salt	Seafood	Seafood/Salt Ratio (w/w)
1	30 ± 1°C	Unstoved	Hoki heads and frames	4/1
2			Snapper heads and frames	3/1
3			Green shell mussels	5/1
4		Stoved	Hoki heads and frames	4/1
5			Snapper heads and frames	3/1
6			Green shell mussels	5/1
7	25 ± 1°C	Unstoved	Hoki heads and frames	4/1
8			Snapper heads and frames	3/1
9			Green shell mussels	5/1
10		Stoved	Hoki heads and frames	4/1
11			Snapper heads and frames	3/1
12			Green shell mussels	5/1
13	18 ± 1°C	Unstoved	Hoki heads and frames	4/1
14			Snapper heads and frames	3/1
15			Green shell mussels	5/1
16		Stoved	Hoki heads and frames	4/1
17			Snapper heads and frames	3/1
18			Green shell mussels	5/1

Each fermentation set had a total number of 27 glass bottles that served as an individual sample volume which were sampled at a various time points during a period of 80 weeks fermentation process. Note that a fixed time frequency for sampling could not be conducted due to the Covid -19 pandemic restrictions.

A total number 486 units of small fermenters were set-up for the laboratory scale fermentations. At each sampling point a single sample bottle was removed from the incubators and stored at -20 ± 1 °C until analysis. The calculations for fish and salt ratios are presented in Appendix 3.

3.3.2 Pilot Scale Fermentation

A pilot scale fermenter set of 30 litres was set up as shown in Figures 9, 10 and 11.

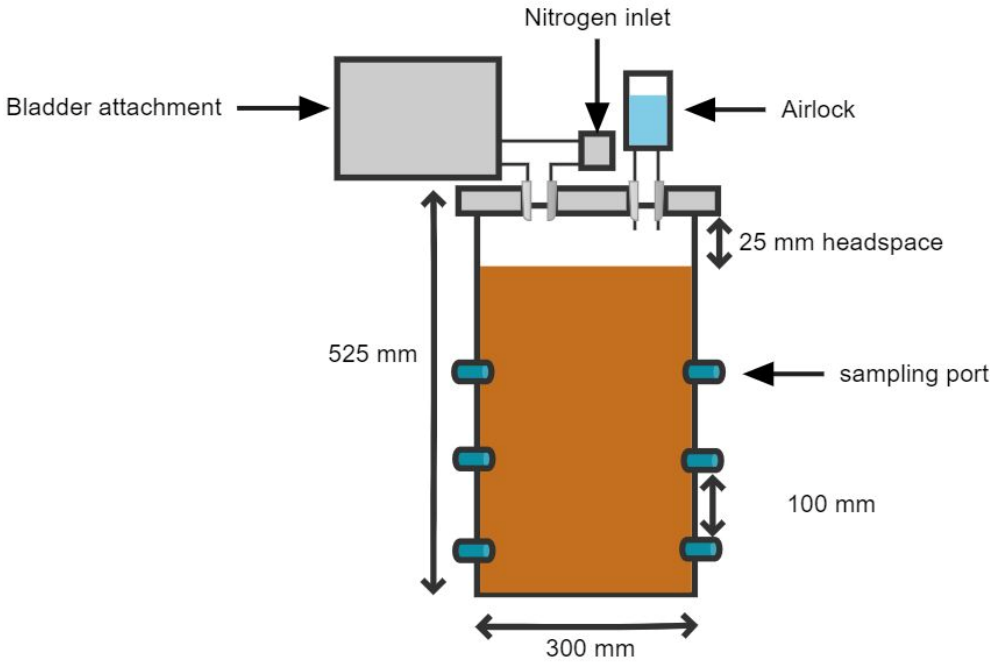
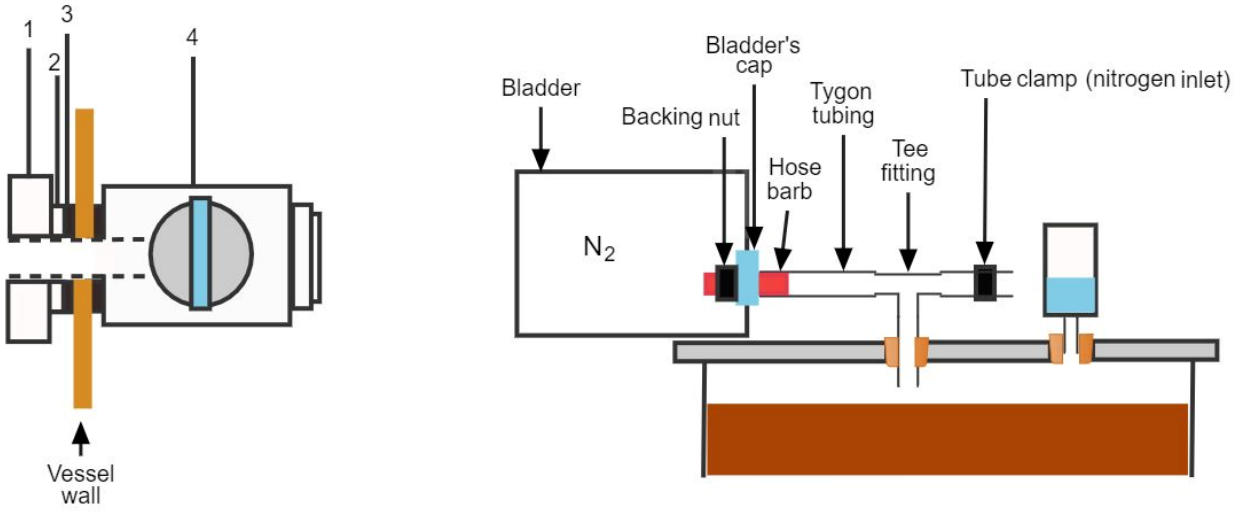


Figure 9: Design sketch of the pilot scale fermenter setup for fish sauce fermentation. The 525 mm x 330 mm fermentation barrel has a bladder attachment filled with nitrogen gas to maintain an anaerobic environment at atmospheric pressure. The sampling ports were positioned at three different heights to allow sampling from different depths of the fermentation unit.

The fermentation barrels with dimensions 525 mm height x 300 mm width made from food grade high density polyethylene (HDPE) had a capacity of 30 L (Beer Shop, Auckland, NZ). Each barrel consisted of six sampling ports positioned at three different heights to provide sampling at three different depths of the fermentation unit. The top sampling ports were positioned at 270 mm below the headspace and the lower ports were vertically positioned at 100 mm below each other. The positions of the sampling ports were determined by estimating the height of the liquid level that would

drop after a total of 12 sampling times were completed to ensure that the fish sauce samples collected were representative of three different depths of the fermentation unit. Detailed calculations can be referred to in Appendix 5.

The fermentation barrel was fitted with a plastic bladder (Beer Shop, Auckland, NZ) filled with nitrogen gas to maintain an anaerobic environment in the headspace, by displacing oxygen gas that may be present. The plastic bladder was made of polyethylene and nylon layers acting as an oxygen barrier from the atmosphere. The barrel was sealed with rubber stopper attached with an airlock.



(a) (b)

Figure 10: A more detailed design sketch of the fermenter set for fish sauce fermentation. (a) shows a sketch of the sampling port attachment drilled into the fermentation barrel, 1 – stainless steel backing nut; 2 – stainless steel washer; 3 – silicone washer; and 4 - John Guest valve. (b) shows a sketch of the nitrogen filled bladder attachment on the fermentation barrel fitted with an airlock.

Table 9 summarizes the fermentation conditions for pilot scale fermentations. The fermentation barrels were placed into an incubated cabinet of 1.9 m length x 0.8 m width x 2.8 m height made from poly panel with the inside temperature set to 30 ± 1 °C as shown in Figure 11 to represent average temperature commonly used in traditional fish sauce fermentation in Southeast Asia.

(a) (b)

Figure 11: (a) Incubation cabinet with a controlled temperature where pilot scale fermentation took place. (b) Pilot scale fermentation set-up using Snapper head and frame with stoved/unstoved solar salt at 30 ± 1 °C .

Table 9: Fermentation conditions of the pilot scale set-up.

Fermentation Barrel	Type of salt	Type of seafood	Temperature	Seafood/Salt Ratio (w/w)
SUS30	Unstoved	Snapper heads and frames	30°C	3/1
SS30	Stoved			

Twenty milliliters of liquid fish sauce sample was collected from one of the two sampling ports at the same height at 3 week intervals over a period of 45 weeks fermentation. The first liquid sample was collected after 3 months of the fermentation period to allow the fish frames and muscles to break down. Prior to sample collection, 10 mL of initial liquid fish sauce was discarded to dispose of the trapped liquid inside the closed valve of the sampling port outlet. The fish sauce samples were stored at -20 ± °C freezer until further analyses.

3.3.3 Fermentation with Mussel Shell

This section describes two different fermentation set-ups: shell-on mussel fermentation and snapper fermentation with added mussel shell. Frozen mussels were thawed at 4 ± 1 °C for 72 hours and then washed twice with tap water. Mussels were deshelled before the meat and shell were ground separately using a hammer mill (Siemens, Germany). snapper paste was prepared as described in Section 3.1.1. The seafood material, shell and salt were thoroughly mixed using different ratios of meat/shell as presented in Table 10. Unstoved salt was added into the different meat/shell mixtures based on salt/water ratio determined as shown in Appendix 4.

Unstoved salt was chosen because it was hypothesized that the salt has existing microbial isolates that may influence/assist the fermentation process (as described in Section 2.8.1).

Table 10: Mussel and snapper fermentation with different meat/shell ratios using unstoved salt at 30 ± 1 °C for 40 weeks.

Fermentation set-up	Meat or paste content	Shell content	Ratio
Shell-on mussel	1	0	1M/0S
	1	0.5	1M/.5S
	1	0.75	1M/.75S
	1	1	1M/1S
Snapper with mussel shell	1	1	1P/1S

Approximately 20 g of the seafood, shell and salt mixture was transferred into 20 ml glass bottles, which were then incubated at 30 ± 1 °C. Each glass bottle was inserted with an inverted Durham tube to observe gas formation during the fermentation. The bottles were each sealed with a rubber stopper attached with an airlock as shown in Figure 8. A single bottle was collected at 2-week intervals for 40 weeks with two sample bottles being drawn at random time-points serving as duplicate samples. Samples bottles were stored at -20 ± 1 °C until further analyses.

3.4 General Chemical Analyses

This section describes the general chemical analyses employed in this study to evaluate the raw materials and fish sauce samples collected.

3.4.1 Protein Analysis for Seafood Materials

Protein analysis on all raw seafood materials were conducted using the Kjeldahl method based on the AOAC Official Method 2001.11 (AOAC, 2005). A 2 g sample of chopped Hoki heads and frames, consisting of a heterogeneous mixture of frames, tails, and head was weighed into the digestion tube. Two Kjeltabs tabs (containing 3.2 g potassium phosphate, K_2SO_4 and 0.00035g selenium, Se, Foss Analytical, United Kingdom) were added and then followed by 25 ml concentrated sulphuric acid, H_2SO_4 (99.5 % (v/v), LabServ Pronalys, Australia). The digestion was carried out using Foss Digestion Unit (Foss Analytical, United Kingdom) with selected mode (Processing mode 3). The fish sample was digested at 420 °C for 2 hours until clear solutions were obtained. The digest was left to cool to room temperature. Steam distillation was carried out using a Kjeltec 8100 Distillation Unit (Foss Analytical AB, Höganäs, Sweden). Seventy millilitres of 40 % sodium hydroxide solution, NaOH, was automatically added into the digestion tube. The distillate was received in a conical flask containing 50 ml of 4% (w/v) boric acid solution (with added 0.1% (w/v) bromocresol green and 0.1% (w/v) methyl red solution made up with alcohol).

Distillation was continued for 4 minutes until a final volume of 50 ml was obtained in the conical flask. Titration was then performed with 0.1 M hydrochloric acid, HCl solution (Thermofisher, New Zealand) as titre to a grey-mauve end point.

The crude protein content of fish sample was calculated using Equations 1 and 2, using a conversion factor of 6.25 (AOAC, 2005). The procedures were repeated for snapper and green-shell mussel samples.

$$\text{Nitrogen (\%)} = \frac{[(A \times B) \times 14 \times 100]}{(1000 \times C)} \quad \text{(Equation 1)}$$

$$\text{Crude protein (\%)} = \text{\% Nitrogen} \times F \quad \text{(Equation 2)}$$

Where A = volume (ml) of HCl used; B = exact molarity of HCl; C = weight (g) of fish sample used; and F = conversion factor (AOAC, 2005).

3.4.2 Gas Chromatography Analysis for Short-Chain Fatty Acids

A gas chromatograph (Nexis GC-2030, Shimadzu Scientific Instrument, Kyoto, Japan) equipped with a flame ionization detector (FID) and an automatic liquid sampler (Shimadzu Scientific Instrument, Kyoto, Japan) was used to determine volatile fatty acids (VFA) present in the fish sauce samples using the modified method of Zhao et al. (2006). One milliliter of fish sauce sample was acidified to pH 2 – 3 by adding 0.1 M hydrochloric acid, HCl to ensure protonation of the VFAs before GC analysis. The acidified sample was filtered using 0.45 μm cellulose acetate syringe filter (Membrane Solutions, Washington, USA) and transferred into 2 ml autosampler vial (9 mm clear vial, with polypropylene cap and bonded PTFE/Si septa, Shimadzu, Kyoto, Japan). The internal standard, pentanoic acid solution (1000 ppm, AccuStandard, Connecticut, USA) was spiked into the sample at a final concentration of 100 ppm. Separation was performed on DB-Wax fused-silica capillary column (30 m × 0.25 mm i.d.; 0.5 μm film; Alltech DB-Wax, Code No. 122-7033). Nitrogen (Instrument grade, BOC, New Zealand) was used as the carrier gas at a flow rate of 20 mL/min. The initial oven temperature was 100 °C, maintained for 0.5 min, raised to 180 °C at 8 °C/min and held for 1.0 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min.

The temperature of the FID and the injection port was 240 °C and 200 °C, respectively. The flow rates of hydrogen, air and nitrogen as makeup gas were 30, 300 and 20 mL/min, respectively. One μL of fish sauce sample was introduced by split/splitless injection (split ratio 1:5), and the run time for each analysis was 17.5 min. Fish sauce sample from the same sample vial was injected three times. Ten percent formic acid (v/v) was injected in between every sample injection to wash off any residual contaminants. An aqueous stock standard solution containing a mixture of acetic acid (1000 ppm, Analytical standard, Ajax Finechem Pty Ltd, Australia), propionic acid (1000 ppm, AccuStandard, Connecticut, USA), butanoic acid (1000 ppm, AccuStandard, Connecticut, USA), pentanoic acid (1000 ppm, AccuStandard, Connecticut, USA) 3-methylbutanoic acid (1000 ppm, AccuStandard, Connecticut, USA), hexanoic acid (1000 ppm, AccuStandard, Connecticut, USA) and heptanoic acid (1000 ppm, AccuStandard, Connecticut, USA) was prepared and stored at 4 ± 1 °C until used. On the day of the analysis, the stock standard solution mix was diluted to 100 ppm and acidified as described above, then injected into GC at the beginning of each batch run. Peak areas of the standard solution mix were used to quantify the VFA concentrations in fish sauce sample. A typical chromatogram of the standard solution mix is shown in Figure 12.

To quantify the peak area in terms of concentration, the relative response factor (RRF) was used. The RRF was calculated using the Equation 3:

$$RRF = \frac{A_{A-100}}{A_{IS-100}} \qquad \text{(Equation 3)}$$

Where $A_{\text{A-100}}$ and $A_{\text{IS-100}}$ represent the peak area of 100 ppm VFA standard solution and internal standard, respectively.

On the basis of RRF, the concentration of the VFA in the sample was calculated as follows:

$$C_A = \frac{A_A \times C_{IS}}{RRF \times A_{IS}} \qquad \text{(Equation 4)}$$

Where A_A = the peak area of the targeted VFA in sample, C_{IS} = the concentration of the internal standard (ppm), and A_{IS} = the peak area of internal standard spiked in the sample (Zhao et al., 2006).

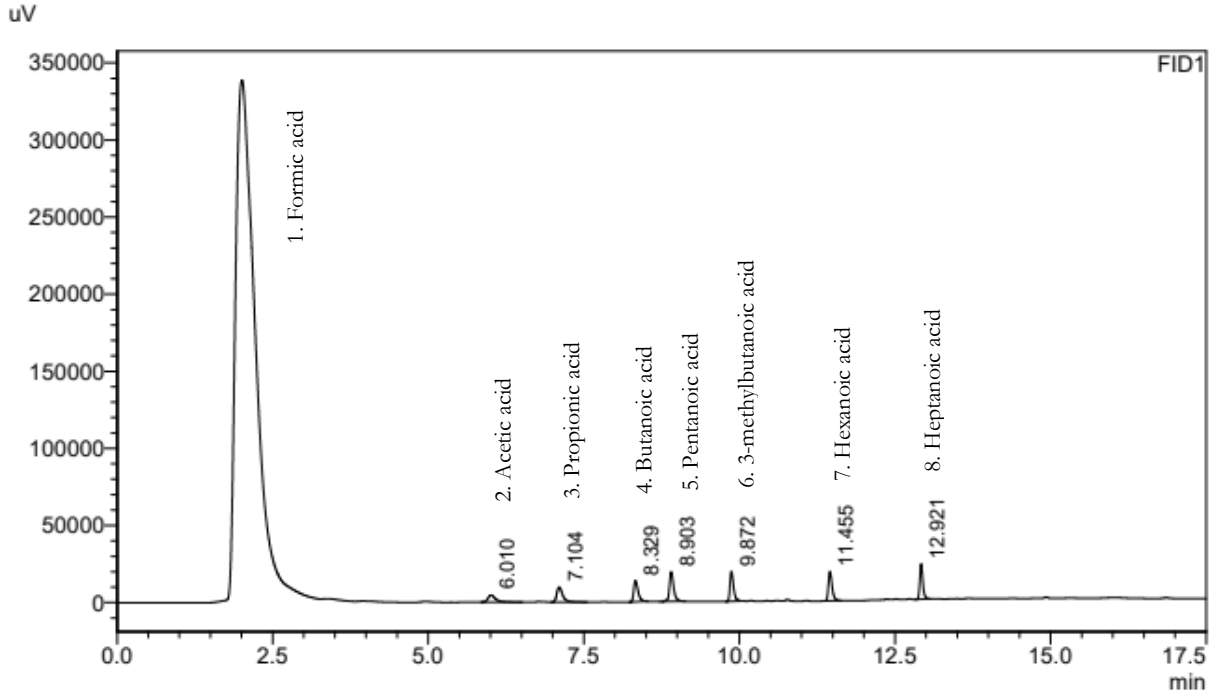


Figure 12: Chromatogram of diluted and acidified standard solution mix at 100 ppm. Formic acid solution (10 % v/v) was injected in between every injection to wash off residual contaminants.

3.5 General Molecular Biology Methods

This section describes the general molecular biology techniques employed in this study. It also outlines three different DNA extraction protocols tested to develop and validate the molecular method to be used in this study. Further discussion on the development and validation of molecular method will be described in the following chapter.

3.5.1 Bacteria Isolation and Growth Conditions

Fish sauce sample from the preliminary fermentation (as described in Section 3.2.1) was streaked onto Lysogeny Broth (LB) (Thermofisher Scientific, New Zealand) agar plates with 10 % NaCl and incubated anaerobically with an Oxoid AnaeroGen (3.5 L sachet, ThermoFisher, New Zealand) at 30 ± 1 °C (similar to fish sauce fermentation temperature) and 37 ± 1 °C (commonly adopted to encourage rapid bacterial growth). The incubation period was extended to two weeks as bacteria under environmental stress typically require longer periods to grow. A single opaque white colony was observed after 72 hours of incubation at 37 ± 1 °C and 96 hours at 30 ± 1 °C. No further colony growth was seen after the extended incubation period. The isolated colony was transferred to LB broth with 10 % NaCl and incubated at 37 ± 1 °C for 72 hours to facilitate further growth, serving as a positive control for the development and validation of a molecular method in this study. The selection of 10 % NaCl LB media was made to provide a suitable growth medium that was not overly selective but remained appropriate for bacteria adapted to the high-salt conditions of fish sauce. The incubation conditions were chosen to accelerate bacterial growth as the primary purpose was to develop and validate molecular methods rather than focusing on optimizing the growth of bacteria present in fish sauce.

The bacterial isolate was subjected to DNA extraction (described in Section 3.5.3) and 16S rRNA sequencing was performed by Macrogen Oceana, Seoul, South Korea using Miseq300 bp PE system and was identified as *Micrococcus luteus*. The isolate was preserved at - 80 °C in glycerol stock (50 % glycerol and 50 % bacteria culture) for long-term storage.

3.5.2 Extraction of Genomic DNA using Wizard Genomic DNA Purification Kit

The extraction of microbial genomic DNA in fish samples were performed using the standard protocol of Wizard Genomic DNA Purification Kit (Technical manual, Promega, USA) for isolating genomic DNA from Gram positive bacteria. One ml of sample was added to a 1.5 ml microcentrifuge tube and centrifuged at 13000 *g* for 2 min to pellet the cells. The supernatant was removed, and the cell pellet was resuspended in 480 µl of 50 mM EDTA (Sigma Aldrich, USA). A 120 µl of 10 mg/ml lysozyme (Sigma Aldrich, USA) was added to the mixture before it was incubated at 37 ± 1 °C for 30 min. The incubated mixture was then centrifuged at 13000 *g* for 2 min and the supernatant was removed. A 600 µl of nuclei lysis solution (Promega, USA)was added to resuspend the cells and the mixture was incubated for 5 min at 80 ± 1 °C to lyse the cells. Once the cell lysate was cooled to room temperature (18 ± 5 °C), 3 µl of RNase solution provided (Promega, USA) was added. The tube was inverted 2 to 5 times to mix the sample mixture before it was incubated at 37 ± 1°C for 60 min. The incubated sample mixture was cooled to room temperature (18 ± 5 °C) and then 200 µl of protein precipitation solution was added to the RNase-treated cell lysate. The mixture was vortexed at high speed for 20 seconds to mix before incubated at 0 ± 1°C for 5 min, followed by centrifugation at 13000 *g* for 3 min. The supernatant containing the DNA was transferred to a clean microcentrifuge tube containing 600 µl isopropanol (Macron Fine Chemicals, USA) (room temperature) and the mixture was inverted gently until the thread-like strands of DNA formed a visible mass.

The mixture was recentrifuged at 13000 *g* from 2 min. The supernatant was then discarded and the tube was drained carefully with adsorbent paper (Kimwipes, Kimtech Science, USA). A 600 µl of 70% ethanol (99.8 % v/v, Macron Fine Chemicals, USA) was added to the tube and it was inverted several times to wash the DNA pellet before it as again recentrifuged at 13000 *g* for 2 min. The supernatant was drained and the pellet was let to air-dry for 15 min.

A 100 µl of DNA rehydration solution provided was added to the DNA pellet and the DNA was rehydrated by incubating at 65 °C ± 2 °C for 1 hour. The DNA solution sample was stored at -20 ± 1 °C until used.

3.5.3 Extraction of Genomic DNA using DNeasy Powersoil Kit

The extraction of microbial genomic DNA in fish samples are performed using the modified protocol of DNeasy Powesoil Kit (Qiagen, Bio-Strategy Ltd, New Zealand). The solutions used were prepared and provided in the kit. Five milliliter of fish sauce sample was vortexed at maximum speed for 2 min to ensure that the homogeneous sample was thoroughly mixed. A 250 µl of fish sauce sample was added to the PowerBead Tube supplied by the kit (Qiagen, Bio-Strategy Ltd, New Zealand) and vortexed for 5s to mix. Sixty microliter of Solution C1 (supplied by kit) was then added into the tube containing the fish sauce sample and vortexed at maximum speed for 10 min. Solution C1 contains sodium dodecyl sulfate (SDS) and disruption agents required for cell lysis. The mixture was then centrifuged at 10 000 *g* for 1 min and the supernatant was transferred to a clean 2 ml collection tube. Two hundred and fifty microliter of Solution C2 (supplied by kit) was added into the collection tube and vortexed for 5s to mix before incubated at 0 ± 1 °C for 5 min. Solution C2 contains reagent that can precipitate non-DNA organic and inorganic material in the sample to increase DNA purity for downstream application. The tube was then centrifuged at 10 000 *g* for 1 min and approximately 600 µl of supernatant was transferred to a new clean collection tube.

Two hundred µl of Solution C3 (supplied by kit) was added into the collection tube and vortexed for 5s to mix before incubated at 0 ± 1°C for 5 min. Solution C3 contains second reagent to precipitate non-DNA organic and inorganic material that may still remain in the sample. The tube was then centrifuged at 10 000 *g* for 1 min and 750 µl of supernatant was transferred into another clean tube before adding 1200 µl of Solution C4 (supplied by kit).

The mixture was vortexed for 5s to mix. A 675 µl of the mixture was loaded onto an MB Spin Column supplied in the kit before being centrifuged at 10 000 *g* for 1 min - this step was repeated twice and flow-through was discarded after every centrifugation. Solution C4 is a high concentration salt solution that adjusted the salt concentration of the DNA solution to allow DNA to bind to the MB Spin Column. Five hundred microliter of Solution C5 (supplied by kit) was added onto the same MB Spin Column and centrifuged again at 10 000 *g* for 1 min with flow-through discarded after centrifugation. Solution C5 was an ethanol based wash solution to further clean the DNA that was bound to the silica filter membrane in the MB Spin Column by removing residual salt and other contaminants. A 100 µl of Solution C6 (sterile elution buffer, supplied by kit) was loaded onto the center of the white filter membrane of the MB Spin Column (supplied by kit) before it was centrifuged again at 10 000 *g* for 1 min to collect isolated DNA. The DNA was stored at -20 ± 1 °C until used for downstream application.

3.5.4 Extraction of Genomic DNA using Phenol-Chloroform-Isoamyl Alcohol (PCI) Method

The extraction of microbial genomic DNA in fish samples are performed using the method of Wen (2016) with modification. One milliliter of fish/fish sauce sample was homogenized in a stomacher for 10 min at maximum speed. The homogenized sample was then centrifuged for 10 min at 16 000 *g* (Thermo Scientific, USA) and the supernatant was discarded.

The harvested cell pellet was resuspended in 500 µl of Buffer A [30 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 50 mM NaCl] then centrifuged for 4 min at 16 000 *g* and the supernatant was discarded. This washing step was repeated once. The final cell pellet was then resuspended in 500 µl of Buffer B [50 mM Tris-HCl (pH 8.0), 25 % (w/v) sucrose, 20 mg/ml lysozyme (Sigma Aldrich, USA) and 1 mM EDTA (pH 8.0)] before incubation at 37 ± 1°C to weaken the bacterial cell wall.

After incubation, a 500 µl of 0.25M EDTA (pH 8.0) was added and the cell suspension was incubated at room temperature (18 ± 5°C) for 5 min. An additional of 200 µl of 20 % (w/v) SDS was added and the mixture was incubated at 65 °C for 1 hour to solubilize the cell membranes. Next, 10 µl of 20 mg/ml of Proteinase K (Omega Bio-tek, USA) was added and the mixture was incubated at 65 °C for 15 min to degrade the proteins. A 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) solution (Thermo Scientific, USA) was added and mixed thoroughly. After centrifugation for 10 min at 16000 *g*, the DNA-containing aqueous phase was carefully transferred to a clean Eppendorf tube and the extraction step was repeated once more. Then 10 µl of DNase-free RNase (Roche Diagnositics, Germany) was added to the aqueous fraction collected after the second extraction step at a final concentration of 100 µg/ml and the mixture was incubated at 37 ± 1°C for 30 min followed by the phenol/chloroform/isoamyl alcohol extractions as previously described until a minimal visible white particulate protein layer at the interface was observed. The DNA was precipitated by adding 500 µl cold 95 % (v/v) ethanol (4 ± 1 °C) into the aqueous fraction and the mixture was incubated at -20 ± 1 °C for 24 hours. One microliter of 20 mg/ml glycogen (Thermo Scientific, USA) was added to every 1 ml of aqueous fraction before it was centrifuged at 16 000 *g* for 10 min and the supernatant was discarded. The DNA pellets were washed in 70 % (v/v) ethanol before centrifuged at 16 000 *g* for 2 min and the supernatant was drained and the DNA pellet was let to air-dry for 15 min. The DNA was rehydrated in 100 µl of 10 mM Tris-HCl (pH 8.0). The DNA solution was stored at -20 ± 1°C until used for downstream application.

3.5.5 Polymerase Chain Reaction (PCR)

The PCR amplifications were carried out using a Biorad T100 Thermal Cycler (Bio-Rad, USA), with 16S rRNA universal primers for bacteria, namely, forward primer 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and reverse primer 1492R (5´-CGG TTA CCT TGT TAC GAC TT-3´)

(Invitrogen, USA). The PCR reaction mixture included 5 µl 10X PCR buffer without $MgCl_2$, 1.5 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 0.2 µl *Taq* DNA Polymerase (5U/µl, Invitrogen, USA), 2.5 µl 10 µM each of the forward and reverse primers, 5 µl of the DNA template, and distilled water to bring the total volume to 50 µl. Initial denaturation hot start of 3 min at 94 °C was followed by 30 cycles of the following incubation pattern: 94 °C for 45s, 53.8 °C for 30s and 72 °C for 135s. A final extension at 72 °C for 10 min concluded the reaction.

3.5.6 Gel Electrophoresis for DNA Separation

The DNA samples extracted from fish sauce sample and PCR products were separated with electrophoresis on 1% (w/v) agarose gels (Hoefer, USA). Five microliter of DNA samples was diluted with 1 µl of 6X TriTrack DNA Loading Dye (#R1161, Thermo Scientific, USA) before being loaded into gel wells DNA and electrophoresis was run at 110V for 45 min. A GeneRuler 1 kb DNA Ladder (Thermo Scientific, NZ) was used for size quantification of DNA samples and PCR products. The band patterns were visualised using a UV transilluminator (Bio-Rad, USA).

3.5.7 Quantitative and Qualitative Assessments of DNA

The DNA concentrations and the quality assessment of the extracted DNA templates were obtained from the NanoDrop Spectrophotometer (Thermo Scientific, USA).

3.5.8 Sequencing process and data analysis

The 16S rRNA sequencing was performed by Macrogen Oceana, Seoul, South Korea using Miseq300 bp PE system.

All DNA samples were subjected to PCR amplifications according to the standard protocol from Macrogen Oceana using the universal primers, Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC).

Sequencing data sets were trimmed using Geneious Prime 9.1.8 (Geneious, Dotmatics, New Zealand) software to remove any remaining adaptors, bases below an average quality of 30 (Q30 – a Phred quality score equivalent to the probability of an incorrect base call of 99.9%) and reads that are less than 100 bp. The high quality reads obtained were aligned and clustered into operational taxonomic units (OTU) defined by 97% similarity before subjected to the BLAST 16s Microbial database (National Centre for Biotechnology Information (NCBI), USA) for taxonomy classification. 16S rRNA gene taxonomy were classified at the genus, family, order, class, and phylum levels based on the respective taxonomic categorizations of the BLAST server.

The microbial diversity was expressed in Chao1 (measure of richness), Shannon-Weaver (measure of evenness) and Simpson's Index (measure of diversity which take into accounts both richness and evenness) and these indices are calculated using the equations:

Chao1 index, $\mathbf{R_{Chao1}} = R_{obs} + \frac{f_1(f_1-1)}{2(f_2+1)}$ (Equation 5)

Where f_1 is the number of species observed only once and f_2 is the number of species observed twice.

Shannon-Weaver index, $\mathbf{R_{Shannon}} = \sum_{i=1}^{k} p_i \log\ (p_i)$ (Equation 6)

Where p_i represents the relative abundances of the i-th taxon.

Simpson's index, $D = \frac{\sum n_i\,(n_i-1)}{N(N-1)}$ (Equation 7)

Where N is the total number of species in the environment and n_i is the total number of organisms of a particular species.

3.6 Statistical Analysis

Analysis of variance (ANOVA) was performed on IBM SPSS Statistics Software (version 21.0; IBM®, United States). Kruskal-Analysis was performed on SigmaPlot Software (version 15.0, SigmaPlot, Grafiti LLC, USA). Non-metric multidimensional scaling (NMDS) and Principal Component Analysis (PCA) were performed using RStudio (Version 4.3.1).

Chapter 4: Development and Validation of Molecular Methods

4. 0 Introduction

To obtain bioinformatics information of the bacteria communities contained in fish fermentations throughout the fermentation time course, protocols for DNA extraction of microbial genomic DNA were developed and validated to suit the nature of the samples in this research. A reliable microbial DNA extraction protocol is important to ensure the efficiency for downstream applications.

The aims of the research described in this chapter were to:

- Evaluate and validate the standard protocols of different microbial DNA extraction kits for fish fermentation samples.
- Investigate the effect of salt and pre-treatment methods on microbial DNA extraction protocols.
- Develop and validate a reliable microbial DNA extraction protocol for fish fermentation samples.

Fish fermentation samples used in experiments conducted in this chapter were either from store-bought commercial fish sauce or preliminary fermentation described in Section 3.2. Note that the development and validation of the molecular method was conducted in parallel time with the fermentation process. Consequently, some control samples were selected based on what was deemed appropriate at the time of the trial, and changes were made as necessary when new information and results were obtained.

4.1 Extraction of Microbial Genomic DNA using Extraction Kit

The extractions of microbial genomic DNA in fish ferments were performed according to the standard protocols as described in Sections 3.3.3 and 3.3.4, and modifications were made based on findings obtained from series of trials to suit the nature of the samples tested. Data obtained from the experiments were subjected to analysis of variance (ANOVA).

ANOVA compares between all mean values to determine whether they are statistically different from each other and is presented as *p*-value. The *p*-value of lower that 0.05 indicates the means compared are statistically significant. Note that the DNA extraction protocol would not be able to differentiate between DNA from seafood material and bacteria. A subsequent PCR amplification targeting the bacterial 16S rRNA region followed by sequencing process were employed to provide bioinformatics information focusing on bacteria communities present in any fish sauce sample.

This section will discuss the workflow employed in investigating the suitable protocols for the fish fermentation samples.

4.1.1 DNA Extraction using Wizard Genomic DNA Purification Kit

A commercial fish sauce [*noucmam* (Vietnamese style fish sauce), Poonsin, Thailand] and fish ferment obtained from the preliminary fermentation set-up were used to evaluate the performance of the microbial genomic DNA extraction protocol as described in Section 3.5.2. A Gram-positive bacterium, *Staphylococcus aureus NCTC 4163* was selected as a control sample. To ensure the reliability of the extraction protocol can be validated, a Gram-positive bacterium was selected due to the presence of bacterial cell wall that may influence the efficiency of an extraction protocol.

DNA extracted from all three samples were subjected to NanoDrop spectrophotometer (Thermo Scientific, USA) to assess the DNA purity and to quantify the DNA concentration in the tested samples as shown in Table 11.

Based on Table 11, the control sample had the highest extractable DNA concentration followed by fish fermentation sample (FS-SS-3) and then *noucmam*. The lowest DNA concentration in the *noucmam* may indicate that the fish sauce may have undergone sterilization and/or filtration processes to increase the shelf life of the product.

Table 11: The DNA concentrations, A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of commercial *noucmam* and fish fermentation samples

Sample	DNA concentration (*ng/µl*)	A_{260}/A_{280} Ratio[3]	A_{260}/A_{230} Ratio[4]
Gram positive bacteria[1]	544.2 ± 67.1^{b}	2.2 ± 0.0^{a}	2.5 ± 0.3^{cd}
Commercial *Noucmam*	1.8 ± 0.4^{a}	4.3 ± 1.9^{a}	0.1 ± 0.1^{a}
FS-SS-3[2]	33.2 ± 1.4^{a}	3.3 ± 0.9^{a}	0.6 ± 0.2^{b}

[1]A Gram positive bacteria, *Staphylococcus aureus NCTC 4163* is the control sample.
[2]FS-SS-3 is fish fermentation sample fermented using snapper heads and frames with iodised sea salt for 3 weeks at 30 ± 2 °C .
[3]A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
[4]A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
Values are presented as means ± standard error of mean (n=3).
Values in the same columns that do not share the same letters are significantly different at $p < 0.05$.

The A_{260}/A_{280} ratio is commonly used to measure protein contamination in a DNA sample (Matlock, 2015). An A_{260}/A_{280} ratio of about 1.8 is generally accepted as pure DNA with a lower value ratio indicates that there is contamination from protein in the sample which may affect the following downstream application (Matlock, 2015). A value of higher than 1.8 for the A_{260}/A_{280} ratio is not indicative of an issue. Based on Table 11, the A_{260}/A_{280} ratios of all samples were above 1.8 indicating that the extracted DNA samples may still be suitable for the downstream applications.

Similar to the A_{260}/A_{280} ratio, an A_{260}/A_{230} is also used as a measure of the DNA purity (Matlock, 2015). Generally, a value ranging between 2.0 to 2.2 for A_{260}/A_{230} is accepted for a pure DNA sample (Matlock, 2015). Table 11 shows that the A_{260}/A_{230} ratios of *noucmam* and FS-SS-3 were statistically lower than the cut-off ratio indicating a possibility of residual phenol used in the extraction protocol that were present in both samples.

A further trial was conducted on the same samples by applying a slight modification to the standard protocol to improve DNA yield. A 1 µl of 20 mg/ml glycogen (Thermo Scientific, USA) was added to the collected supernatant (800 µl) containing DNA after treatment with RNase and protein precipitation solutions. Glycogen is a highly purified polysaccharide that precipitates nucleic acid (Hengen, 1966).

Table 12 shows that DNA concentration of *noucmam* increased from 1.8 ± 0.4 ng/µl (refer Table 11) to 3.4 ± 0.3 ng/µl. However, the DNA concentration extracted from the control and FS-SS-3-II samples had decreased from previous trial (refer Table 11). This indicated that glycogen was not able to precipitate the nucleic acid and improve DNA yield in the tested samples. The A_{260}/A_{280} ratio of FS-SS-3-II was 5.4 ± 0.7 – statistically higher than the cut-off ratio for pure DNA. However, a A_{260}/A_{280} ratio > 1.8 is not indicative of an issue for the use of DNA sample for downstream application (Matlock, 2015). Table 12 shows that A_{260}/A_{230} ratios for all samples were statistically different than the cut-off range. This may indicate the presence of organic contaminants, presumably from glycogen or residual phenol.

Table 12: The DNA concentrations, A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of commercial *noucmam* and fish sauce samples after a slight modification to the original standard DNA extraction protocol by adding glycogen.

Sample	DNA concentration (*ng/µl*)	A_{260}/A_{280} Ratio[3]	A_{260}/A_{230} Ratio[4]
Gram positive bacteria[1]	489.1 ± 45.0[b]	1.9± 0.2[a]	2.6 ± 0.1[d]
Commercial *Noucmam*	3.4 ± 0.3[a]	3.1 ± 0.6[a]	0.1 ± 0.1[a]
FS-SS-3-II[2]	16.3 ± 1.7[a]	5.4 ± 0.7[b]	0.3 ± 0.0[b]

[1]A Gram positive bacteria, *Staphylococcus aureus NCTC 4163* is the control sample.
[2]FS-SS-3 is fish fermentation fermented using snapper heads and frames with iodised sea salt for 3 weeks at 30 ± 2 °C .
[3]A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
[4]A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
Values represent means ± standard error of mean (n=3).
Values in the same columns that do not share the same letters are significantly different at $p < 0.05$.

All DNAs extracted from the two separate trials were subjected to gel electrophoresis to allow visual representations of the DNA fragments present. However, there were negative visuals on the gels.

DNA extraction from the salts used in this study was attempted using the Wizard Genomic DNA Purification Kit, as described in Section 3.5.2. However, the method did not yield recoverable nucleic acid, with NanoDrop spectrophotometer readings showing A_{260}/A_{280} ratios < 0.5 and A_{260}/A_{230} ratios < 0.1, indicating the samples were unsuitable for downstream applications.

4.1.2 DNA Extraction using DNeasy Powersoil Kit

Further experimental trial was conducted using DNeasy Powersoil Kit according to protocols described in Section 3.5.3 to determine the performance of different DNA extraction protocols on seafood pastes (Hoki, snapper and mussel pastes), salts (stoved and unstoved salts) and fish fermentation samples. The DNeasy Powersoil Kit was selected because it includes mechanical lysis using microbeads technology in addition to chemical lysis to ensure maximum bacterial cells lysis (DNeasy Powersoil Handbook, 2020). The DNA kit also allows a DNA extraction from environmental sample, thus, increasing the potential of the kit in extracting DNA from the fish fermentation samples. A Gram-positive bacterium, *Staphylococcus aureus NCTC 4163* was used as a control sample.

Prior to extraction, all tested samples were homogenized to maximize the number of bacteria lysed to give a representative and homogenous mixture of sample. Samples were placed in sterile stomacher bags containing buffered peptone water at a ratio of 1/10 sample/buffer. Homogenization was performed at the highest intensity for 1 min. A stomacher was used to mechanically extract bacteria that may be present on the surface/biofilms of the seafood pastes and fish ferments. Aliquot samples collected from the homogenization process were subjected to DNA extraction.

Salts were diluted in distilled water at a ratio of 1/3 salt/water before centrifuged at 10,000 g for 1 hour. Precipitate collected was used for the following DNA extraction.

Table 13 shows that mussel paste had the highest DNA concentration with the value of 10.3 ± 0.7 ng/µl, followed by Hoki and snapper pastes with the values of 2.5 ±0.4 ng/µl and 2.6 ± 0.6 ng/µl, respectively – no significant difference between Hoki and snapper pastes. DNA concentration in unstoved salt was significantly higher from stoved salt with values of 2.9 ± 0.2 ng/µl and 1.4 ± 0.2 ng/µl, respectively (Table 13). Unstoved salt refers to the untreated crude solar salt collected directly from the salt ponds, then washed with saturated brine to wash off mud before naturally dried out (Reid, 1976). Meanwhile, stoved salt refers to the crude solar salt that has undergone a high temperature dry heat treatment to kill halophilic bacteria (Reid, 1976). The higher DNA concentration in unstoved salt than in stoved salt may indicate a higher bacteria number on the surface of the untreated solar salt than that on the heat-treated salt.

Table 13: The DNA concentrations, A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of extracted DNA from three different seafood pastes, two types of salt and two different fish fermentation samples.

Category	Sample	DNA concentration (ng/µl)	A_{260}/A_{280} Ratio[4]	A_{260}/A_{230} Ratio[5]
Control	Gram positive bacteria[1]	490.1 ± 40.2^{e}	1.7 ± 0.1^{a}	0.8 ± 0.1^{c}
Seafood paste	Hoki	2.5 ± 0.2^{ab}	2.1 ± 0.4^{ab}	0.8 ± 0.1^{c}
	snapper	2.5 ± 0.3^{ab}	4.0 ± 0.2^{b}	0.4 ± 0.0^{a}
	Mussel	10.3±0.3^{d}	1.7 ± 0.6^{a}	1.5 ± 0.0^{e}
Salt	Unstoved	2.9 ± 0.2^{b}	4.0 ± 0.2^{b}	0.7 ± 0.1^{bc}
	Stoved	1.4 ± 0.2^{a}	3.4 ± 1.0^{ab}	0.8 ± 0.4^{c}
Fish ferment	FS-SS-30[2]	5.7 ± 0.2^{c}	1.8 ± 0.1^{a}	0.6 ± 0.3^{ab}
	FS-TS-30[3]	6.4 ± 0.9^{c}	2.9 ± 0.3^{ab}	1.0 ± 0.2^{d}

[1]A Gram positive bacteria, *Staphylococcus aureus NCTC 4163* is the control sample.
[2]FS-SS-3 is fish sauce sample fermented using snapper heads and frames with iodised sea salt for 30 weeks at 30 ± 2 °C .
[3] FS-TS is fish sauce sample fermented using snapper heads and frames with iodised table salt for 30 weeks at 30 ± 2 °C .
[4]A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
[5]A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
Values represent means ± standard error of mean (n=3).
Values in the same columns that do not share the same letters are significantly different at $p < 0.05$.

Table 13 also shows no significant difference between DNA concentrations in snapper fermentations with either sea salt (FS-SS-30) or table salt (FS-TS-30). However, significant increase in DNA concentrations were observed in both snapper fermentations when compared to that in snapper paste indicating bacterial growth during the 30 weeks fermentation – which was expected.

The A_{260}/A_{280} ratios of all samples except snapper paste and unstoved salt were not statistically different than the cut-off ratio for pure DNA (Table 13). On the other hand, A_{260}/A_{230} ratios of all samples were statistically lower than cut-off range for pure DNA. A low A_{260}/A_{230} may be the result of phenol, guanine and/or glycogen from the extraction protocol (Matlock, 2015). Although the purity ratios are important indicators for the quality of the extracted DNA samples, but the best indicator of the samples is the functionality in the downstream application of interest (Matlock, 2015). All extracted DNAs were subjected to gel electrophoresis, but negative visuals were obtained.

4.2 The Effect of Salt on the Genomic Microbial DNA Extraction

From Section 4.1, negative results were obtained from gel electrophoresis to visualize DNA fragments from tested fish fermentation samples. Hence, it was postulated that the high salt content in the samples may have contributed to the current findings. An experiment was conducted to investigate the effect of salt concentration on the DNA extraction protocol. Four concentrations of salt solution ranging between 5 to 25 % (v/w) was added into *Pseudomonas fluorescens* strain SBW25 bacterial suspension at a ratio of 1/1 salt/bacterial suspension. All samples were subjected to genomic microbial DNA extractions as described in Section 3.5.3. The effect of salt concentration on DNA extraction method were assessed based on DNA concentration and purity. Table 14 shows no significant difference in DNA concentration and A_{260}/A_{280} ratio between all samples regardless of the salt concentration. DNA concentrations of all samples ranged at 83 – 93 ng/μl.

The A_{260}/A_{230} ratio of only sample with 5 % (v/w) salt was significantly lower than the cut-off range for pure DNA but not for other samples with higher salt concentrations. This may be due to technical error when handling the sample. All DNAs extracted were subjected to PCR amplification as described in Section 3.5.5 for visualization and the result is presented in Figure 13.

Overall, based on DNA concentration and purity assessment, the salt content did not affect the efficiency of the DNA extraction method employed.

Table 14: The DNA concentrations, A_{260}/A_{280} and A_{260}/A_{230} ratios of extracted DNA from samples with four different salt contents.

Sample	DNA concentration (ng/µl)	A_{260}/A_{280} Ratio[2]	A_{260}/A_{230} Ratio[3]
SBW25[1] (control)	83.1 ± 1.5a	2.2 ± 0.6[a]	1.6 ± 0.1[a]
SBW25 + 5 % (v/w) salt	85.3 ± 0.9[a]	2.1 ± 0.8[a]	1.7 ± 0.2[a]
SBW25 + 10 % (v/w) salt	82.9 ± 1.8[a]	2.1 ± 0.2[a]	1.9 ± 0.3[b]
SBW25 + 20 % (v/w) salt	92.6 ± 0.4[a]	2.1 ± 0.1[a]	2.0 ± 0.1[b]
SBW25 + 25 % (v/w) salt	91.2 ± 0.7[a]	2.1 ± 0.5[a]	2.0 ± 0.0[b]

[1] *Pseudomonas fluorescens* strain SBW25 bacterial suspension

[2]A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.

[3]A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.

Values represent means ± range (n=2).

Values in the same columns that do not share the same letters are significantly different at $p < 0.05$.

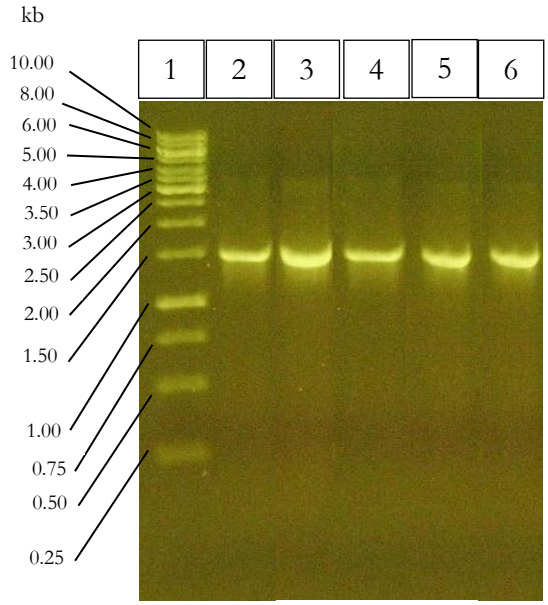


Figure 13: Visual representation from gel electrophoresis after PCR amplification [forward primer 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and reverse primer 1492R (5´-CGG TTA CCT TGT TAC GAC TT-3´)] of DNA extracted from samples with different salt concentrations. Column 1: DNA ladder (ThermoFisher 1kb); 2: *Pseudomonas fluorescens* strain SBW25 (positive control), 3: SBW25 + 5 % (w/w) salt; 4: SBW25 + 10 % (w/w) salt; 5: SBW25 + 20 % (w/w) salt; 6: SBW25 + 25 % (w/w) salt.

4.3 The Effect of Pre-treatment on the Genomic Microbial DNA Extraction

Findings from Section 4.1 found that only small quantities of DNA (between 2 – 10 ng/μl) were extracted from fish fermentation samples. Another set of experiment as shown in Figure 15 was conducted to determine if pre-treatment methods can increase DNA yield using DNA extraction protocol described in Section 3.5.3. To validate the efficiency of DNA extraction protocol used, fish ferments were spiked with *Micrococcus luteus* bacterial suspension isolated from preliminary fermentation as described in Section 3.5.1. As the development and validation of molecular method progressed, it was thought that using *Micrococcus luteus* as the positive control was most appropriate. The spiking method was employed due to low DNA yield extracted from fish ferment alone. In theory, DNA concentration from spiked ferment samples should either be close or more than DNA concentration from only *Micrococcus luteus* bacterial suspension (control).

Table 15 shows no significant difference in DNA concentration between samples after pre-treatments A (control) and B (no filtration) with the values of 100.1 ± 1.5 and 103.1 ± 0.9, respectively. DNA concentrations of samples after pre-treatments C (filtered with cheesecloth) and D (filtered with 22 μm filter paper) were significantly lower than non-filtered samples. This indicate that filtration step decreased DNA yield – presumably because some bacteria on the surface of large solid materials from fish ferments were filtered out.

Table 15 shows no significant difference in A_{260}/A_{280} ratio between all samples regardless of the pre-treatment methods and the cut-off value for pure DNA. The A_{260}/A_{230} ratio of only sample without filtration was significantly lower than the cut-off range for pure DNA but not samples with other pre-treatment methods.

Overall, pre-treatment method did not significantly increase DNA yield indicating that the DNA extraction protocol used may not be suitable for current fish fermentation samples.

Table 15: The DNA concentrations, A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of extracted DNA from samples after different pre-treatment methods.

Sample	Pre-treatment	DNA concentration (ng/µl)	A_{260}/A_{280} Ratio[2]	A_{260}/A_{230} Ratio[3]
A	Control[1]	100.1 ± 1.5[a]	2.2 ± 0.2[a]	1.58 ± 0.09[a]
B	Homogenization	103.1 ± 0.9[a]	2.1 ± 0.3[a]	1.70 ± 0.02[a]
C	Homogenization + filtration (cheesecloth)	78.9 ± 1.8[b]	2.1 ± 0.2[a]	1.90 ± 0.03[b]
D	Homogenization + filtration (22 µm filter paper)	76.5 ± 0.4[b]	2.0 ± 0.3[a]	1.96 ± 0.01[b]

[1] *Micrococcus luteus* bacterial suspension

[2] A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.

[3] A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.

Values represent means ± range (n=2).

Values in the same columns that do not share the same letters are significantly different at $p < 0.05$

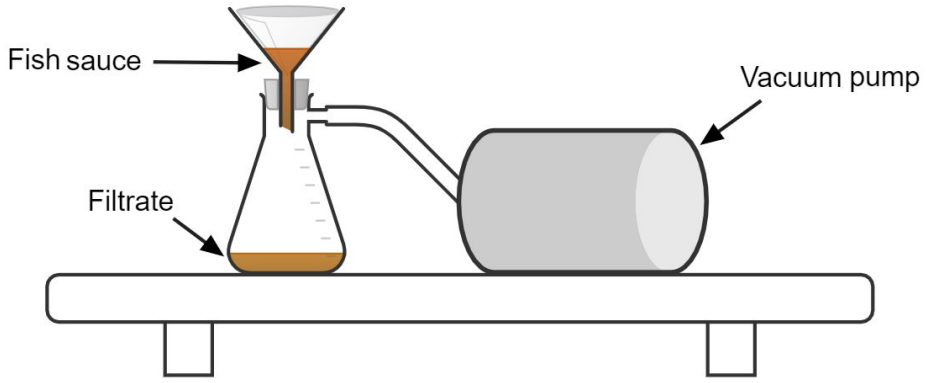
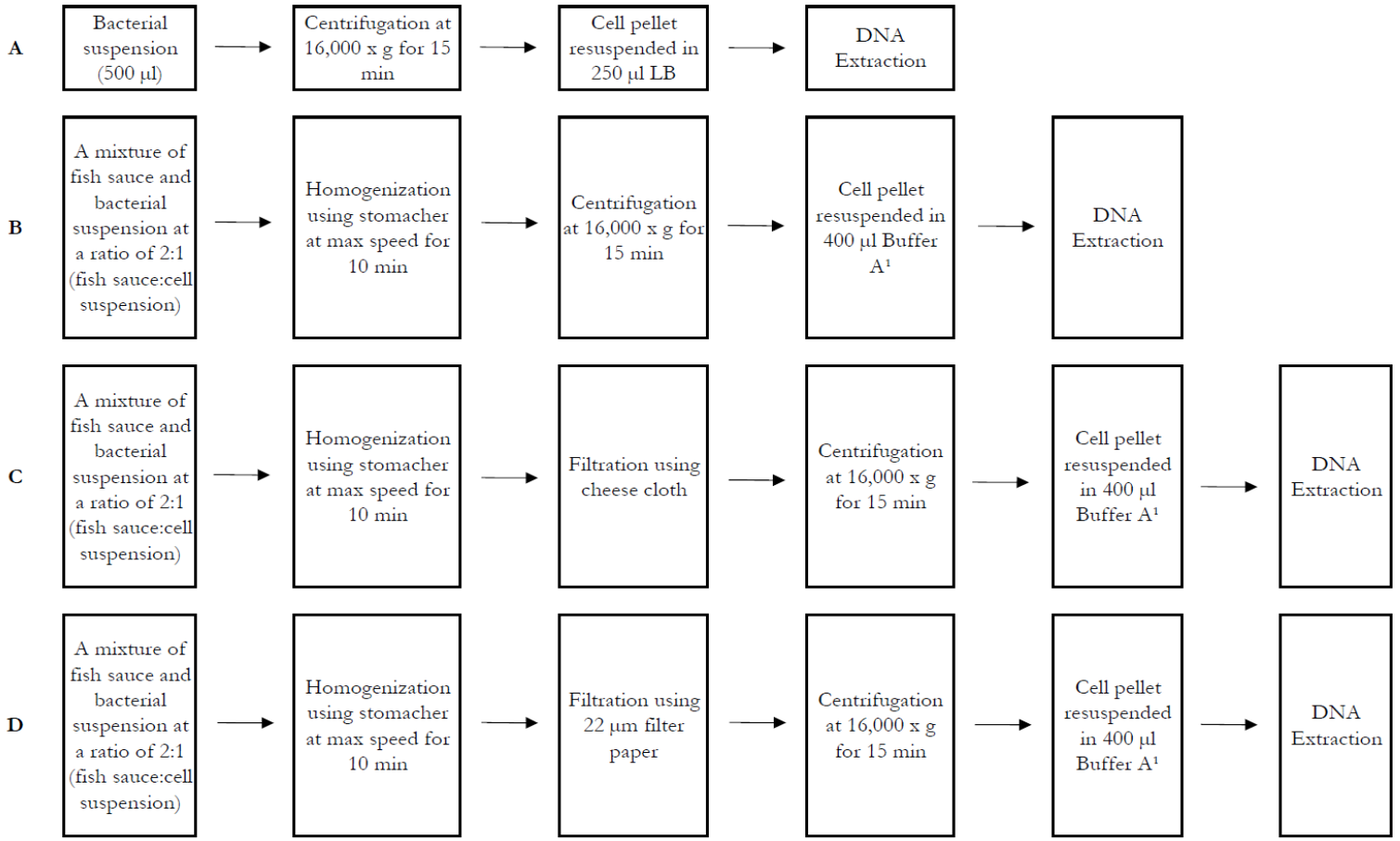


Figure 14: Filtration set-up used in the pre-treatment method prior to DNA extraction.



All experiment was conducted in duplicate.
DNA extraction was performed using DNeasy Powersoil Kit protocol described in Section 3.3.4.
Micrococcus luteus bacterial suspension was used. LB is lysogeny broth.
[1]Buffer A is 20% (w/v) NaCl solution.

Figure 15: Experimental design to investigate the effect of pre-treatment methods on the genomic microbial DNA yield and purity from fish fermentation samples.

4.4 DNA extraction method using Phenol-Chloroform-Isoamyl Alcohol (PCI) Method

Results from Sections 4.1 – 4.3 suggest that DNA extraction kits used (Wizard Genomic DNA Purification Kit and DNeasy Powersoil Kit) were not suitable for DNA extractions of current fish fermentation samples. Phenol-chloroform-isoamyl alcohol (PCI) method as described in Section 3.5.4 was tested to determine its efficiency for DNA extraction on seafood pastes (Hoki, snapper and mussel pastes), salts (stoved and unstoved salts) and fish ferments (described in Section 3.1.1).

Table 16 shows that DNA concentration of all samples ranged between 85 – 275 ng/µl – much higher than any DNA concentration obtained from previous extraction protocols (refer to Sections 4.1.1 and 4.1.2). This suggests that PCI method was more effective in extracting microbial DNA from current fish fermentation samples.

Table 16: The DNA concentrations, A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of extracted DNA using phenol-chloroform-isoamyl alcohol (PCI) method.

Category	Sample	DNA concentration (ng/µl)	A_{260}/A_{280} Ratio[3]	A_{260}/A_{230} Ratio[4]
Control	*Micrococcus luteus*	274.8 ± 1.2^{f}	2.0 ± 0.2^{a}	1.8 ± 0.2^{a}
Seafood paste	Hoki	102.0 ± 1.2^{b}	2.6 ± 0.5^{c}	1.8 ± 0.3^{a}
	snapper	115.6 ± 0.8^{c}	1.9 ± 0.1^{a}	2.4 ± 0.0^{c}
	Mussel	201.7 ± 1.0^{e}	2.1 ± 0.3^{b}	2.5 ± 0.1^{e}
Salt	Unstoved	98.8 ± 1.1^{b}	1.9 ± 0.2^{a}	2.2 ± 0.0^{b}
	Stoved	84.7 ± 0.8^{a}	2.0 ± 0.0^{a}	1.8 ± 0.2^{a}
Fish ferment	S-US-W8[1]	107.0 ± 1.4^{bc}	1.9 ± 0.2^{a}	2.6 ± 0.4^{c}
	S-US-W44[2]	120.63 ± 1.3^{d}	1.8 ± 0.1^{a}	2.0 ± 0.3^{b}

[1]S-US-W8 is snapper ferment with unstoved salt for 8 weeks at 30 ± 1 °C .
[2]S-US-W44 is snapper ferment with unstoved salt for 44 weeks at 30 ± 1 °C .
[3]A value of A_{260}/A_{280} ratio that is significantly higher or lower than 1.8 indicates the presence of protein contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
[4]A value of A_{260}/A_{230} ratio that is not in between 2.0 – 2.2 indicates the presence of organic contaminants in samples. The indicative value is subjected to ANOVA to determine whether the value is statistically different.
Values represent means ± range (n=2).
Values in the same columns that do not share the same letters are significantly different at $p < 0.05$.

The A_{260}/A_{280} ratios of all samples except Hoki and mussel pastes were significantly higher than the cut-off ratio for pure DNA (Table 16).

However, A_{260}/A_{280} ratio > 1.8 is not indicative of an issue and the extracted DNA may still be suitable for downstream applications – as already discussed in Section 4.1.1. Based on A_{260}/A_{230} ratio, Hoki paste and stoved salt were significantly lower than the value range for pure DNA, indicating a possibility of organic contaminants. Meanwhile, three samples – snapper and mussel pastes as well as 30 °C unstoved snapper ferment after 44 weeks fermentation time, they all had A_{260}/A_{230} ratio significantly higher than the value range for pure DNA. All extracted DNA were subjected to PCR amplification as described in Section 3.5.5 to determine their suitability for downstream applications and the gel visualization is presented in Figure 16.

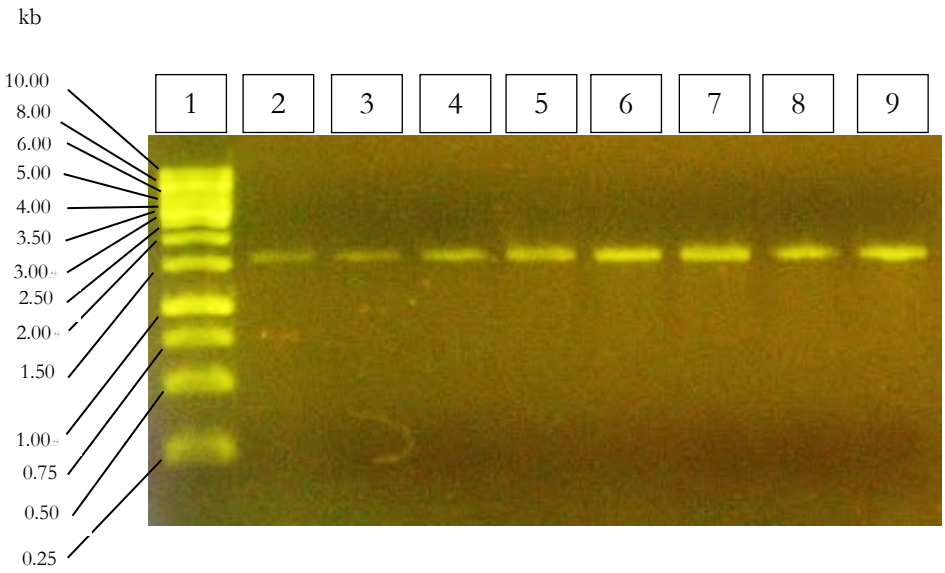


Figure 16: Visual representation after PCR amplification [forward primer 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and reverse primer 1492R (5´-CGG TTA CCT TGT TAC GAC TT-3´)] of DNA extracted using phenol chloroform isoamyl alcohol (PCI) method. Column 1: DNA ladder (ThermoFisher 1kb); 2: *Micrococcus luteus* (positive control), 3: Hoki paste; 4: Snapper paste; 5: Mussel paste; 6: Unstoved salt; 7: Stoved salt; 8: Snapper ferment with unstoved salt after 8 weeks fermentation at 30 ± 1 °C , 9: Snapper ferment with unstoved salt after 44 weeks fermentation at 30 ± 1 °C.

Figure 3 shows clear bands at 1.5 kb for all samples indicating the extracted DNAs from PCI method were suitable for downstream applications. Based on these results, PCI method was selected for extraction of microbial genomic DNA for this current study.

Chapter 5: Fish Sauce Fermentation using Hoki (*Macruronus novaezelandiae)* Heads and Frames at Laboratory Scale

5.0 Introduction

The commercial fish harvested in the largest amount in New Zealand waters is Hoki (*Macruronus novaezelandiae*) (Mohtar, 2013). Hoki is a fish species related to cod and hake that lives in a cold-water habitat from a depth of 200 to 600 m (Connell et al., 2010; Mohtar, 2013). Hoki fishing takes place at several fishing grounds around New Zealand, predominantly the Stewart-Snare shelf south of Stewart Island, the west coast of the South Island and in the Cook Strait (MPI, 2016). Hoki is known to be a good source of omega-3 fatty acids making it an excellent dietary choice and it has a delicate white flesh without any lateral bone making it a safe product for consumption (Mohtar, 2013). In 2019, Hoki exports contributed more than NZD 200 million to the New Zealand economy (Lundquist, 2023). A consequence of the growing fishing industry is increasing generation of fish waste and by-products (MPI, 2016). As mentioned in Section 2.1, current industrial practices often further process the fish waste into low value products such as fish meal and natural fertiliser (Mohtar, 2013). In recent years, fishing industries have spent more effort and time into funding research in developing by-products to manage waste more sustainably and to increase the returns (income) on their current catch.

The aim of this chapter is to understand the natural fermentation process to fish sauce using Hoki heads and frames with two types of solar salt (stoved and unstoved salts), at three different temperatures (18, 25 and 30 °C) and for 80 weeks.

The physical appearance, pH, bacterial composition and the formation of volatile fatty acids (VFA) of and in the Hoki ferments will be monitored throughout the fermentation time to understand the influence of the raw materials used on the fermentation process.

Hypotheses that will be tested in this chapter are:

1. The microbiological composition and diversity present during the fermentation period are affected by the different fermentation conditions for the Hoki fermentations.
2. The concentration of volatile fatty acids produced will increase as the fermentation time increases.
3. Halophilic and halotolerant bacteria pre-existing in the salt are a key inoculum source that will dominate the Hoki fermentation as opposed to bacteria from the fish.
4. Unstoved salt provides a more diverse and/or abundant source of halophiles and/or halotolerant bacteria compared to the stoved salt as the key inoculum source for fish sauce fermentation.

The laboratory scale fermentation set-up allowed sample being withdrawn from the solid and liquor parts of the fish sauce providing a good representation of the entire fermentation. The 27 glass bottles, each representing an individual sample volume, were sampled at different time points during the 80-week fermentation. This set-up allowed the natural fermentation to occur independently within each bottle.

Please note that there are missing data between Week 12 until Week 42 of the laboratory scale fermentation because samples were unable to be collected due to the Covid-19 pandemic restrictions.

5.1 Experimental Method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in Figure 17.

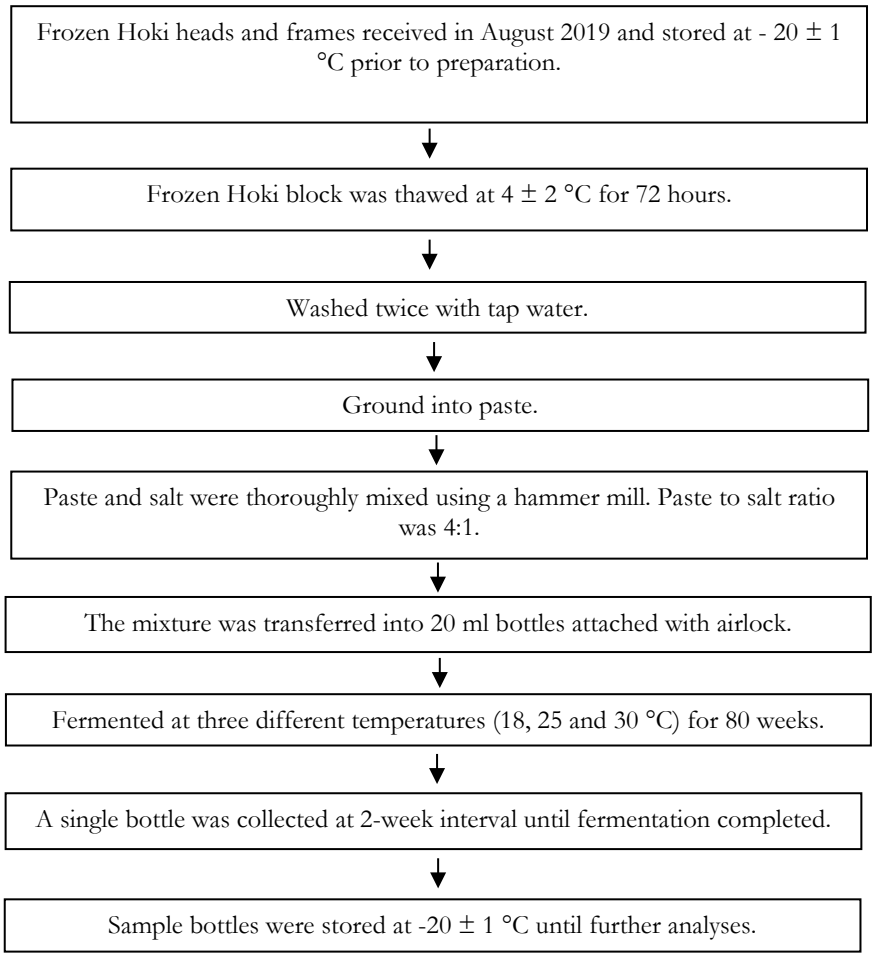


Figure 17: Experimental method flowchart of Hoki heads and frames fermentation at laboratory scale. The experiment was started in November 2020. Design of the laboratory scale fermentation was described in Section 3.3.1.

Although there was a time gap between receiving the raw materials and initiating the fermentation process, appropriate precautions were taken as described in Section 3.1 to minimize the effect of the delay on the final outcomes of this study.

5.2 Visual Observation of the Hoki Fish Sauce

Similar visual appearances were observed in all fermentations at the same temperature regardless of the type of salt used (Tables 1 and 2). The overall colour of the Hoki ferment changed from dark grey at Week 0 (similar to the colour of ground Hoki heads and frames) to dark brown/reddish brown at the final fermentation time, Week 80. The colour changed to light brown with a reddish hue at Week 44 for ferment fermented at 30 °C, and at Week 50 and Week 56 for ferments from 25 °C and 18 °C, respectively. The colour of the ferments intensified to a darker brown-reddish colour as the fermentation progressed until it reached the final colour recorded in Tables 17 and 18.

The texture of the ferments changed from firm solid homogenous mixture (at Week 0) to a soft solid with a light brown liquid layer on the top after Week 44 for sample fermented at 30 °C, and at appeared similarly at Week 50 and 56 for samples fermented at 25 °C and 18 °C, respectively. At the end of the fermentation period (week 80), the textures of all ferments were soft with visible small bones and scales settled at the bottom layer. Based on the visual observations when handling the samples, the changes in physical appearances in Hoki fermentations took place at an earlier period for samples fermented at higher temperature regardless of the type of salt used. The colour and texture changes are common phenomenon during fish sauce fermentation and are important indicators of protein degradations and enzymatic reactions involved during the process (Faisal et al., 2015). According to Saisithi (1994), the colour changes during fish sauce fermentation may be attributed to non-enzymatic browning reaction between the amino acids in the fish sauce and ribose, a by-product of ATP degradation. At the start of the fermentation period (Week 0), the aroma of all fermentations were fishy and marine-like. As the fermentation progressed, the ferments developed a salty, pungent, fishy and ammonia-like aroma. Further investigation on the formation of the key volatile compounds during the fish sauce fermentation were explored and further discussed in the following sections.

Table 17: Summary of the visual observations on the fish sauce ferments from Hoki heads and frames with stoved salt fermented at three different temperatures.

Fermentation temperature, (°C)	**Fermentation time (week)**	**Sample photo**	**Description**
N/A	0		The homogenous mixture of fish frames/heads and salt are in firm texture. The dark grey colour of the mixture is similar to that of the ground Hoki frames and heads.
18	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.
25	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.
30	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.

Table 18: Summary of the visual observations on the fish sauce ferments from Hoki heads and frames with unstoved salt fermented at three different temperatures.

Fermentation temperature, °C	**Fermentation time (Week)**	**Sample photo**	**Description**
N/A	0		The homogenous mixture of fish frames/heads and salt are in solid form. The dark grey colour of the mixture is similar to that of the ground Hoki frames and heads.
18	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.
25	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.
30	80		A dark brown/reddish brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected.

5.3 pH in Hoki Fermentations

The pH values of all Hoki ferments were monitored throughout the fermentation process and are presented in Figure 18.

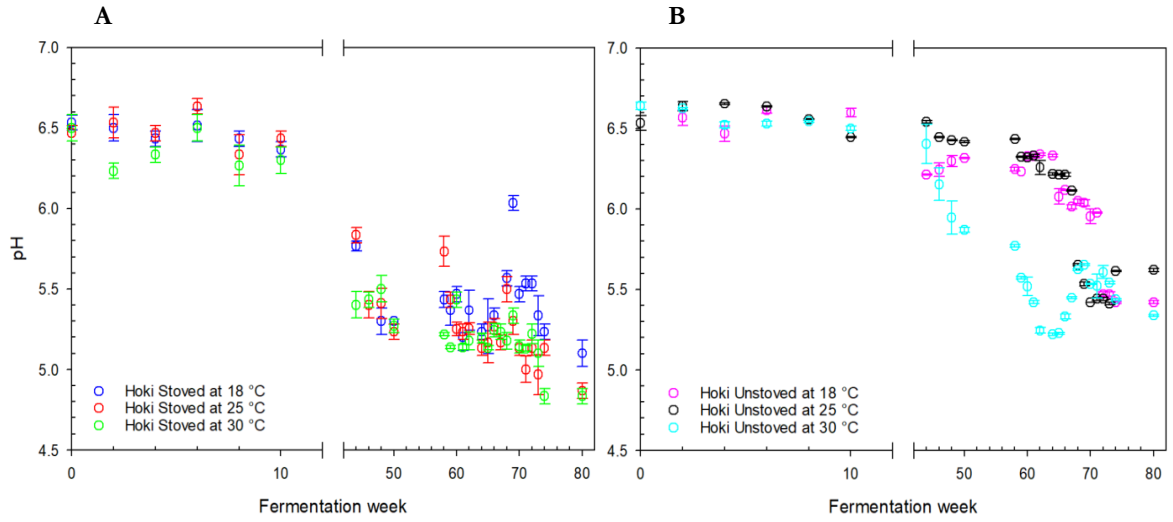


Figure 18: The pH values of fermentations from Hoki heads and frames fermented with stoved (A) and unstoved (B) salts at three different temperatures. Values represent means ± standard mean of error(n=3). n is the number of readings from the same sample bottle. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

The ferments at Week 0 represents triplicate samples with either stoved or unstoved salts as no fermentation had taken place yet and samples at all temperatures should be identical. The pH values at Week 0 of all ferments was 6.5 ± 0.1. Figure 18 shows that pH of all ferments ranged at 6.2 – 6.6 during early fermentation period (Weeks 2 – 10). Decreasing trends observed in all stoved ferments for the remainder of the fermentation period with a "bump" noticed only in 18 °C stoved between Weeks 68 – 72. Unstoved fermentations were slightly different – for 30 °C unstoved ferment, a decreasing trend observed from Week 44 with a "bump" noticed between Weeks 67 – 73. For ferments at lower temperatures, decreasing trends took place at later periods (at Weeks 62 – 64) with no "bumps" observed during the fermentation time course.

The final pH of all Hoki ferments ranged at 4.8 – 5.4 which fell within the standard pH range of a traditionally fermented fish sauce underlined by CODEX as described in Section 2.6.

Statistical analysis was conducted to determine how significantly different all the datasets were to each other. Results obtained from all pairwise comparisons based on Tukey test was presented in Figure 19. Figure 19 revealed that pH values of 18 °C and 25 °C unstoved Hoki ferments were significantly different to all stoved ferments fermented at all temperatures. Only 30 °C stoved ferment was significantly different to all unstoved ferments. The statistical analysis revealed that the type of salt regardless of the fermentation temperature may influence the pH of the fish sauce.

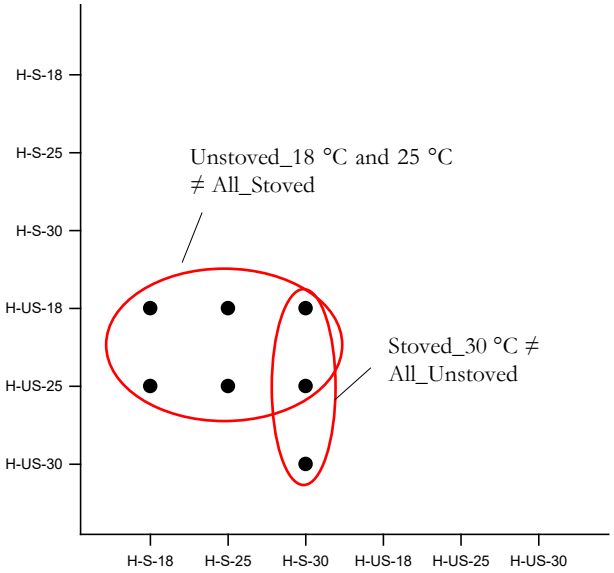


Figure 19: All pairwise comparisons based on Tukey test comparing pH values in all Hoki fermentations. The x- and y-axis labels represent each ferment using stoved and unstoved salts fermented at three different temperatures where "H" is abbreviated for Hoki, the "S" is abbreviated for stoved salt, the "US" is abbreviated for unstoved salt, and the following number is the fermentation temperature used. The "•" symbol indicates that the two compared Hoki samples are significantly different to each other (p-value < 0.05). The "≠" symbol is used to indicate "significantly different".

5.4 Volatile Fatty Acid Formation in the Fish Sauce from Hoki Frames and Heads

In this study, the concentration of VFA in fish ferments were determined throughout the fermentation time course to assess the quality of the product using gas chromatography based on the modified method of Zhao et al. (2006) as described in Section 3.4.2.

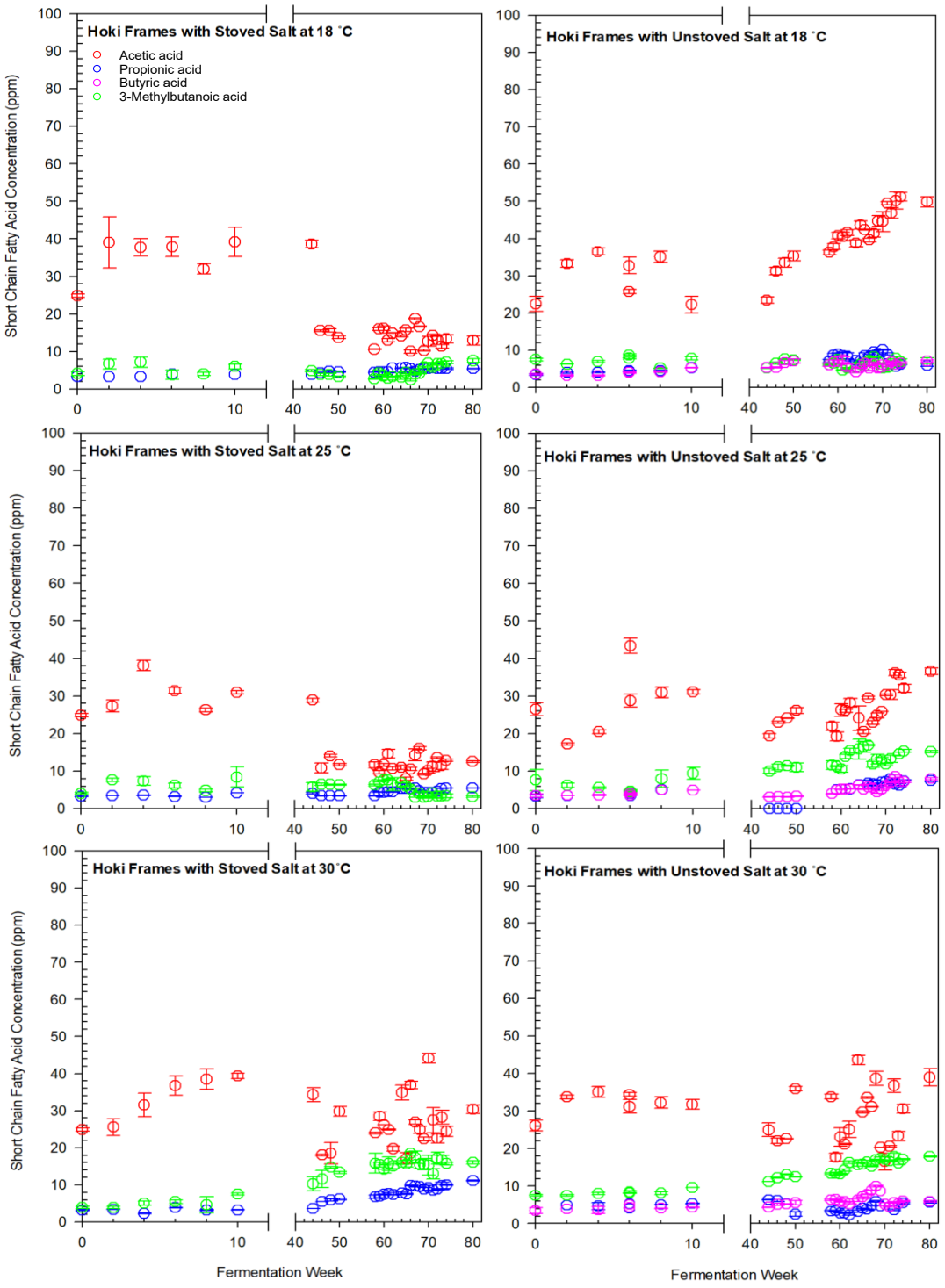


Figure 20*:* Short chain volatile fatty acids (VFA) in fish sauce samples from Hoki heads and frames with stoved and unstoved salt fermented at three different temperatures. Values represent means ± standard error of mean (n=3). n is the number of injections from the same sample vial. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9 ppm, 2 ppm, 3 ppm and 2 ppm, respectively. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

Four VFA were measured in all Hoki ferments: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Figure 20 shows that acetic acid had the highest concentration in all Hoki ferments followed by 3-methylbutanoic acid and then propionic acid. Butanoic acid was observed only in unstoved ferments. The ferments at Week 0 represents a triplicate set of samples with stoved salt plus a separate triplicate set of samples for unstoved salt, since no fermentation has yet occurred at Week 0. Apart from salt type, samples can be expected to be identical. The high level of agreement observed for all analytes across these triplicates indicates consistency in sample preparation and analysis and gives confidence in measurements made on other samples which were not checked by duplicate.

Figure 20 shows that acetic acid in stoved and unstoved Hoki ferments at Week 0 was measured at 24 ± 1 ppm and 26 ± 2 ppm, respectively. Acetic acid in all ferments hovered between 22 – 38 ppm during early fermentation period (Weeks 2 – 10) – with an outlier observed at Week 6 in the 25 °C unstoved ferment. Acetic acid in three ferments: 25 °C and 30 °C unstoved ferments as well as 30 °C stoved ferment ranged at 16 – 44 ppm during the remainder of the fermentation time course (Figure 20). Meanwhile, stoved ferments with lower temperatures fluctuated at a lower range (8 – 18 ppm) during the same fermentation period (Weeks 44 – 80), suggesting a hint of two phases of fermentation in these fermentations. An increasing trend was observed in acetic acid in the 18 °C unstoved ferment during Weeks 44 – 80 with the highest value recorded was 51 ± 1 ppm at Week 74. Based on Figure 20, acetic acid behaved differently during the fermentation time course depending on the type of salt used.

Figure 20 shows that 3-methylbutanoic in stoved and unstoved Hoki ferments at Week 0 were 4 ± 2 ppm and 8 ± 1 ppm, respectively. In 18 °C stoved and unstoved ferments as well as 25 °C stoved ferment, 3-methylbutanoic acid ranged at 3 – 8 ppm throughout entire fermentation time course.

For the other three fermentations, 3-methylbutanoic acid fluctuated at the same range of 3 – 8 ppm during early fermentation (Weeks 2 – 10), then the values rose to 10 – 20 ppm for the remainder of the fermentation period (Figure 20). There was a hint of two phases fermentation observed for higher temperature fermentations regardless of the type of salt used. As shown in Figure 20, propionic acid in all Hoki ferments at Week 0 was measured at 3 ± 0 ppm. Propionic acid in all ferments ranged at 3 – 8 ppm throughout the entire fermentation time course. Interestingly, propionic acid was not observed above the recording threshold during Weeks 44 – 50 in 25 °C unstoved ferment. Butanoic acid was observed only in unstoved fermentations and it measured 5 ± 1 ppm at Week 0. Figure 20 shows that butanoic acid fluctuated at 5 – 9 ppm during the entire fermentation time course.

Kruskal-Wallis test was conducted to determine any significant difference in all VFA identified in all Hoki fermentations and the results are presented in Figure 21. Kruskal-Wallis test is a non-parametric statistical analysis used to compare the medians of independent groups (Frost, 2024). The non-parametric method is followed by Tukey *post-hoc* test where p-value < 0.05 indicates that there is a significant difference between the datasets compared (Forest, 2024). All statitical analysis was performed using SigmaPlot Software (Systat Software Inc, California, USA).

Figure 21(A) shows that acetic acid in 18 °C unstoved ferment was significantly different from 25 °C unstoved ferment and all stoved ferments. Acetic acid in 18 °C and 25 °C stoved ferments were significantly different from 30 °C stoved ferment and unstoved ferments with the same temperatures – exception to 18 °C stoved ferment where there was no significant difference in acetic acid from 25 °C unstoved ferment. Figure 21(B) shows that 18 °C unstoved ferment and 30 °C stoved ferment were significantly different from 18 °C and 25 °C stoved ferments as well as 30 °C unstoved ferment. Figure 21(C) shows that stoved and unstoved ferments at 18 °C as well as 25 °C stoved ferment were significantly different from three fermentations: stoved 30 °C ferment, 25 °C and 30 °C unstoved ferments.

Statistical analysis also revealed that there was no significant difference between butanoic acid in unstoved Hoki fermentations.

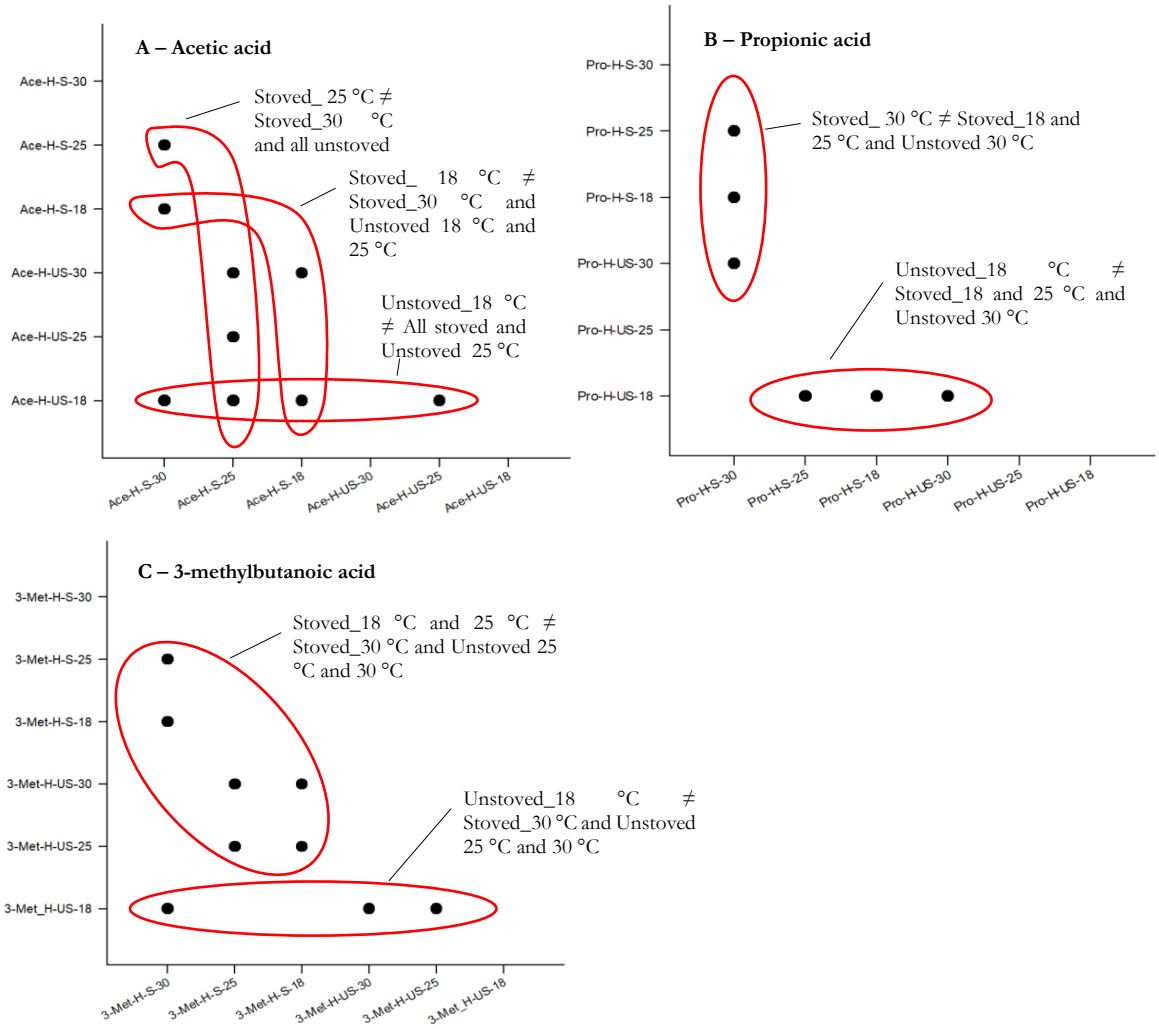


Figure 21: All pairwise comparisons based on Tukey test comparing each volatile fatty acid identified in all Hoki fermentations. Each graph represents one of three different volatile fatty acids identified and the x- and y-axis labels represent each fermentation using stoved and unstoved salts fermented at three different temperatures where "Ace" = acetic acid; "Pro" = propionic acid; "3-Met" = 3-methylbutanoic acid, "But" = Butanoic acid, the first "H" is abbreviated for Hoki, the second "S" is abbreviated for stoved salt, the "US" is abbreviated for unstoved salt and the following number is the fermentation temperature used. The "•" symbol indicates that the two compared mussel ferments are significantly different to each other. The "•" symbols are clustered and annotated to visualise the statistical results obtained. The "≠" symbol is used to indicate "significantly different".

Overall, Figure 21 revealed that three out of four VFA identified in Hoki fermentations were influenced by the type of salt used and the fermentation temperatures independently and in combination.

5.5 Bacterial Composition in the Hoki Fermentation

To understand the influence of different fermentation conditions on Hoki fermentations and on the bacterial communities, a metagenomic approached was adopted as discussed in Section 3.5.8.

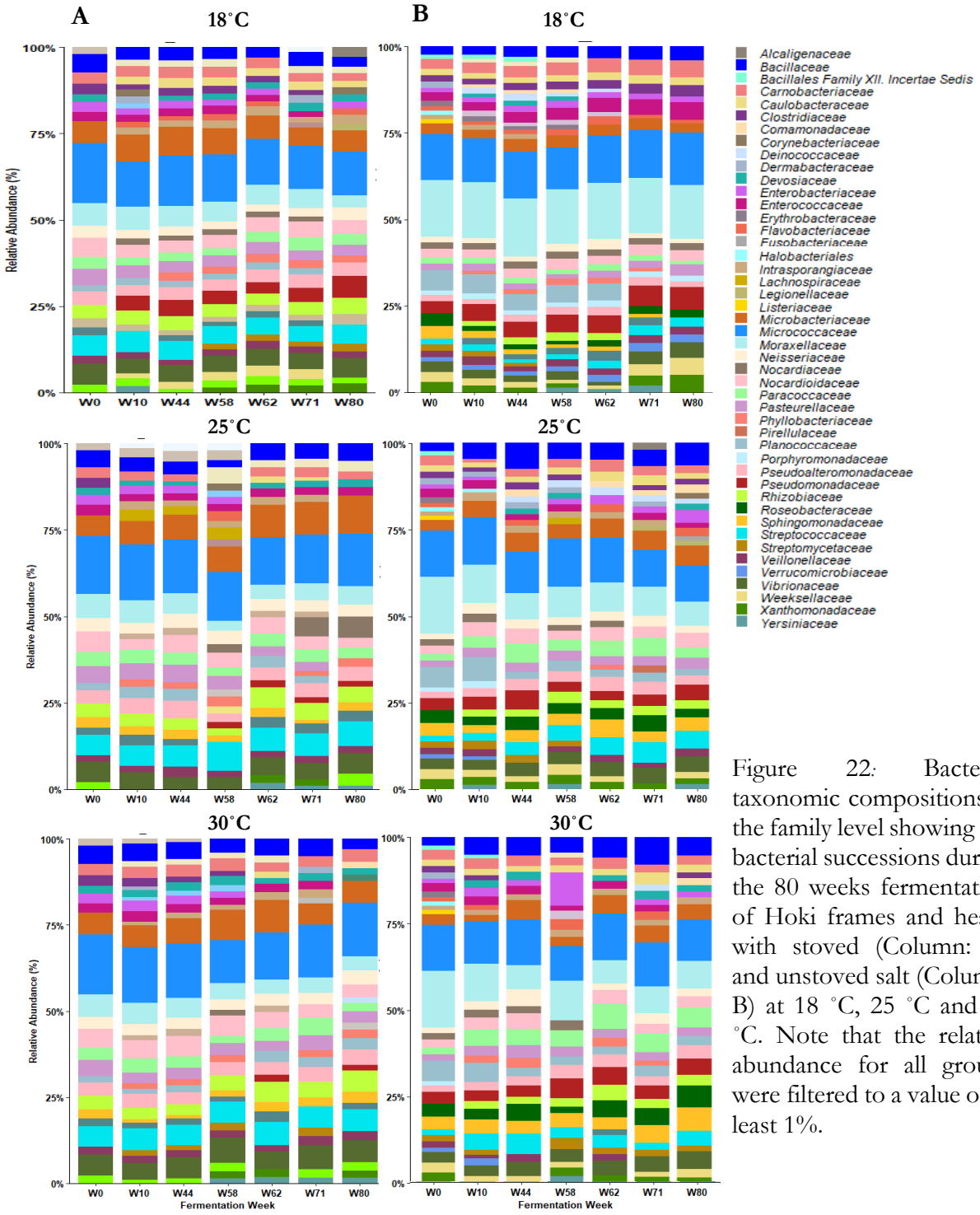


Figure 22: Bacterial taxonomic compositions at the family level showing the bacterial successions during the 80 weeks fermentation of Hoki frames and heads with stoved (Column: A) and unstoved salt (Column: B) at 18 ˚C, 25 ˚C and 30 ˚C. Note that the relative abundance for all groups were filtered to a value of at least 1%.

The 16S rRNA genes metagenomic approach does not require isolation and cultivation of microorganisms – providing a tremendous amount of bioinformatic information about naturally occurring microbial communities in a fermented fish sauce. The bacterial 16s rRNA sequencing reads were classified at the family level using the Geneious Prime 9.1.8 Software (Geneious, Dotmatics, New Zealand) to analyse the bacterial communities taxonomically in Hoki fermentations. The bacterial communities identified were expressed quantitatively in relative abundance percentages – in this current study, is referring to percentage of a bacterial family group to the total number of organisms in the environment (Hoki fermentations). The laboratory scale fermentation set-up allowed sample being withdrawn from the solid and liquor parts of the fish sauce providing a good representation of the biofilm and planktonic bacterial communities in the fermentation.

Figure 22 shows that *Micrococcaceae* predominated at Week 0 in stoved and unstoved Hoki ferments with relative abundances (RA) of 17.2 ± 0.5 % and 13.3 ± 0.4 %, respectively. *Micrococcaceae* remained predominant throughout the entire fermentation time course in all ferments. Figure 22(B) shows that *Moraxellaceae* predominated at Week 0 in all unstoved ferments (RA: 16.3 ± 0.4 %) and remained predominant throughout the entire fermentation time course – note that 18 °C unstoved ferment had the highest overall relative abundance (RA: 15.2 – 17.0 %). In contrast, *Moraxellaceae* in all stoved ferments fluctuated at much lower range (3.6 – 7.0 %) during the 80 weeks fermentation [Figure 22(A)].

Figure 22 shows that *Streptococcaceae* was steady at relative abundances between 4.8 – 8.3 % in all stoved ferments during the entire fermentation time course while the group fluctuated at a lower range (RA: 1.0 – 4.8 %) in unstoved Hoki fermentations. Similar observations made for *Microbacteriaceae* where the family group fluctuated at a higher range (RA: 6.1 – 9.5 %) in stoved fermentations than unstoved fermentations (RA: 2.5 – 5.5 %) and *Microbacteriaceae* was predominant during late fermentation period (Weeks 62 – 80) in 25 °C stoved ferment (Figure 22).

Figure 22(B) shows that *Roseobacteriaceae* was present only in unstoved Hoki fermentations with relative abundances between 1.0 – 6.0 % during the entire fermentation time course. Similarly, *Halobacteriales* was present only in unstoved Hoki ferments (RA: 1.0 - 1.9 %) but disappeared after Week 10 [Figure 22(B)].

Overall, Figure 22 shows that there are some differences in bacterial communities present between Hoki fermentations with stoved and unstoved salt. It was also observed that Bacterial diversities in stoved fermentations at final fermentation period (Week 80) were higher than at the initial (Week 0), while the opposite was observed in bacterial diversity in unstoved fermentations.

5.5.1 Heatmaps of the Hoki Fermentations

The relative abundances of all bacterial family groups identified in Hoki fermentations were visualised on heatmaps presented in Figures 23 and 24. The relative abundance of each family group is represented by a colour grid with darker green indicating higher relative abundance. The closer the distance of the cluster, in the hierarchical clustering on the y-axis indicates that the Hoki fermentations were similar in terms of the microbial composition present.

Figure 23 shows that *Micrococcaceae* predominated in all stoved ferments throughout the entire fermentation time course – as already revealed in Figure 22. *Microbacteriaceae* in 25 °C stoved ferment had higher relative abundances during late fermentation period (Weeks 62 – 80) compared to that at earlier fermentation time and compared to that in stoved ferments at other temperatures (Figure 23). Relative abundances of *Moraxellaceae, Streptococcaceae* and *Vibrionaceae* were fairly consistent throughout the entire fermentation time course of stoved ferments.

Figure 24 shows that *Micrococcaceae* and *Moraxellaceae* predominated during the 80 weeks of Hoki unstoved fermentations. No other groups predominated during any time-points in unstoved fermentations.

Overall, Figures 23 and 24 shows that almost all Hoki were clustered based on fermentation temperatures regardless of the fermentation week suggesting that bacterial communities were influenced by fermentation temperatures. In addition, it was found that the bacterial compositions in all fermentations at the initial fermentation (Week 0) were similar and clustered close to each other which was predicted as these samples were directly stored at -20 ± 2 °C prior to further analyses and no fermentation had taken place yet.

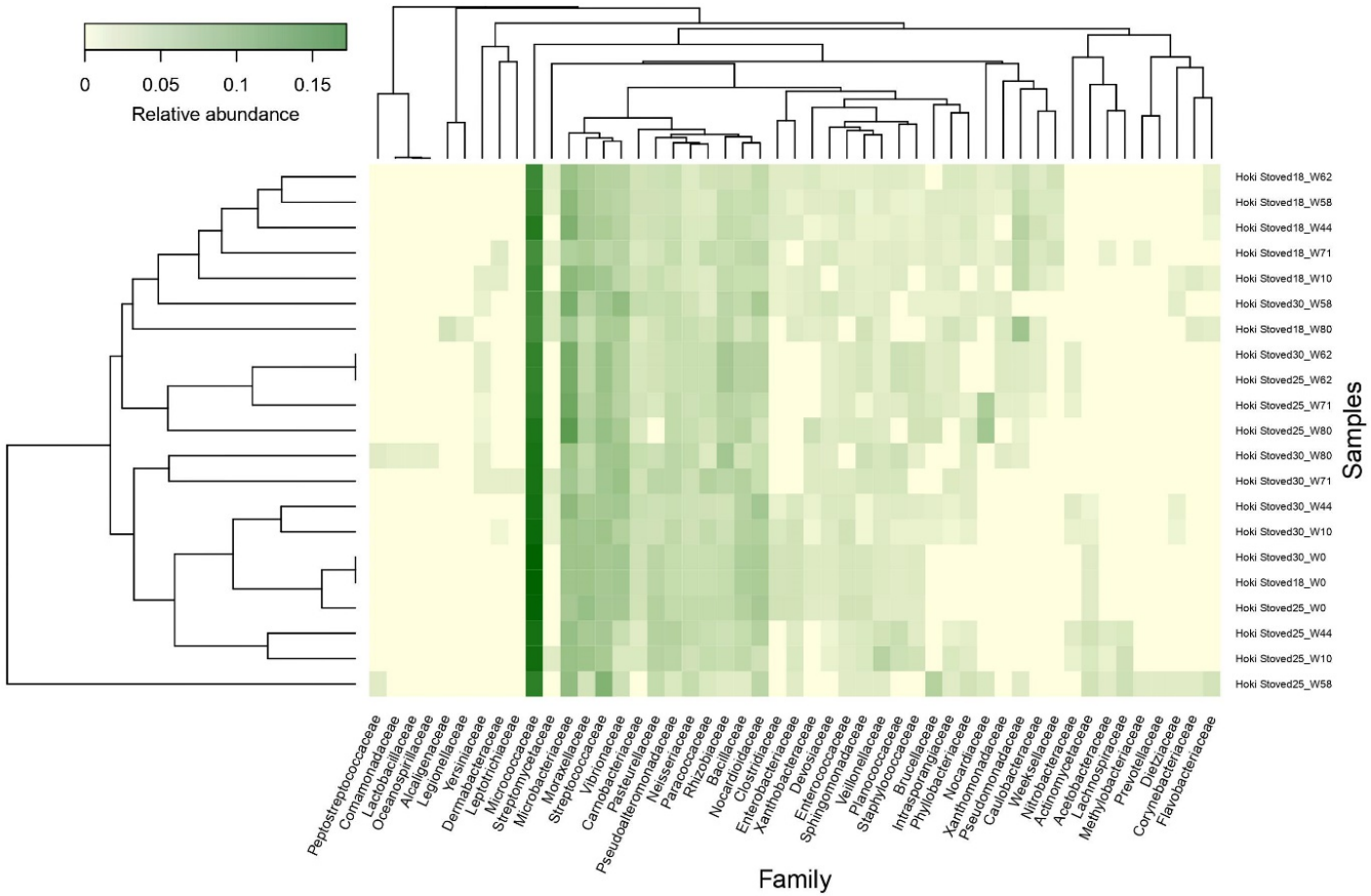


Figure 23: Heatmap of relative abundance at the family level across all Hoki samples fermented with stoved salt at 18, 25 and 30 °C for 80 weeks. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of at least 0.01. The right y-axis labels represent individual Hoki samples where the number following the "Hoki Stoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

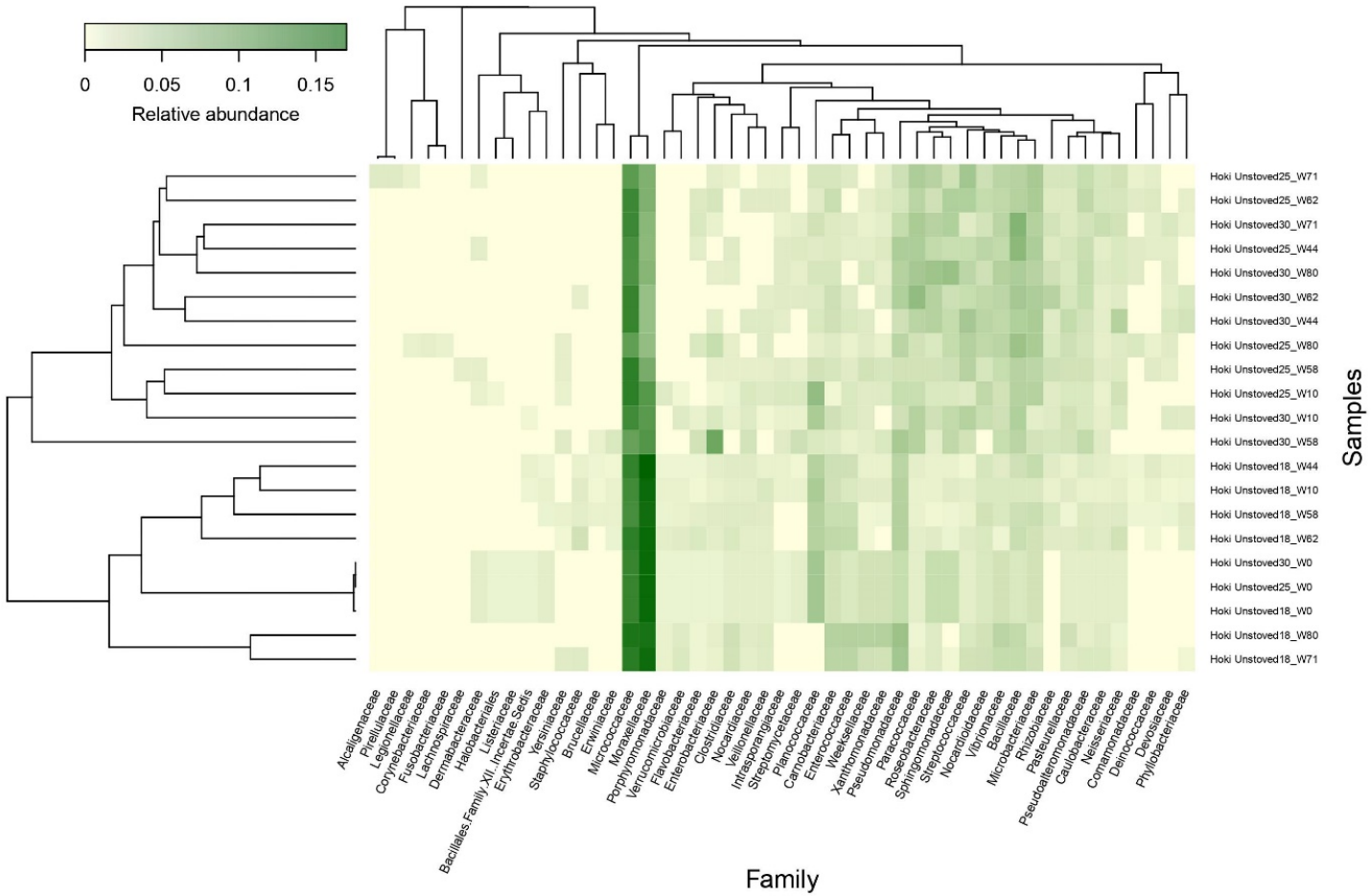


Figure 24: Heatmap of relative abundance at the family level across all Hoki samples fermented with stoved salt at 18, 25 and 30 °C for 80 weeks. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of at least 0.01. The right y-axis labels represent individual mussel samples where the number following the "Hoki Unstoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

5.5.2 Binary Comparison of Initial and Final Bacterial Composition in Hoki Fermentation

Binary comparison graphs were employed to spotlight differences in the bacterial communities between Week 0 and Week 80 and are presented in Figure 25. Note that this approach providing a comparison between only two fermentation time-points. The overview of the overall bacterial compositions in all Hoki ferments can be referred to Figures 22, 23 and 24.

The coordinates for all bacterial family groups were plotted based on the relative abundances at both Week 0 (x-axis) and Week 80 (y-axis). Family groups positioned below the 45° line indicates that the respective groups had lower relative abundance at Week 80 compared to that at Week 0. Conversely, any groups positioned above the 45° line indicates that the respective groups had higher relative abundance at Week 80 compared to that at Week 0. Family groups positioned on the 45° line indicates no difference in relative abundance at the two compared time-points.

Figure 25 shows that *Micrococcaceae* (no. 62) predominated in all Hoki ferments at the initial and final fermentation period and it was observed that its relative abundance was higher at Week 0 than at Week 80 – except in 18 °C unstoved ferment where the opposite was observed. Figure 25 also shows that *Moraxellaceae* (No. 63) predominated in all unstoved ferments at both time-points, however, significant decrease in its relative abundance was observed in ferments at higher temperatures. In 18 °C and 25 °C stoved ferments, *Pseudomonadaceae* (No. 88) was present only at Week 0 with fairly high relative abundance (RA: 4 – 6 %) while *Pseudomonadaceae* was present only at Week 80 in 30 °C stoved ferment (Figure 25). *Pseudomonadaceae* was fairly consistent in unstoved Hoki fermentations.

More than three groups were present only at Week 0 in stoved ferments and more than three groups were present only at Week 80. With unstoved salt, more than nine groups were present exclusively at Week 0 including *Bacillales Family XI. Incertae Sedis* (No. 11), *Dermabacteraceae* (No. 26), *Erythrobacteraceae* (No. 35), *Halobacteriales* (No. 44), and *Listeriaceae* (No. 57) while less than eight groups were present exclusively at Week 80.

Overall, Figure 25 shows that number of groups present exclusively at Week 0 in all stoved ferments were higher compared to that present exclusively at Week 80 indicating that bacterial diversity diminished across the fermentation period. In contrast, the opposite observation was made for all unstoved ferments suggesting that bacterial diversity at final fermentation period is higher than initial. In addition, family groups present at Week 0 were similar in ferments with the same type of salt used regardless of the fermentation temperature which was predicted as discussed in Section 5.5.1.

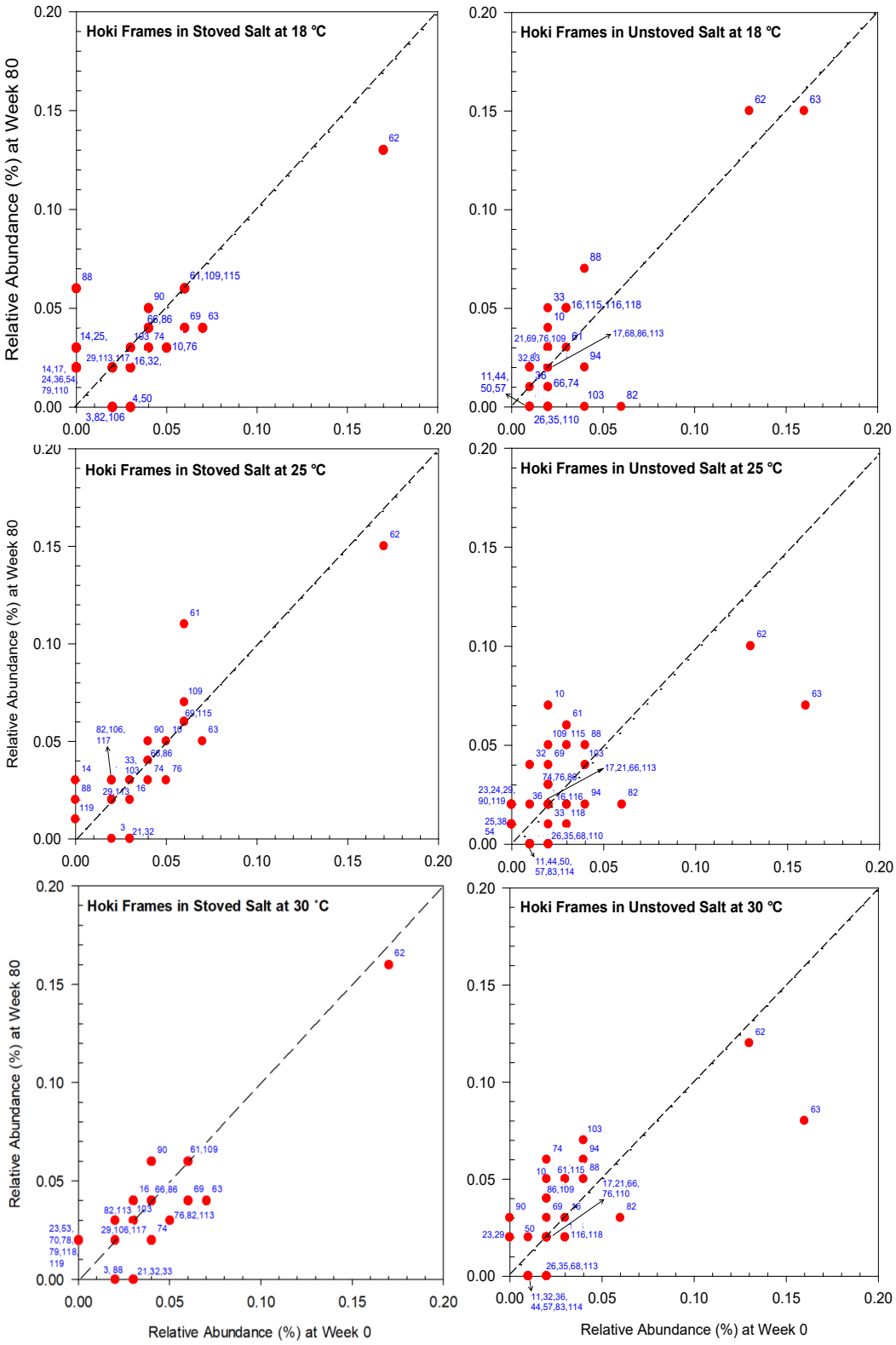


Figure 25: Binary comparison of relative abundance at the family level between two fermentation weeks in Hoki fermentations with stoved and unstoved salts fermented at three different temperatures. Family that are positioned below the 45° line indicate that the percentage of relative abundance is lower at Week 80 compared to that at Week 0 whereas family that are positioned above the 45° line indicate that the percentage of relative abundance is higher at Week 80 compared to that at Week 0. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of at least 0.01

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

5.5.3 Alpha Diversity Analysis of the Hoki Fermentations

The sequencing datasets were trimmed using Geneious Prime 9.1.8 software (Geneious, Dotmatics, New Zealand) to obtain high-quality reads which were then aligned and clustered into operational taxonomic units (OTU) before subjected to the BLAST 16s Microbial database [National Centre for Biotechnology Information (NCIB), USA] for taxonomy classification (Section 3.5.8). Alpha diversity is a statistical tool used to describe the ecological complexity within a sample – expressed in Chao1, Shannon-Weaver and Simpson's Diversity indices which were calculated using Equations 5, 6 and 7 (Section 3.5.8) (Calle, 2019). Chao1 index is a measure of species (or OTU) richness present in a community where a high value indicates a high diversity (Hughes et al., 2001). Shannon-Wiener's Index or evenness measures the abundance homogeneity of different species in a community where the closer the index number to 1, the similar the abundances of different species in the community (Calle, 2019).

Simpson's index takes into account the richness and evenness of a species in a community where a higher number indicates higher diversity (Calle, 2019). Tables 19 and 20 summarize the sequencing data reads from Hoki fish sauce samples post-software trimming procedures and alpha diversity indices calculated from the obtained sequence reads.

Chao1 and Simpson's indices in Table 19 show that bacterial diversities in stoved ferment at 18 °C, 25 °C and 30 °C was highest at Week 80, Week 62 and Week 71, respectively. Table 19 also revealed that bacterial communities in stoved ferments were more diverse during late fermentation period compared to the earlier weeks – supporting the results shown in Figures 22 and 23.

On the other hand, Chao1 and Simpson's indices in Table 20 show that bacterial diversities in all unstoved ferments were highest at Week 0. Table 20 also shows that bacterial communities were more diverse at the start of the fermentation period – as already revealed in Figures 22 and 24.

The Shannon indices of all Hoki ferments had values close to 1 (D values ≥ 0.97), indicating that the abundances of the bacterial species within each sample were similar.

Table 19: Summary of the sequencing data sets derived from fish sauce samples fermented from Hoki frames and heads with stoved salt at three different temperatures for 80 weeks and alpha diversity l analysis of bacterial diversity.

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp*	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	33256	467	122	175.62	0.98	1.82
	10	36159	467	103	142.00	0.97	1.79
	44	40125	468	105	139.21	0.97	1.77
	58	41225	467	109	140.58	0.97	1.81
	62	39784	467	110	140.87	0.98	1.80
	71	28603	469	112	142.88	0.98	1.81
	80	45703	469	146	326.07	0.98	1.89
25 ± 1°C	0	32855	468	123	176.71	0.98	1.83
	10	33154	469	102	142.32	0.97	1.83
	44	40125	469	105	143.25	0.97	1.81
	58	40325	468	104	151.83	0.98	1.84
	62	41371	467	129	225.25	0.98	1.85
	71	41125	470	128	215.15	0.98	1.84
	80	39897	470	115	201.00	0.98	1.82
30 ± 1°C	0	32158	469	122	177.01	0.98	1.82
	10	34569	468	127	178.36	0.97	1.84
	44	39785	469	130	180.11	0.97	1.85
	58	40221	471	124	238.07	0.98	1.84
	62	41371	467	129	225.25	0.98	1.85
	71	42588	467	131	303.50	0.98	1.86
	80	41051	471	125	215.05	0.98	1.84

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.

*bp = base pairs

*OTU = operational taxonomic unit.

Table 20: Summary of the sequencing data sets derived from fish sauce samples fermented from Hoki frames and heads with unstoved salt at three different temperatures for 80 weeks and alpha diversity analysis of bacterial diversity.

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp*	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	32789	466	140	214.01	1.88	0.98
	10	33125	467	120	210.52	1.87	0.98
	44	40125	468	119	212.78	1.87	0.98
	58	37158	467	121	210.98	1.86	0.97
	62	38469	467	122	211.85	1.87	0.98
	71	37891	470	115	208.21	1.86	0.98
	80	42105	471	110	198.21	1.85	0.98
25 ± 1°C	0	31398	466	145	223.06	1.89	0.98
	10	33256	467	141	219.00	1.85	0.97
	44	37299	468	147	215.33	1.83	0.98
	58	34653	467	123	214.33	1.84	0.97
	62	36190	467	101	218.00	1.85	0.97
	71	32056	468	99	211.67	1.83	0.98
	80	44505	501	112	208.32	1.80	0.98
30 ± 1°C	0	32125	467	141	209.12	1.88	0.98
	10	33546	467	115	205.25	1.83	0.97
	44	32953	467	133	208.59	1.82	0.98
	58	39041	467	128	205.77	1.78	0.97
	62	42577	468	144	203.89	1.83	0.98
	71	42187	471	131	204.24	1.82	0.98
	80	54719	471	108	203.00	1.81	0.98

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.

*bp = base pair

*OTU = operational taxonomic unit.

5.6 Bacterial Composition in Raw Hoki Heads and Frames Paste and Salts

To understand the influence of the Hoki frames and heads used in this current study on the bacterial succession during the fermentation process, 16S rRNA sequencing was employed to determine the bacterial composition in the raw material prior to fermentation process. The preparation of the Hoki fish heads and frames was described in Section 3.1.1 and the paste was subjected to DNA extraction and subsequent sequencing analysis as described in Sections 3.5.4 and 3.5.8. The bacterial composition in Hoki paste is presented in Figure 26.

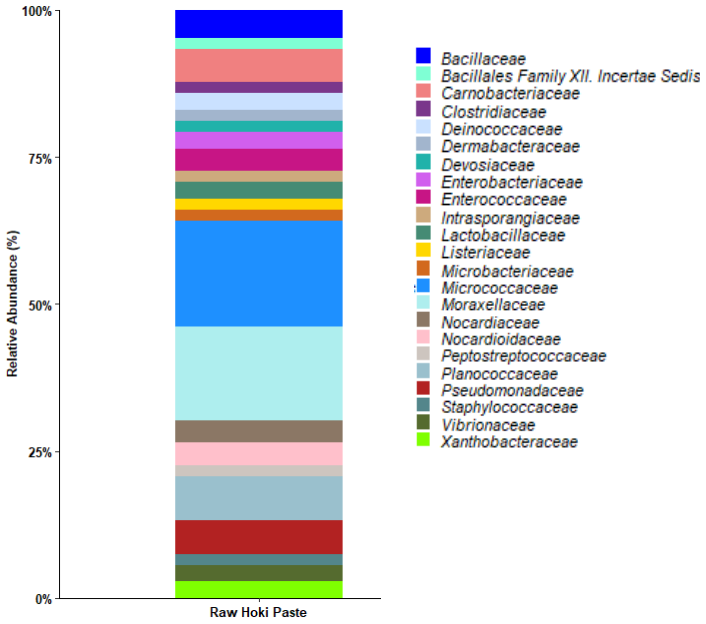


Figure 26: Bacterial taxonomic compositions at the family level showing the bacterial composition in raw material Hoki heads and frames paste. Note that the relative abundance for all groups were filtered to a value of at least 1%.

Figure 26 shows that the predominant family groups in Hoki paste were *Micrococcaceae* and *Moraxellaceae* with values of 17.9 % and 16.0 %, respectively. These two family groups were present in all Hoki ferments throughout the fermentation time course, particularly for unstoved ferments, both *Micrococcaceae* and *Moraxellaceae* were the predominant groups (Figure 22).

Other groups that present in Hoki paste and persisted throughout the Hoki fermentation time course included the LAB family groups *Carnobacteriaceae, Enterococcaceae* and *Pseudomonadaceae; Bacillaceae, Planococcaceae,* and *Vibrionaceae* (Figures 22 and 26). Figure 26 also shows that several groups present exclusively in Hoki paste but not in any of the Hoki ferments including *Lactobacillaceae, Peptostreptococcaceae, Staphylococcaceae* and *Xanthobacteraceae.* Meanwhile, more than ten family groups that were not present in Hoki paste but appeared in Hoki ferments either at a certain stage during the fermentation or throughout the entire fermenation period, this included *Halobacteriales, Roseobacteriacea, Rhizobiaceae* and *Xanthomonadaceae*. Alpha diversity indices were determined and are presented in Table 21 to further understand the bacterial diversity in Hoki paste and to compare to the overall bacterial diversities in all Hoki fermentations.

Table 21: Summary of the sequencing data sets derived from Hoki paste and statistical analysis of bacterial diversity (alpha-diversity).

Sample	**High quality reads***	**Average read length, bp***	**OTU***	**Chao1**	**Shannon index, *H***	**Simpson's index, *D***
Hoki frames and heads paste	35623	467	40	53.6	1.40	0.94

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.

*bp = base pair

*OTU = operational taxonomic unit.

Table 21 shows that the Chao1 and Shannon indices of Hoki paste were 53.6 and 1.40, respectively, which were much lower than that determined in all Hoki fermentations (Tables 19 and 20) indicating that the bacterial diversity in Hoki paste was less diverse than that found in all Hoki fermentations.

The 16S rRNA sequencing approach was also employed to determine the bacterial composition in both stoved and unstoved salts to investigate the influence of the raw materials on the bacterial successions in Hoki fermentations. However, negative sequencing results were obtained with no bacterial groups detected to be present in either salt used in this current study.

The findings prevent further conclusions to be made in regard to the hypotheses 1) the salt biofilms are the key inoculum source that will dominate the Hoki fish sauce fermentation and 2) unstoved salt provides a more diverse and/or abundant halophiles and/or halotolerant bacteria compared to the stoved salt as the key inoculum source for the Hoki fish sauce fermentation.

It is speculated that the DNA samples extracted from both of the salts were of fungi taxa. As described in Section 3.5.8, the DNA samples were subjected to PCR amplifications using the universal primers targeting the V3 – V6 region of the 16S rRNA gene of bacteria. This may explain the limitation of the universal primers used in this current study in amplifying the DNA of any fungi that may present in both stoved and unstoved salts for subsequent sequencing. It was reported that different primers was used for PCR amplification to determine the fungi compositions in fermented soy bean products (Ding et al., 2023). This current study was focused on the bacterial diversity and dynamics during fish sauce fermentation hence no further experiment was conducted to investigate the findings obtained. The focus on bacteria compositions and diversity was due to the dominant role of bacteria in the fermentation process especially in the production of key VFAs and metabolites (Han et al., 2023; Jung et al., 2012; Lopetcharat et al., 2001). However, the possibility that fungi may also influence the overall quality of a fish sauce product has not been disregarded and further studies should be conducted to understand the complex dynamic of the fermentation process. According to Han et al. (2023), *Saccharomyces, Kodamaea, Candida, Debaryomyces* and *Torulospora* are ordinary yeasts that predominated in fermented fish products after 3 months of fermentation. It was also reported that *Kodamaea ohmeri* yeast isolated from fish sauce had a strong biogenic amines degradation activity at 30 °C which could enhance the quality of the product (Yang et al., 2012).

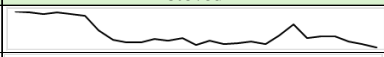
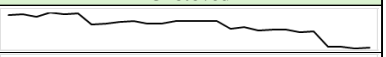
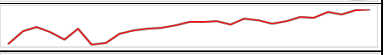
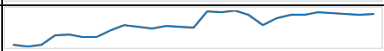
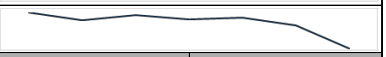
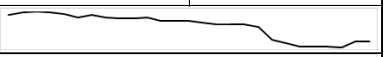
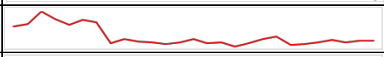
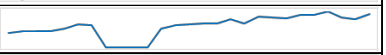
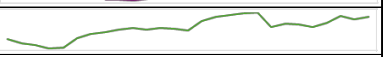
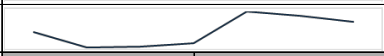
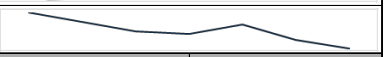
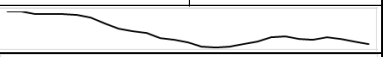
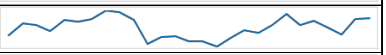
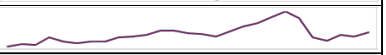
5.7 Discussion

Summary of the overall results from Hoki fermentation is presented in Table 22. Figure 18 shows that a "bump" in pH values was observed during the late fermentation period (Weeks 67 – 73) in 18 °C stoved and 30 °C unstoved ferments which may influence the presence of *Bacillaceae* in these ferments. Some genera from *Bacillaceae* family are known to be tolerant to high pH (Parkouda et al, 2014). Figure 22 shows *Bacillaceae* increased at Week 71 in 18 °C stoved ferment and there was a gradual increase of the respective group between Weeks 58 – 71 in 30 °C unstoved ferment – both observations may be related to higher pH during these fermentation weeks.

Similar findings were reported in a study on Indian fish sauce, *ngari,* where the high pH during the later stage of the fermentation was associated with the emergence of the genus Bacillus (Bacillaceae family) and its ability to ferment in alkaline conditions (Devi et al., 2015). A higher pH in fish sauce could also be attributed to the buffering capacity of non-protein nitrogen compounds, particularly biogenic amine and ammonia production during the fermentation process (Devi et al., 2015).

Findings made in Section 5.5 showed that there were differences in microbial compositions between Hoki fermentations using stoved and unstoved salts. The microbial communities in a fish sauce can influence the formation and concentration of VFA which consequently influence the overall sensory profile and quality of the final product (Ma et al., 2022). Table 23 summarizes the bacterial taxa known to possess the metabolic pathways leading to the production of volatile fatty acids including acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Based on Table 23, *Enterobacteriaceae* was associated with the production of butanoic acid. The presence of butanoic acid in all Hoki unstoved ferments may be explained by the high overall relative abundances of *Enterobacteriaceae* in the respective samples (Figure 22). In contrast, *Enterobacteriaceae* disappeared at Week 58 – 62 in all stoved Hoki ferments and coherently, no formation of butanoic acid was detected in these samples (Figures 20 and 22).

Table 22: Summary of the results from analyses of Hoki fermentation.

Temperature	Parameter	Hoki			
		Stoved		Unstoved	
18 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	W0	W80	W0	W80
		Microbacteriaceae *Micrococcaceae* *Moraxellaceae* *Vibrionaceae*	*Microbacteriaceae* *Micrococcaceae* *Pseudomonadaceae* *Vibrionaceae*	*Micrococcaceae* *Moraxellaceae* *Planococcaceae*	*Micrococcaceae* *Moraxellaceae* *Pseudomonadaceae*
25 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	W0	W80	W0	W80
		Microbacteriaceae *Micrococcaceae* *Moraxellaceae* *Nocardioidaceae* *Vibrionaceae*	*Microbacteriaceae* *Micrococcaceae* *Nocardiaceae* *Vibrionaceae*	*Micrococcaceae* *Moraxellaceae* *Planococcaceae*	*Bacillaceae* *Micrococcaceae* *Moraxellaceae*
30 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	W0	W80	W0	W80
		Microbacteriaceae *Micrococcaceae* *Moraxellaceae* *Vibrionaceae*	*Microbacteriaceae* *Micrococcaceae* *Rhizobiaceae* *Streptococcaceae* *Vibrionaceae*	*Micrococcaceae* *Moraxellaceae* *Planococcaceae*	*Micrococcaceae* *Moraxellaceae* *Paracoccaceae* *Roseobacteriaceae* *Sphingomonadaceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

Table 23 shows that *Veillonellaceae* is associated with the production of propionic acid via sugar catabolism. Figure 20 shows that propionic acid was not observed above the recording threshold between Weeks 44 – 50 in 25 °C unstoved ferment which may be explained by the disappearance of *Veillonellaceae* at Week 44 in the respective sample. Conversely, *Veillonellaceae* was present throughout the entire fermentation time course in other Hoki ferments either with stoved or unstoved salts and concurrently, propionic acid was detected at values of 3 – 8 ppm in these samples.

Figure 23 shows that *Micrococcaceae* and *Moraxellaceae* predominated throughout the entire fermentation time course in all Hoki fermentations. *Micrococcaceae* is commonly isolated in food products and are able to grow at reduced water activity, as low as 0.85 (Nuñez, 2014). *Micrococcaceae* was found to be the predominant group throughout the curing process of a dry-salted smoked beef from the Northern Spain (*cenina)* (Nuñez, 2014). It was reported that the genus *Micrococcus* which belongs to the *Micrococcaceae* family was one of the predominant genera in fermented Indian fish sauce, *ngari,* where the relative abundance of the respective genus found to be fluctuating throughout the fermentation period (Sarojnalini and Schitra, 2009). Another study on *Ngari* has found that *Micrococcaceae* is one of the predominant family groups during the fermentation period, alongside with *Staphylococcaceae, Lactobacillaceae* and *Enterococcaceae* (Devi et al., 2015). On the other hand, *Moraxellaceae* family encompasses the genera *Acinetobacter, Moraxella* and *Psychrobacter* (Zoqratt and Han, 2020). A study by Ijong and Ohta (1996) found that *Moraxella* present in fermented fish paste and fermented shrimp sauce. The genus *Acinetobacter* was found to be one of the predominant genera in the Chinese fish sauce fermented at 35 °C and was reported to be associated with the formation of key flavour compounds including 2-methyl-2-butenal, 3-methylbutanal, 1,3-cis,5-cis-octatriene, decamethyl-cyclopentasiloxane and methyl benzoate (Han et al., 2023).

Although there was no indicator of direct relationship between *Micrococcaceae* and *Moraxellaceae* and VFA production in this current study, however, it was not disregarded that these predominant groups play a role in a fermentation process in general and more specifically in Hoki fish sauce.

Table 23: Summary of the bacterial family groups known to possess metabolic pathways leading to the production of volatile fatty acids.

Volatile fatty acid	Metabolic pathway/substrate	Bacterial taxa	Optimum pH for bacterial growth	Reference
Acetic acid	L-lysine fermentation	*Peptostreptococcaceae* *Clostridiaceae* *Fusobacteriaceae* *Porphyromonadaceae*	4.8 – 8.0 6.5 - 7.0 6.9 – 7.7 5.0 – 7.6	Barker and Hendrick, 1982; Costilow et al., 1966; Mechichi et al., 2000
	L-glutamate fermentation	*Clostridiaceae* *Fusobacteriaceae* *Lachnospiraceae* *Oscillospiraceae*	6.5 - 7.0 6.9 – 7.7 6.5 – 7.5 5.2 – 7.9	Buckle, 1974; Hetzel et al., 2003
	L-alanine degradation	*Clostridiaceae*	6.5 - 7.0	Mechichi et al., 2000
Propionic acid	Theonine degradation	*Enterobacteriaceae*	5.4 – 8.0	Bell and Turner, 1977
	Isoleucine degradation	*Bacillaceae* *Burkholderiaceae* *Enterobacteriaceae* *Pseudomonadaceae*	6.0 – 7.0 5.0 – 7.0 5.4 – 8.0 5.0 – 6.0	Conrad et al., 1974; Massey et al., 1976
	Valine degradation	*Bacillaceae* *Burkholderiaceae* *Streptomycetaceae* *Pseudomonadaceae*	6.0 – 7.0 5.0 – 7.0 4.5 – 5.5 5.0 – 6.0	Marshall and Sokatch, 1972; Gonzales-Garcia et al., 2017
	Sugars catabolism (glucose, lactate and succinate)	*Clostridiaceae* *Veillonellaceae* *Propionibacteriaceae*	6.5 - 7.0 6.5 - 7.0 6.5 - 7.0	Gonzales-Garcia et al., 2017
Butanoic acid	L-glutamate degradation	*Enterobacteriaceae* *Clostridiaceae*	5.4 – 8.0 6.5 - 7.0	Baker et al., 1974
3-methylbutanoic acid	L-leucine degradation via oxidative Stickland reaction	*Clostridiaceae* *Peptostreptococcaceae*	6.5 - 7.0 4.8 – 8.0	Elsden and Hilton, 1978; Sanceda et al., 2002

Overall, the findings made on microbial compositions in all Hoki fermentations support the hypothesis that the microbial dynamics and diversity present during the fermentation period are affected by the different fermentation conditions. Figure 20 shows that all VFA identified in all Hoki fermentations were higher by the end of the fermentation time compared to their concentration at Week 0 supporting the hypothesis that the concentration of VFA increases as the fermentation time increases

5.8 Conclusion

In conclusion, the different fermentation conditions (type of salt and temperature) influenced the bacterial diversity and succession, and the VFA produced in the Hoki fish sauce samples during the fermentation process. The findings from this current study supported by literature findings confirm that the bacteria family groups identified in the Hoki fish sauce samples are common in other fish sauce products although the roles of these microorganisms especially the key family groups are yet to be further investigated and validated. It is also important to note that the natural fermentation induced a complex microbial population during fermentation and the interactions between the bacterial communities and metabolites formations during the process should be further investigated to better understand the complex process thus allowing more consistent and higher quality final products for commercialization.

Chapter 6: Fish Sauce Fermentation from Snapper (*Pagrus auratus*) Heads and Frames at Laboratory Scale

6.0 Introduction

Snapper (*Pagrus auratus*) are demersal fish commonly found in central and northern areas of New Zealand at depths of 15 to 60 metres (MPI, 2024). Snapper fish occupy a wide range of habitats including rocky reefs, areas of sand and mud bottoms (MPI, 2021). Sexual maturity occurs at an age of 3 to 4 years when they reach 20 to 28 cm long (MPI, 2024). Snapper fisheries are one of the largest and most valuable inshore fisheries in New Zealand (MPI, 2021). In 2008, New Zealand's export earnings from snapper fisheries was valued at 32 million NZD (MPI, 2008). In 1986, the Quota Management System (QMS) was introduced in New Zealand as an ongoing effort of the country for a sustainable fishery (MPI, 2020). There are 98 marine species listed in the QMS and one of them is snapper fish with a total allowable commercial catch (TACC) of 6,907,300 kg per year (MPI, 2023). With a quota limit implemented, it has come into realization that fully utilizing the commercial allowance catch is important in generating a profitable and sustainable income for the country's economy (MPI, 2023). Mainly, snapper fillets are exported with heads and frames rendered to oil and fish meal (Mohtar, 2013). Hence, heads and frames are available for added value applications in New Zealand.

Chapter 6: Fish Sauce Fermentation from Snapper (*Pagrus auratus)* Heads and Frames at Laboratory Scale

The aim of this chapter is to understand the natural fermentation process using snapper heads and frames with two types of solar salt (stoved and unstoved salts), at three different temperatures (18, 25 and 30 °C) for 80 weeks. The physical appearance, pH, bacterial composition and the formation of volatile fatty acids (VFA) of and in snapper ferments will be monitored throughout the fermentation time course to understand the influence of the raw materials used on the fermentation process.

Hypotheses that will be tested in this chapter are:

1. The microbiological composition and diversity present during the fermentation period are affected by the different fermentation conditions for the snapper ferments.
2. The concentration of volatile fatty acids produced will increase as the fermentation time increases.

Please note that there are missing data between Week 12 until Week 42 of the laboratory scale fermentation because samples were unable to be collected due to the Covid-19 pandemic restrictions.

Chapter 6: Fish Sauce Fermentation from Snapper (*Pagrus auratus)* Heads and Frames at Laboratory Scale

6.1 Experimental Method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in Figure 27.

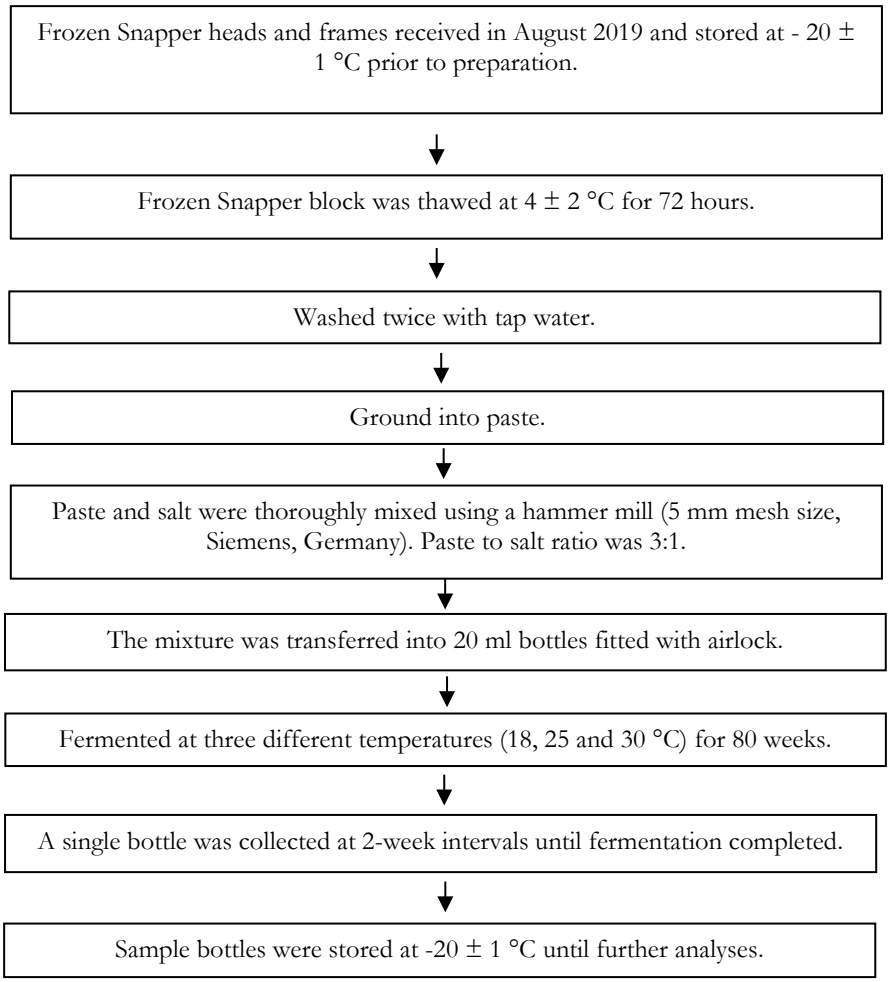


Figure 27: Experimental method flowchart of Snapper heads and frames fermentation at laboratory scale. The experiment was started in November 2020. Design of the laboratory scale fermentation was described in Section 3.3.1.

6.2 Visual Observation of the Snapper Fermentations

Based on overall observations, similar visual changes were observed in fermentations at the same temperatures regardless of the type of salt used (Tables 23 and 24).

A distinct difference was noted in the colour of ferments fermented at 30 °C where it changed from dark-grey (similar to the colour of ground snapper heads and frames) at Week 0 to dark-brown by the end of the fermentation time whereas samples fermented at lower temperatures changed to reddish-brown by Week 80. The reddish hue appeared at Week 44 for 30 °C ferments and at Week 60 and Week 64 for ferments fermented at 25 °C and 18 °C, respectively. The colour of the samples intensified as the fermentation progressed until it reached the final colour recorded in Tables 24 and 25.

The texture of the ferments changed from a firm solid homogenous mixture at the initial fermentation time to soft solid with a light brown liquid layer on the top after Week 44 for 30 °C ferments and appeared similarly at Week 60 and Week 64 for 25 °C and 18 °C ferments. At the end of the fermentation (Week 80), the textures of all ferments were soft with visible bones and scale residue settled at the bottom layer. At the start of the fermentation, the aroma of all snapper ferments was described as marine-like and fishy. As the fermentation progressed, the ferments developed a salty, pungent, fishy and ammoniacal aroma. Further investigations were conducted to determine the volatiles fatty acids associated with snapper fermentations and results obtained will be discussed in the following sections.

Table 24: Summary of the visual observations on the fish sauce ferments from snapper heads and frames with stoved salt fermented at three different temperatures.

Fermentation temperature, (°C)	**Fermentation time (week)**	**Sample photo**	**Description**
N/A	0		The homogenous mixture of fish heads/frames and salt were firm in texture. The dark grey colour of the mixture was similar to that of the ground snapper heads and frames.
18	80		A reddish brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.
25	80		A reddish brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.
30	80		A dark brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.

Table 25: Summary of the visual observations on the fish sauce ferments from snapper heads and frames with unstoved salt fermented at three different temperatures.

Fermentation temperature, °C	**Fermentation time**	**Sample photo**	**Description**
N/A	0		The homogenous mixture of fish heads/frames and salt are in solid form. The dark grey colour of the mixture was similar to that of the ground snapper heads and frames.
18	80		A reddish brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.
25	80		A reddish brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.
30	80		A dark brown liquid layer on top of solid sediment observed. Pungent aroma and strong fishy smell was detected.

6.3 pH in Snapper Fermentations

The pH values of all snapper ferments were monitored throughout the fermentation process and are presented in Figure 28.

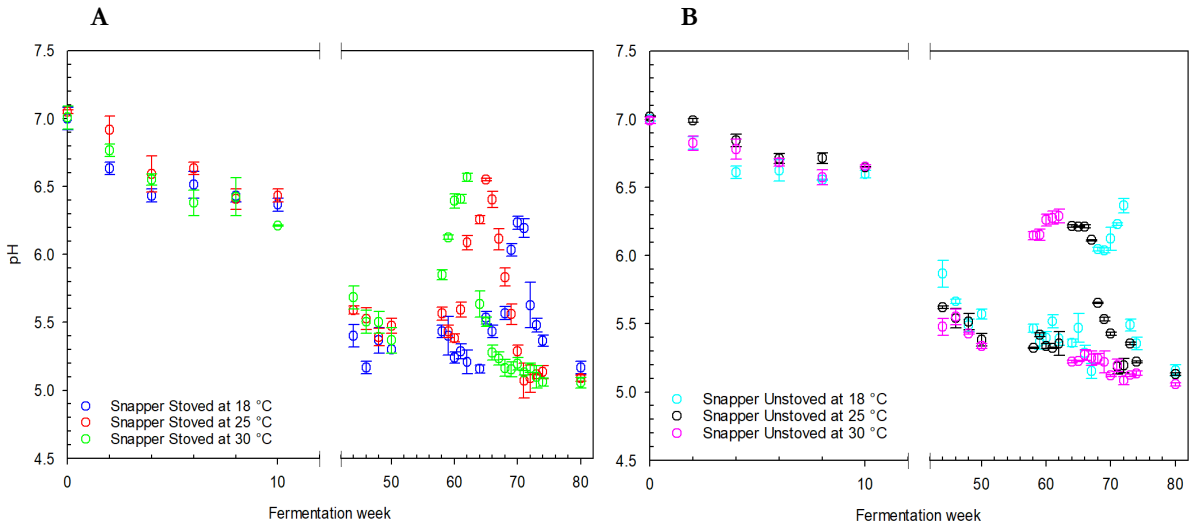


Figure 28: The pH of fermentations from Snapper heads and frames fermented with stoved (A) and unstoved (B) salts at three different temperatures. Values represent means ± standard error of mean (n=3). n is the number of readings from the same sample bottle. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

The ferments at Week 0 represents triplicate samples with either stoved or unstoved salts as no fermentation had taken place yet and samples at all temperatures should be identical. The pH values at Week 0 of all ferments was 7.0 ± 0.1. This implied that salt type had no significant effect on the starting pH. Decreasing trends were observed in all ferments during early fermentation (Weeks 2 – 10). A "bump" was observed in all ferments at different time-points depending on the fermentation temperature: Weeks 68 – 72 for 18 °C stoved/unstoved ferments; earlier fermentation weeks for higher temperatures stoved/unstoved ferments (Figure 28). The pH in all snapper ferments ranged at 5.1 – 5.4 for the remainder of the fermentation period.

The final pH values of all snapper ferments fell within the standard pH range of a traditionally fermented fish sauce outlined by CODEX as described in Section 2.6.

Statistical analysis was conducted to determine how significantly different all the datasets were to each other. Results obtained from all pairwise comparisons based on Tukey test determined that there were no significant differences between all pH values of all snapper fermentations. The statistical analysis results may indicate that the different fermentation conditions of snapper fish sauce did not influence the pH of the samples. Further analyses on other variables used in assessing the quality and progress of the fermentations in this current study will be discussed in the following sections.

6.4 Volatile Fatty Acid Formation in Snapper Fermentations

Four VFA were measured in snapper ferments: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Figure 29 shows that 3-methylbutanoic acid had the highest concentration in all snapper ferments followed by acetic acid and then propionic acid. Butanoic acid was observed only in unstoved ferments.

The 3-methylbutanoic acid concentrations in stoved and unstoved snapper ferments at Week 0 were measured at 19 ± 1 ppm and 5 ± 1 ppm, respectively (Figure 29). During early fermentation (Weeks 2 – 10), 3-methylbutanoic acid in all snapper ferments fluctuated between 30 – 70 ppm – except for 25 °C unstoved ferment where a sharp increase from 20 ppm (Week 0) to 150 ppm (Week 10) was observed. Then, 3-methylbutanoic acid in all stoved ferments and 30 °C unstoved ferment hovered between 60 – 200 ppm during Weeks 44 – 80. For unstoved ferments at lower temperatures, 3-methylbutanoic acid hovered at a higher range of 100 – 450 ppm during the same fermentation period. A hint of two phases of fermentation was observed in 3-methylbutanoic acid in all ferments.

As shown in Figure 29, acetic acid in stoved and unstoved ferments at Week 0 were measured at 14 ± 2 ppm and 5 ± 2 ppm, respectively. Acetic acid in 18 °C and 25 °C stoved ferments as well as 30 °C unstoved ferment remained steady between 14 – 30 ppm during the entire fermentation time course.

For unstoved ferments with lower temperatures (18 °C and 25 °C), a hint of two phases of fermentation was observed where the values during Weeks 50 – 80 had higher ranges of 35 – 58 ppm compared to earlier weeks with ranges between 5 – 30 ppm. In 30 °C stoved ferment, acetic acid was steady between 14 – 28 ppm during Weeks 2 – 58, then, a sharp increase was observed during the remainder of the fermentation weeks with the highest value measured of 154 ppm at Week 74 (Figure 29).

Propionic acid in stoved ferments at Week 0 were measured at 5 ± 1 ppm. Propionic acid in all stoved ferments fluctuated between 4 – 11 ppm throughout the entire fermentation time course. Propionic acid was not detected above the recorded minimum threshold at Week 0 in any unstoved ferment and was first detected at Week 4, Week 8 and Week 58 in ferments at 18 °C, 25 °C and 30 °C, respectively. Then, the values fluctuated between 3 – 11 ppm for the remainder of the fermentation period.

Butanoic acid was only present in unstoved snapper ferments – consistent with the findings made in Hoki ferments discussed in Section 5.4. Butanoic acid ranged between 4 – 13 ppm throughout the entire fermentation time course. The presence of butanoic acid exclusively in unstoved snapper fermentations may be associated with the presence/high abundance of bacterial groups unique to the ferments or the unstoved salt used.

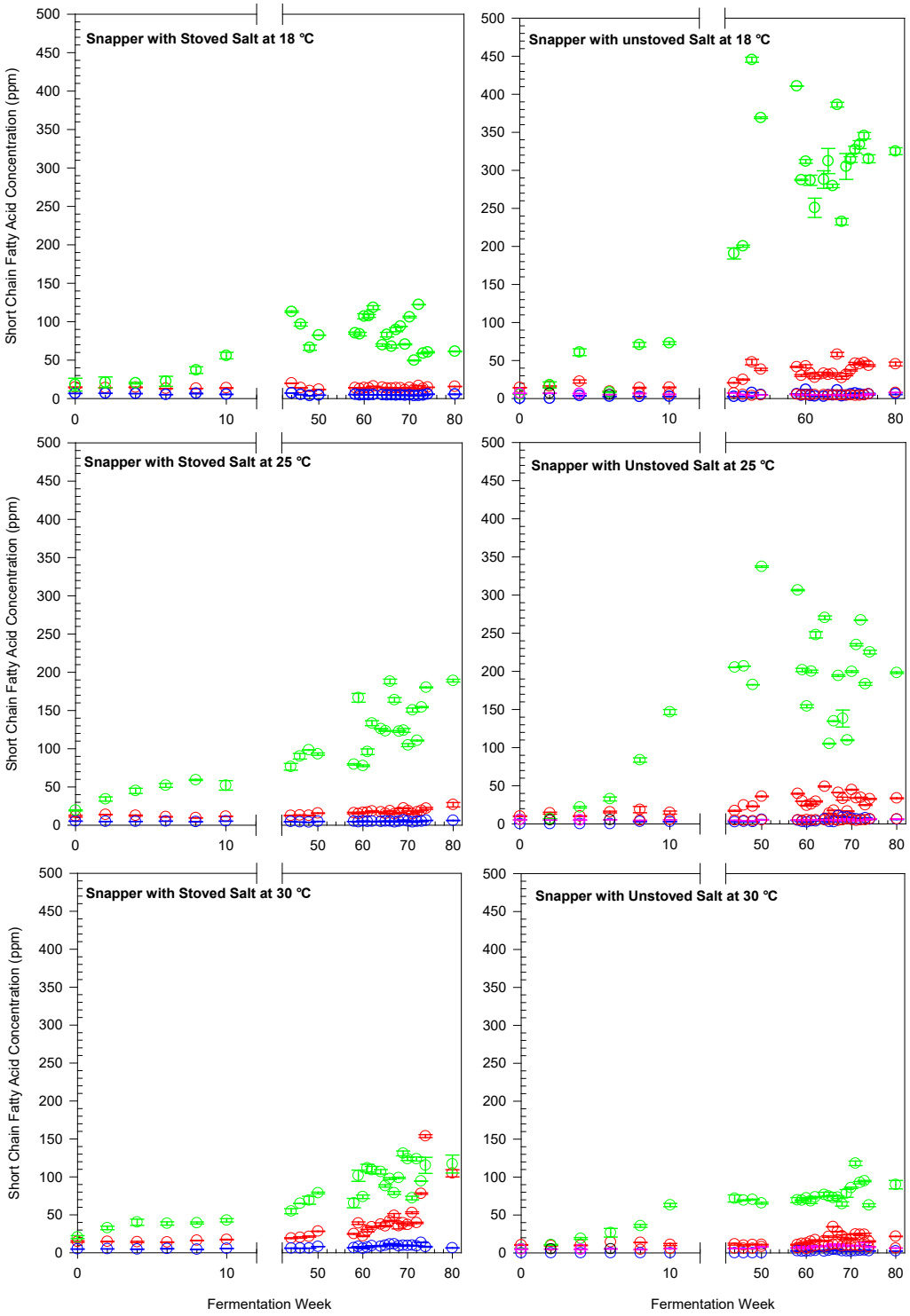


Figure 29: Short chain volatile fatty acids (VFA) in fish sauce samples from snapper heads and frames with stoved and unstoved salt fermented at three different temperatures. Values represent means ± standard error of mean (n=3). n is the number of injections from the same sample vial. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9 ppm, 2 ppm, 3 ppm and 2 ppm, respectively. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

Statistical analysis was conducted to determine any significant difference in all VFA identified in all snapper fermentations and the results are presented in Figure 30.

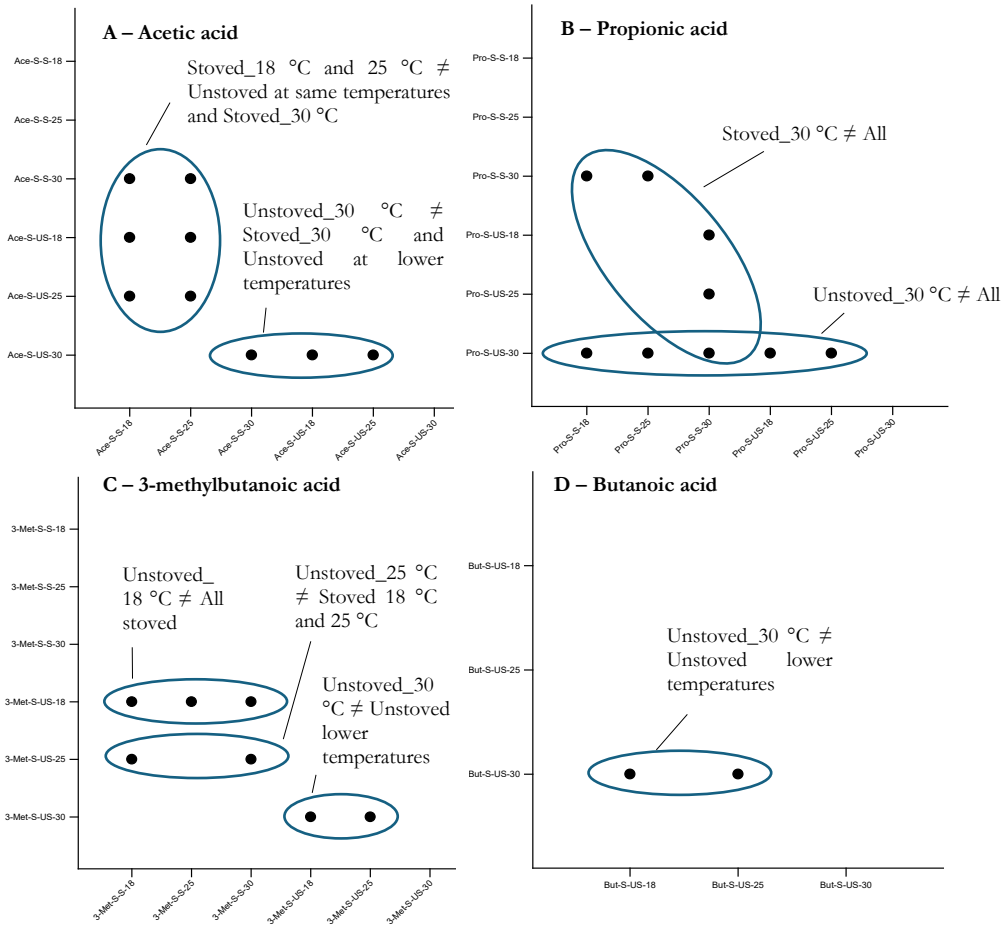


Figure 30: All pairwise comparisons based on Tukey test comparing each volatile fatty acid identified in all snapper fermentations. Each graph represents one of four different volatile fatty acids identified and the x- and y-axis labels represent each fermentation using stoved and unstoved salts fermented at three different temperatures where "Ace" = acetic acid; "Pro" = propionic acid; "3-Met" = 3-methylbutanoic acid, "But" = Butanoic acid, the first "S" is abbreviated for snapper, the second "S" is abbreviated for stoved salt, the "US" is abbreviated for unstoved salt and the following number is the fermentation temperature used. It is to note that butanoic acid is only detected in unstoved snapper fermentations. The "•" symbol indicates that the two compared snapper ferments are significantly different to each other. The "•" symbols are clustered and annotated to visualise the statistical results obtained. The "≠" symbol is used to indicate "significantly different".

Figure 30(A) shows that acetic acid in 18 °C and 25 °C stoved ferments were significantly different than that in unstoved ferments at the same temperatures and 30 °C stoved ferment.

Acetic acid in 30 °C unstoved ferment was significantly different than that in stoved ferment at the same temperature and in unstoved ferments at lower temperatures. Based on Figure 30(A), it may be suggested that acetic acid was influenced by the type of salt used and the fermentation temperatures independently and in combination.

Figure 30(B) shows that propionic acid in both stoved and unstoved fermentations fermented at 30 °C was significantly different than all other ferments. Figure 30(B) indicates that high fermentation temperature may influence the accumulation of propionic acid in snapper fermentations regardless of type of salt used.

Figure 30(C) shows that 3-methylbutanoic acid in 18 °C and 25 °C unstoved were significantly different to all stoved ferments – except that 25 °C unstoved ferment was not significantly different from stoved ferment at the same temperature. Figure 30(C) also shows that 3-methylbutanoic acid in 30 °C unstoved ferment was significantly different from unstoved fermentations at lower temperatures. Figure 30(D) revealed that butanoic acid in 30 °C unstoved ferment was significantly different than that in unstoved ferments at lower temperatures.

Overall, the statistical analysis suggests that the combination of different factors of the fermentation conditions may influence the VFA accumulations in snapper fermentations.

6.5 Bacterial Composition in Snapper Fish Sauce

To understand the influence of different snapper fermentation conditions on the bacterial communities, metagenomic approaches were adopted as discussed in Section 3.5.8.

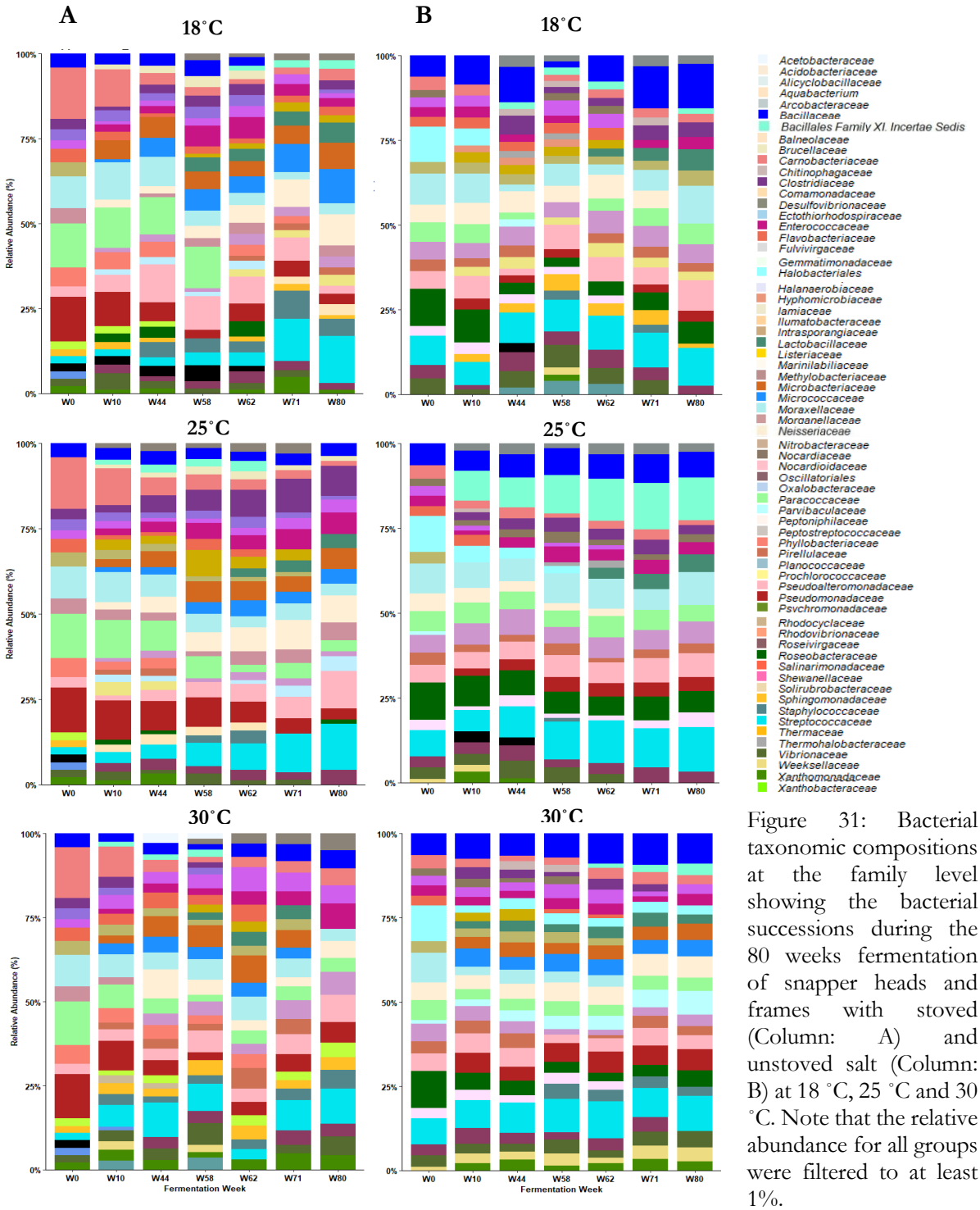


Figure 31: Bacterial taxonomic compositions at the family level showing the bacterial successions during the 80 weeks fermentation of snapper heads and frames with stoved (Column: A) and unstoved salt (Column: B) at 18 ˚C, 25 ˚C and 30 ˚C. Note that the relative abundance for all groups were filtered to at least 1%.

Figure 31(A) shows that there were four predominant groups at Week 0 in all stoved snapper ferments: *Carnobacteriacea* [Relative abundance (RA) = 15.1 ± 0.2 %], *Moraxellaceae* (RA = 9.3 ± 0.2 %), *Paracoccaceae* (RA = 13.0 ± 0.1 %) and *Pseudomonadaceae* (RA = 13.1 ± 0.5 %). These four groups predominated in all stoved ferments until about Week 10 – 58 before their relative abundances decreased by the end of the fermentation period.

Figure 31(A) shows that the relative abundance of *Streptococcaceae* in all stoved ferments increased gradually during the fermentation time course and it eventually became the predominant group by Week 80. It was also observed that *Micrococcaceae* was present above the recorded threshold in all stoved ferments from Week 10 and was one of the predominant groups at Week 80 in the 18 °C stoved ferment [Figure 31(A)]. Other groups that appeared from Week 10 onwards were *Enterococcaceae, Microbacteriaceae* and *Neisseriaceae* where their relative abundances gradually increased during the remainder of the fermentation time.

Figure 31(B) shows that there were also five predominant groups at Week 0 in all unstoved snapper ferments: *Bacillaceae* (RA = 6.4 ± 1.0 %), *Halobacteriales* (RA = 10.6 ± 0.5 %), *Moraxellaceae* (RA = 9.0 ± 1.0 %), *Roseobacteriaceae* (RA = 11.1 ± 0.2 %) and *Streptococcaceae* (RA = 9.0 ± 0.5 %). Only *Bacillaceae* and *Streptococcaceae* remained as the predominant groups throughout the entire fermentation time course in all unstoved ferments while others either decreased in relative abundance or disappeared by Week 80 [Figure 31(B)].

It is important to point out that *Halobacteriales* was only present in unstoved ferments which was consistent with results obtained in Hoki fermentations as discussed in Section 5.5. *Halobacteriales* remained present throughout the entire fermentation time course only in 30 °C unstoved ferment while it disappeared at Week 44 and Week 58 in 18 °C and 25 °C unstoved ferments, respectively.

Figure 31 also shows that *Lactobacillaceae* appeared during late fermentation period in all stoved and unstoved ferments.

Overall, Figure 31 shows that there are some differences in bacterial communities present between snapper fermentations with stoved and unstoved salt, particularly the predominant family groups. It was also observed in snapper fermentations with stoved salt that particular family groups predominated during the early fermentation period and were replaced by others during the later fermentation period, in contrast to the findings made for fermentations with unstoved salt where same family groups predominated throughout the fermentation period. Bacterial diversities in all snapper fermentations were higher during the mid-fermentation period (between Week 44 and 62) compared to that during the initial and final fermentation periods.

6.5.1 Heatmaps of the Snapper Fish Sauce

The relative abundances of all bacterial family groups identified in all snapper fermentations were further visualised on heatmaps presented in Figures 32 and 33. Figure 32 shows that *Carnobacteriaceae, Moraxellaceae, Paracoccaceae* and *Pseudomonadaceae* were the predominant groups in all stoved ferments during the early fermentation period and their relative abundances were lower during the late fermentation period as already revealed in Section 6.5. *Streptococcaceae* predominated during late fermentation period in all stoved ferments (Figure 32). It was also revealed that *Clostridiaceae* had higher relative abundances during the late fermentation period (between Week 58 until 80) in stoved ferments fermented at lower temperatures.

Figure 33 shows that *Bacillaceae* and *Streptococcaceae* were the predominant groups throughout the entire fermentation period in all unstoved ferments as revealed in Section 6.5. Figure 33 shows clearly that the *Bacillales Family XI. Incertae Sedis* was a predominant group from Week 10 until Week 80 in 25 °C unstoved ferment while hardly appearing under other conditions.

Overall, Figures 32 and 33 show that the bacterial diversities in all snapper fermentations were higher during mid fermentation compared to that during initial and final fermentation period supporting the findings made in Section 6.5. There was no noticeable difference in bacterial diversities between Week 0 and Week 80 in all snapper fermentations however, it was noted that the family groups may differ between the two time-points. Figures 32 and 33 also revealed that the snapper fermentations were clustered based on fermentation temperatures regardless of the fermentation week suggesting that the bacterial communities were influenced by the fermentation temperatures. In addition, it was found that the bacterial compositions in all fermentations at the initial fermentation (Week 0) were similar and clustered close to each other which was predicted as these samples were directly stored at -20 ± 2 °C prior to further analyses and no fermentation had taken place yet.

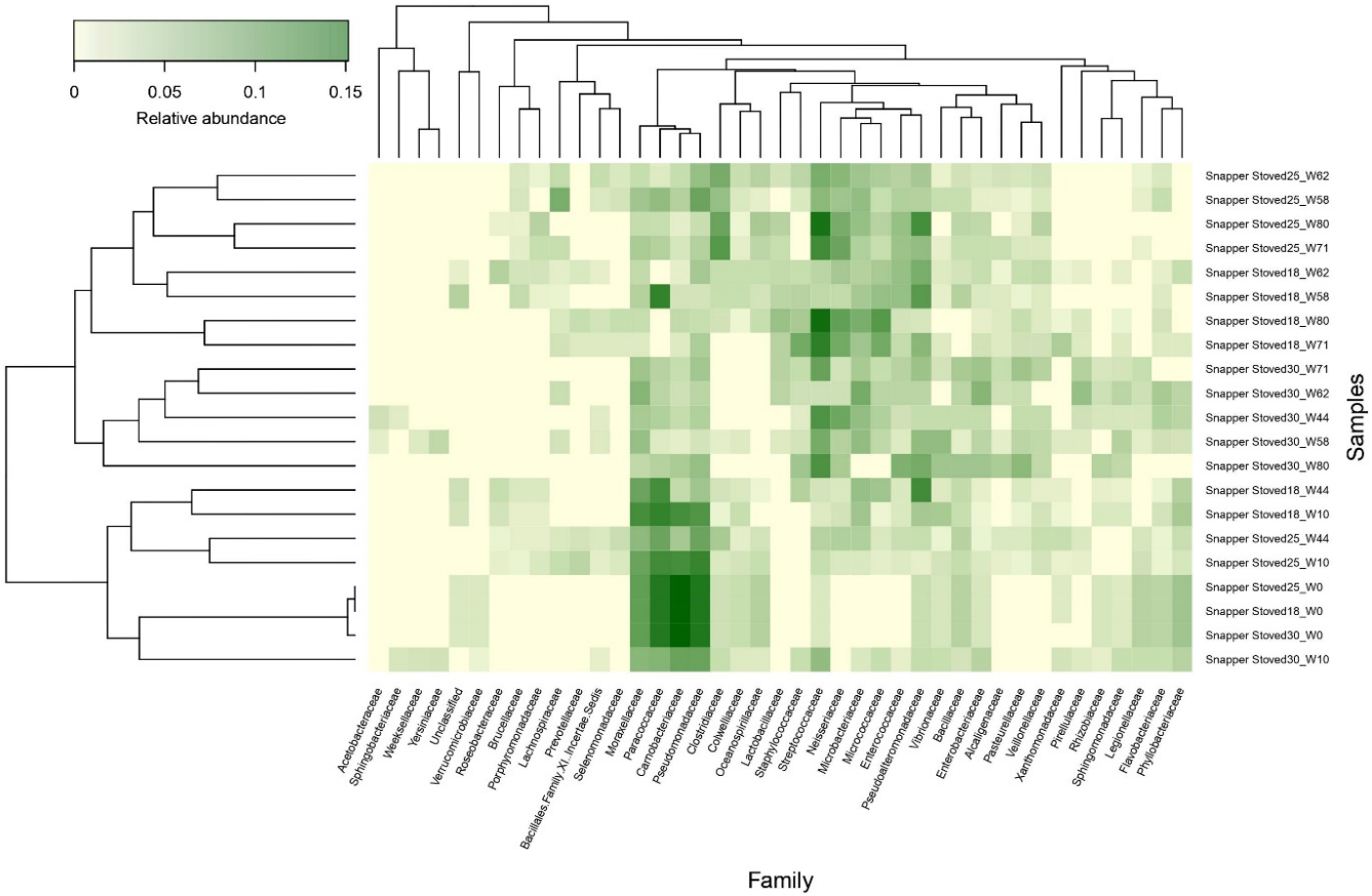


Figure 32: Heatmap of relative abundance at the family level across all snapper samples fermented with stoved salt at 18 °C, 25 °C and 30 °C for 80 weeks. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual snapper fermentations where the number following the "snapper Stoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

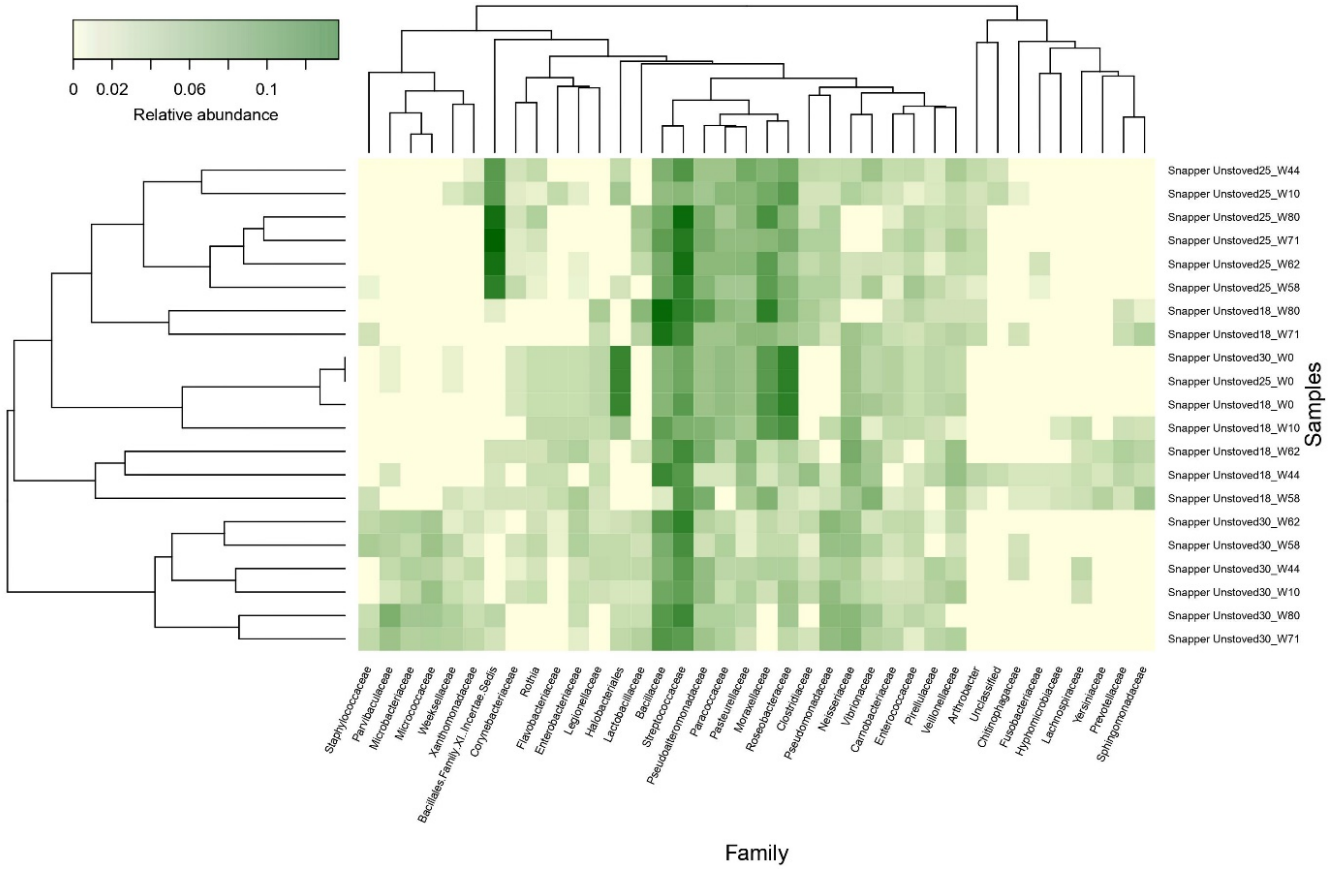


Figure 33: Heatmap of relative abundance at the family level across all snapper samples fermented with unstoved salt at 18 °C , 25 °C and 30 °C for 80 weeks. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual snapper fermentations where the number following the "snapper Unstoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

6.5.2 Binary Comparison of Initial and Final Bacterial Composition in Snapper Fermentations

Binary comparison graphs were employed to spotlight differences in the bacterial communities between Week 0 and Week 80 and are presented in Figure 34. Relative abundances of *Streptococcaceae* (no. 109) were consistently higher at Week 80 than Week 0 in all snapper ferments, particularly with stoved salt where *Streptococcaceae* became the predominant group by Week 80. With unstoved salt, *Streptococcaceae* was already the predominant family group at Week 0.

Figure 34 shows that *Carnobacteriaceae* (no. 16), *Moraxellaceae* (no. 63), *Paracoccaceae* (no. 74) and *Pseudomonadaceae* (no. 88) were the predominant groups at Week 0 in stoved ferments and all showed noticeably lower relative abundances by Week 80 (positioned below the 45° line). Figure 34 also shows that *Microbacteriaceae* (no. 61), *Micrococcaceae* (no. 62) and *Neisseriaceae* (no. 66) were not present at Week 0 in any stoved ferments but were the predominant groups at Week 80 as revealed in Figures 31, 32 and 33. *Halobacteriales* (No. 44) predominated at Week 0 in all unstoved ferments and remained present by Week 80 only in 30 °C unstoved ferment.

More than seven family groups were present exclusively at Week 0 in all stoved ferments, including *Legionellaceae* (no. 54), *Phyllobacteriaceae* (no. 79) and *Verrucomicrobiaceae* (no. 114) while more than six family groups were present exclusively at Week 80 including *Enterococcaceae* (no. 33), *Pasteurellaceae* (no. 76) and *Veinollaceae* (no. 113). With unstoved salt, there were more than five family groups that were present only at Week 0 while more than four family groups were present only at Week 80. Figure 34 shows that there were no apparent differences between the number of groups that were present exclusively at both Week 0 and Week 80 in all snapper ferments indicating that the bacterial diversities between the two time-points may not be very different.

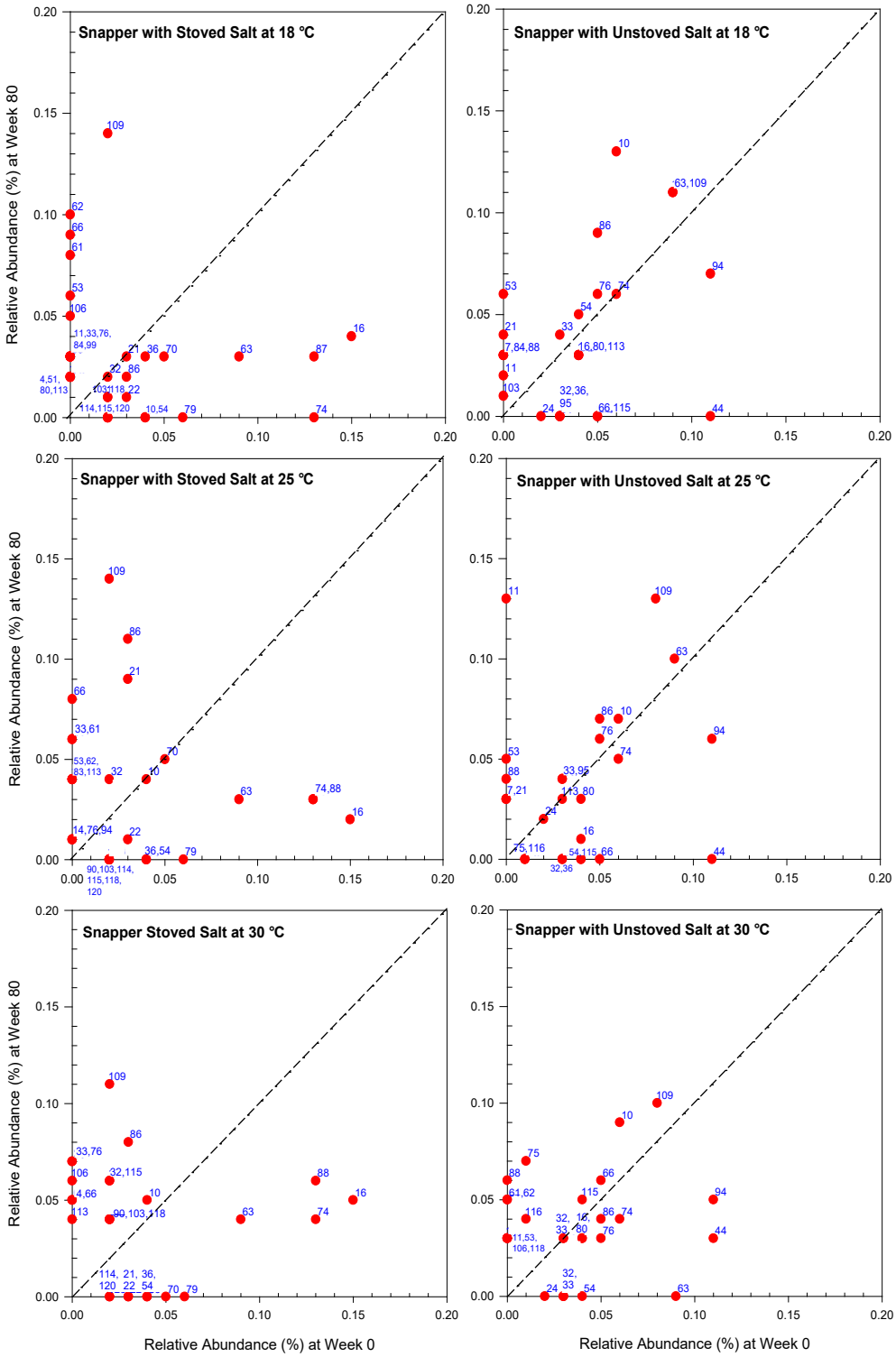


Figure 34: Binary comparison of relative abundance at family level between two fermentation weeks in Snapper samples with stoved and unstoved salts fermented at three different temperatures. A position below the 45° line indicates relative abundance is lower at Week 80 compared to Week 0 whereas a family positioned above the 45° line has relative abundance higher at Week 80 compared to Week 0. The relative abundance of the bacterial taxon at family level was filtered to a value of at least 0.01.

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

Despite this, the bacterial groups found only at Week 80 differ across the three different temperatures suggesting that the bacterial compositions during fermentation was influenced by the fermentation temperatures as already revealed. Overall, Figure 34 shows that the predominant groups at Week 0 were different compared to that at Week 80 indicating that the predominant groups at initial fermentation were eventually replaced by other groups by the end of fermentation week.

6.5.3 Alpha Diversity Analysis of the Snapper Fermentations

Tables 26 and 27 summarize the sequencing data reads from snapper fermentations post-software trimming procedures and alpha diversity indices calculated from the obtained sequence reads. Based on the Chao1 indices in Table 26, there were small differences in the bacterial diversities in all snapper fermentations with stoved salt between Week 0 and Week 80. The Chao1 indices show that bacterial diversities in stoved ferments fermented at 18 °C, 25 °C and 30 °C were highest at Week 62 (Chao1 = 201.41), Week 62 (Chao1 = 180.23) and Week 71 (Chao1 = 172.36), respectively. Consequently, the Simpson's indices shows that the highest values were obtained in stoved ferments at 18 °C (H = 1.85), 25 °C (H = 1.85) and 30 °C (H = 1.83).

Similar to results represented in Table 26, it is shown in Table 27 that there were small differences in Chao1 indices obtained for Week 0 and Week 80 for all unstoved snapper ferments. The highest Chao1 indices for unstoved ferments at 18 °C, 25 °C and 30 °C were obtained at Week 58 (Chao1 = 201.25), Week 62 (Chao1 = 183.74) and Week 62 (Chao1 = 198.72), respectively. Consequently, the highest Simpson's indices for unstoved ferments at 18 °C, 25 °C and 30 °C were determined at Week 58 (H = 1.85), Week 62 (H = 1.84) and Week 62 (H = 1.83), respectively. The Chao1 and Simpson's indices obtained for all snapper ferments indicate that the bacterial communities were more diverse during mid fermentation period compared to that during the earlier and final periods which supports the findings discussed previously in Sections 6.5.1 and 6.5.2.

The Shannon indices of all snapper fish sauce have values close to 1 (D values ≥ 0.97), indicating that the abundances of the bacterial species within each sample are similar.

Table 26: Summary of the sequencing data sets derived from fermentations of snapper heads and frames with stoved salt at three different temperatures for 80 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	35261	466	115	160.23	0.98	1.78
	10	33245	467	120	155.51	0.99	1.77
	44	35986	467	124	154.62	0.98	1.78
	58	40125	466	137	200.12	0.99	1.84
	62	41365	471	136	201.41	0.98	1.85
	71	39001	471	125	211.32	0.98	1.85
	80	38798	467	120	161.33	0.98	1.80
25 ± 1°C	0	35211	467	123	152.41	0.98	1.81
	10	36256	467	120	153.41	0.98	1.80
	44	37412	466	122	156.45	0.98	1.81
	58	37256	465	132	171.23	0.97	1.84
	62	36458	471	131	180.23	0.99	1.85
	71	40123	471	127	164.84	0.99	1.83
	80	41025	471	122	157.22	0.98	1.80
30 ± 1°C	0	36997	467	125	161.23	0.98	1.78
	10	36554	467	122	162.32	0.98	1.77
	44	35412	470	127	159.87	0.97	1.76
	58	35245	471	133	171.69	0.97	1.80
	62	35612	466	134	172.36	0.97	1.83
	71	36211	467	136	170.25	0.98	1.82
	80	35412	467	121	158.92	0.98	1.77

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

Table 27: Summary of the sequencing data sets derived from fermentations of snapper heads and frames with unstoved salt at three different temperatures for 80 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	38256	469	125	176.21	0.98	1.77
	10	37442	471	130	180.12	0.98	1.81
	44	36554	468	127	177.36	0.97	1.76
	58	37444	468	140	201.25	0.98	1.85
	62	31254	466	137	189.87	0.98	1.84
	71	35967	468	133	188.77	0.98	1.83
	80	40012	470	128	177.52	0.97	1.79
25 ± 1°C	0	40123	471	124	176.24	0.98	1.80
	10	38754	472	125	175.35	0.96	1.78
	44	39745	472	131	180.12	0.98	1.81
	58	38425	471	133	181.23	0.97	1.83
	62	37415	469	134	183.74	0.97	1.84
	71	38794	469	132	182.65	0.98	1.82
	80	35661	467	126	177.15	0.98	1.77
30 ± 1°C	0	40127	467	121	173.25	0.98	1.76
	10	41236	466	127	173.74	0.98	1.76
	44	38975	465	133	177.36	0.99	1.78
	58	36945	471	135	180.01	0.97	1.80
	62	35784	470	140	198.72	0.97	1.83
	71	36451	470	133	183.45	0.98	1.85
	80	37891	468	125	175.15	0.97	1.82

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

6.6 Bacterial Composition in Raw Snapper Frames and Heads Paste

To understand the influence of the snapper heads and frames used in this current study on the bacterial compositions during the fermentation process, 16S rRNA sequencing was employed to determine the bacterial composition in the snapper paste prior to fermentation process. The preparation of the snapper paste was described in Section 3.1.1.1 and the paste was subjected to the DNA extraction and subsequent sequencing analysis as described in Section 3.3.5 and Section 3.3.9. The bacterial composition in raw snapper paste is presented in Figure 35.

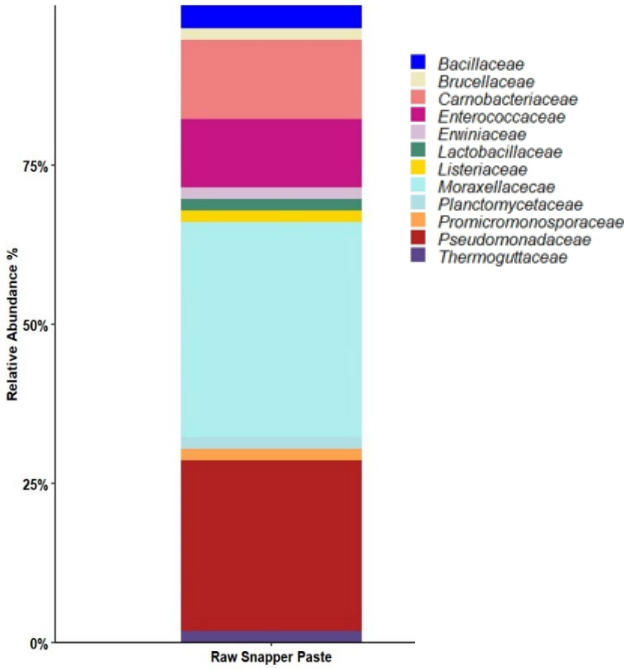


Figure 35: Bacterial taxonomic compositions at the family level showing the bacterial composition in raw material Snapper heads and frames paste. Note that the relative abundance for all groups were filtered to a value of equal and higher than 1%.

Figure 35 shows that the predominant family groups in raw snapper pastes were *Moraxellaceae* and *Pseudomonadaceae* with the relative abundances of 33.9 % and 26.8 %, respectively. *Moraxellaceae* was one of the predominant groups in all snapper ferments at Week 0 and it remained as one throughout the fermentation time course in 18 °C and 25 °C unstoved ferments (refer to Figure 31).

Pseudomonadaceae was also one of the predominant groups in all stoved ferments during Weeks 10 – 58 before the relative abundances gradually decreased during the remainder of the fermentation period. Meanwhile, *Pseudomonadaceae* was not present at Week 0 in all unstoved ferments and only appeared from Week 10 onwards.

Figure 35 also shows that *Erwiniaceae, Promicromonosporaceae* and *Thermoguttaceae* were exclusively present in snapper paste but not in any of the snapper fermentations. Alpha diversity indices were determined and presented in Table 28 to further understand the bacterial diversity in the snapper paste and to compare that to the overall bacterial diversities in all snapper fermentations.

Table 28: Summary of the sequencing data sets derived from snapper paste and statistical analysis of bacterial diversity (alpha-diversity).

Sample	**High quality reads***	**Average read length, bp**	**OTU***	**Chao1**	**Shannon index, *H***	**Simpson's index, *D***
Snapper heads and frames paste	38625	468	12	22.5	0.80	0.80

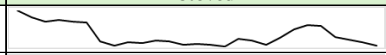
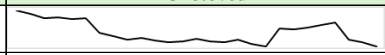
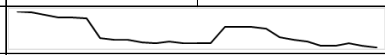
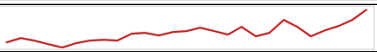
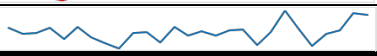
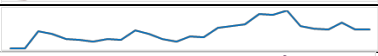
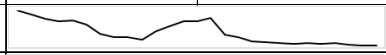
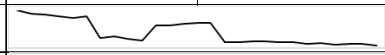
*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

Table 28 shows that the Chao1 and Shannon indices of snapper paste was 22.5 and 0.80, respectively, which were much lower that that determined in all snapper fermentations (Tables 26 and 27) indicating that the bacterial diversity in snapper paste was less diverse than that found in all snapper fermentations.

6.7 Discussion

Summary of the overall results from snapper fermentation is presented in Table 29. Findings made in Section 6.5 showed that there were differences in microbial compositions between snapper fermentations using stoved and unstoved salts. The microbial communities in a fish sauce can influence the formation and concentration of VFA which consequently influence the overall sensory profile and quality of the final product (Ma et al., 2022). Figure 29 shows that butanoic acid was only present in unstoved ferments but not in stoved ferments. In a study on the microbial communities during the fermentation of a Chinese shrimp paste, it was reported that *Bacillus* genus which belongs to the *Bacillaceae* family was associated with the production of butanoic acid (Dai et al., 2018). The presence of butanoic acid in all snapper fermentations with unstoved salt may be explained by the high relative abundances of *Bacillaceae* in the respective samples. In contrast, *Bacillaceae* was present at noticeably lower relative abundances in all snapper fermentations with stoved salt throughout the 80 weeks fermentation period and coherently, no formation of butanoic acid was detected in these samples (Figures 29 and 31). Figure 29 also shows that the concentrations of 3-methylbutanoic acid were the highest in all snapper ferments and this may be explained by the high relative abundances of LAB family groups in all fermentations during the fermentation process. LAB family groups include *Carnobacteriaceae, Streptococcaceae, Lactobacillaceae* and *Enterococcaceae.* It was found that these four groups were among the predominant family groups in all snapper ferments either during certain fermentation weeks or throughout the entire fermentation and their aggregated relative abundances were more than 10.0 % in all ferments (Figure 31). In a study on flavour-formation mechanisms in LAB, it was found that the bacterial groups were involved in metabolic pathways leading up to the production of 3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid via amino acid degradations (Liu et al., 2014).

Table 29: Summary of the results from analyses of snapper fermentation.

Temperature	Parameter	Snapper			
		Stoved		Unstoved	
18 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Carnobacteriaceae *Moraxellaceae* *Paracoccaceae* *Pseudomonadaceae*	*Lactobacillaceae* *Microbacteriaceae* *Neisseriaceae* *Streptococcacea*	*Bacillaceae* *Halobacteriales* *Moraxellaceae* *Roseobacteriaceae* *Streptococcaceae*	*Bacillaceae* *Lactobacillaceae* *Moraxellaceae* *Paracoccaceae* *Pseudoalteromonadaceae* *Streptoccaceae*
25 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Carnobacteriaceae *Moraxellaceae* *Paracoccaceae* *Pseudomonadaceae*	*Clostridiaceae* *Enterococcaceae* *Microbacteriaceae* *Neisseriaceae* *Streptococcacea*	*Bacillaceae* *Halobacteriales* *Moraxellaceae* *Roseobacteriaceae* *Streptococcaceae*	*Bacillaceae* *Bacillales Family XI. Incertae* *Carnobacteriaceae* *Moraxellaceae* *Pasteurellaceae* *Pseudoalteromonadaceae* *Roseobacteriaceae* *Streptoccaceae*
30 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Carnobacteriaceae *Moraxellaceae* *Paracoccaceae* *Pseudomonadaceae*	*Enterococcaceae* *Pesteurellaceae* *Pseudoalteromonadaceae* *Pseudomonadaceae* *Streptococcacea*	*Bacillaceae* *Halobacteriales* *Moraxellaceae* *Roseobacteriaceae* *Streptococcaceae*	*Bacillaceae* *Neisseriaceae* *Parvibaculaceae* *Pseudomonadaceae* *Streptoccaceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

The metabolic pathways leading up to the flavour compounds are presented in Figure 36. *Pseudomonadaceae* is associated with the production of propionic acid via isoleucine and valine degradations (refer to Table 22, Section 5.7). The absence of propionic acid in snapper fermentations with unstoved salt between Week 0 until Week 8 as presented in Figure 29 may be explained by the absence of *Pseudomonadaceae* at Week 0 in these samples (Figure 31).

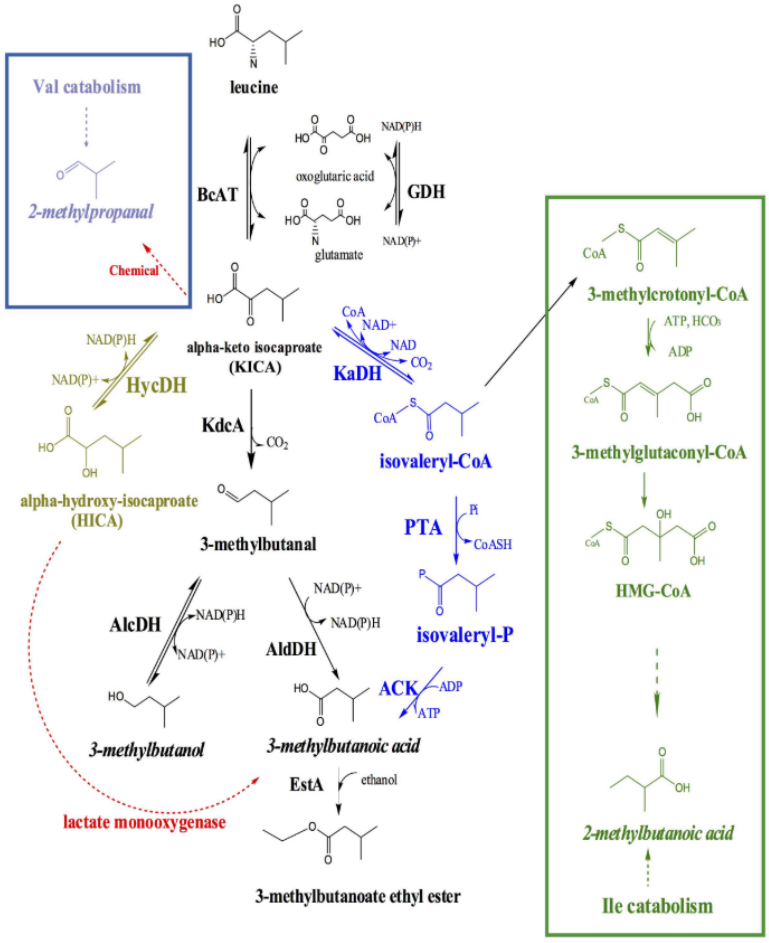


Figure 36: Leucine degradation pathways in lactic acid bacteria (LAB) including the inter-conversion pathways between leucine degradation and valine catabolism (framed by blue box) and between leucine degradation and isoleucine catabolism (framed by green box). Three branches of subsequent degradation of alpha-keto isocaproate (KICA) are depicted in three different colours where gold depicts degradation to alpha-hydroxy-isocaproate (HICA); black depicts the conversion to the corresponding aldehyde, alcohol or carboxylic acid via alpha-keto acid decarboxylation; and blue depicts the oxidative decarboxylation producing isovaleryl-CoA. Enzymes names are: BcAT, branched-chain aminotransferase; GDH, glutamate dehydrogenase; HycDH, hydroxyacid dehydrogenase; KdcA, alpha-ketoacid decarboxylase; AlcDH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; EstA, esterase A; KaDH, alpha-ketoacid dehydrogenase complex; PTA, phosphotransacylase; ACK, acyl kinase. (Liu et al., 2014).

It was observed that *Pseudomonadaceae* appeared at Week 10 and remained present until Week 80 in all unstoved ferments and coherently, propionic acid was present in these ferments at Week 10 until Week 80 with the values ranged at 3 – 11 ppm during the fermentation time course (Figures 29 and 31).

The bacterial dynamics are complex and often involve intricate interactions between different microbial groups (Giraffa, 2004). The presence/abundance of some family groups may affect the presence/abundance of another groups (Jung et al., 2011). The complex and interesting microbial relationships may be depicted by the presence/abundance of *Paracoccaceae* during snapper fermentations in correlation to the presence/abundance of *Pseudomonadaceae. Paracoccaceae* was reported to be able to utilize propionic acid (Kim et al., 2019). The absence of propionic acid in unstoved ferments during early fermentation which was assumed due to the absence of *Pseudomonadaceae,* may also explain the low relative abundances of *Paracoccaceae* during these periods (refer to Figure 31). On the other hand, the high relative abundances of *Paracoccaceae* in stoved ferments may be due to the consistent presence of propionic acid at values ranging between 4 – 7 ppm during the entire fermentation time course (Figures 29 and 31). However, no further conclusions can be drawn to confirm the dynamics between *Pseudomonadaceae* and *Paracoccaceae* due to limited information from literature. The relationships between the family groups can be either symbiotic, competitive or neutral and understanding these dynamics is crucial to develop a more comprehensive understanding of microbial ecosystems and their roles during fish sauce fermentation in order to produce high quality products (Giraffa, 2004).

The pH may also influence the bacterial compositions in an environment and is deemed as an indicator of the quality of the fish sauce (Jung et al., 2011; Mueda, 2015).

Figure 31 shows that *Lactobacillaceae* appeared during mid-fermentation (at Week 58/62) in all snapper ferments and remained present until the end of the fermentation (Week 80). In many of the fermentations of different conditions, the relative abundances of *Lactobacillaceae* increased as the fermentation time increased (Figure 31). The presence/abundance of *Lactobacillaceae* may be due to the decrease in pH values during the fermentation process as presented in Figures 28 and 31. In a study on kimchi fermentation, it was reported that *Leuconostocaceae* predominated when the pH was higher (pH 5.4 – 5.5) during early fermentation while the relative abundance of *Lactobacillaceae* increased during late fermentation period where the pH values were lower (pH 4.9 – 5.0) (Jung et al., 2011).

As discussed in Sections 6.5.3, the bacterial communities in all snapper fermentations were more diverse during mid-fermentation (between Week 58 - 71) compared to that during initial and final weeks. The high bacterial diversity may be explained by the "bumps" in the pH values observed between Weeks 60 and 71 in all fermentations as presented in Figure 28. In some cases, near neutral pH is considered favourable for bacterial diversity as these conditions are often associated with a broader range of available nutrients and a more stable environment (Wu et al., 2013). Many bacterial groups can adapt to this relatively mild pH, leading to increased diversity as various organisms find suitable niches for growth (Wu et al., 2013). However, it is also important to emphasize that bacteria exhibit a wide range of pH tolerances and different species may prosper under specific conditions hence, further investigation should be conducted to better understand the bacterial diversity during the fermentation process of snapper fish sauce.

Overall, the findings made on the microbial compositions in all snapper fermentations support the hypothesis that the microbial dynamics and diversity present during the fermentation period are affected by the different fermentation conditions.

Figure 29 shows that the concentration of 3-methylbutanoic acid in all snapper fermentations showed an increasing trendline during the fermentation time supporting the hypothesis that the concentration of VFA increases as the fermentation time increases. It was noted that the concentrations of other VFA identified in all snapper fermentations fluctuated during the fermentation time. However, in most fermentations, the concentrations of acetic, propionic and butanoic acids were higher by the end of the fermentation time compared to their concentrations at Week 0.

6.8 Conclusion

In conclusion, the different fermentation conditions both type of salt and temperature influenced the bacterial diversity and compositions as well as the VFA produced in the snapper fermentations during the fermentation process. The findings from this current study also imply that there may be correlations in the presence/abundance between specific bacterial groups and the others. Further investigations should be conducted to further understand these relationships that may be unique to snapper fish sauce.

Chapter 7: Green-shell Mussel (*Perna canaliculus*) Fermentation at Laboratory Scale

7.0 Introduction

Green-shell mussels (*Perna canaliculus*) are filter-feeding molluscs unique to New Zealand making them one of the most iconic seafoods in the country (MPI, 2019). The green-shell mussels are distributed throughout New Zealand but most common in central and northern parts where they frequently form dense beds of up to 100 m^2 (MPI, 2019). The species is a dioecious (unisexual) broadcast spawner in which the gonadal development takes place at temperatures above 11 °C (MPI, 2019). Most spawning occurs in late spring to early autumn and sexual maturity of a green-shell mussel is commonly observed when the shell reaches 40 mm in length (MPI, 2019). New Zealand's mussel industries rely on catching wild spat (juvenile mussels) around the coastline which then grow on longline technology that uses continuous crop ropes ranging between 3,000 and 4,200 meters in length (Sanford Ltd., 2024). New Zealand currently processes approximately 100,000 tonnes of green-shell mussels for export and domestic supply per annum (MPI, 2023). The aquaculture industry dominated by mussel is projected to grow from NZ$ 600 million in 2019 to NZ$ 3 billion by 2035, making it a highly prospective industry for economic growth (MPI, 2023).

The aim of this chapter is to understand the natural fermentation process using green-shell mussels with two types of solar salt (stoved and unstoved salts), at three different temperatures (18, 25 and 30 °C) for 80 weeks. The physical appearance, pH, bacterial composition and the formation of volatile fatty acids (VFA) of and in the mussel ferments will be monitored throughout the fermentation time course to understand the influence of the raw materials used on the fermentation process.

Hypotheses that will be tested in this chapter are:

1. The microbiological composition and diversity present during the fermentation period are affected by the different fermentation conditions for the mussel ferments.
2. The concentration of volatile fatty acids produced will increase as the fermentation time increases.

Please note that there are missing data between Week 12 until Week 42 of the laboratory scale fermentation because samples were unable to be collected due to the Covid-19 pandemic restrictions.

7.1 Experimental Method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in Figure 37.

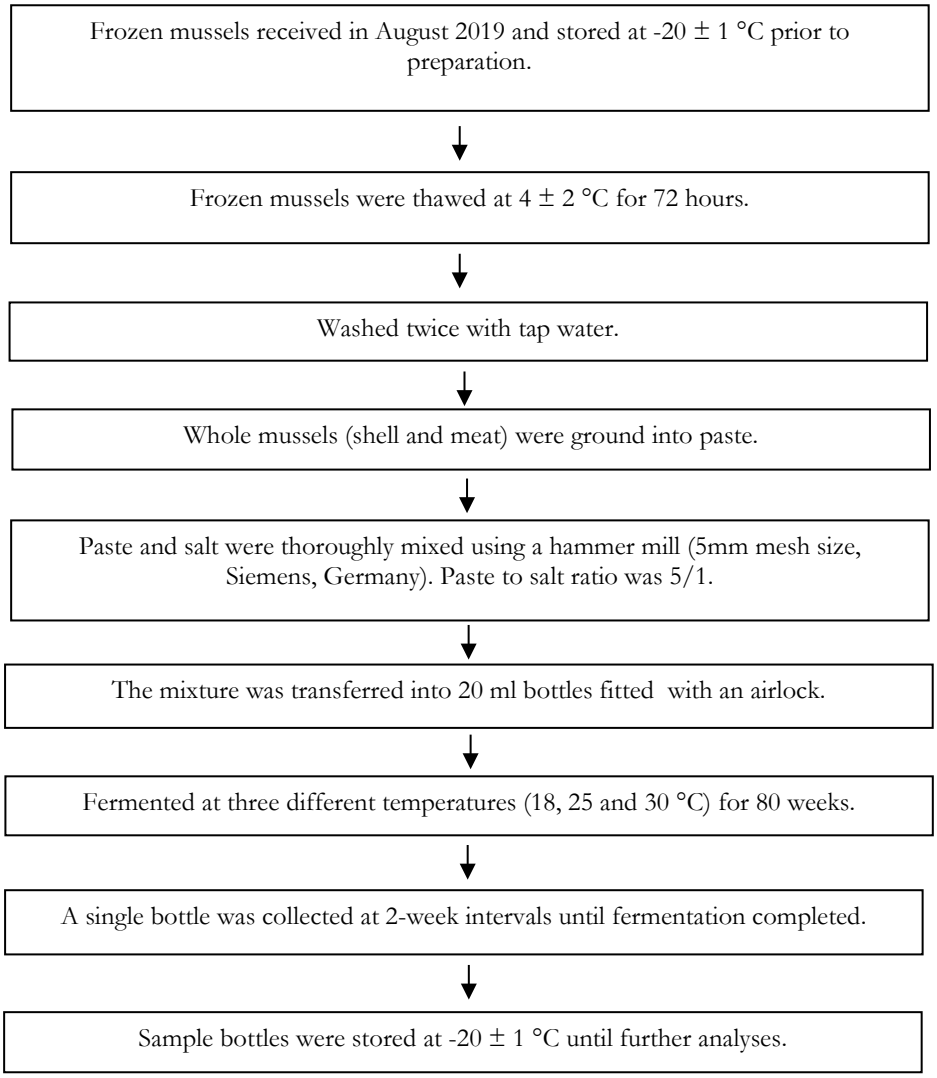


Figure 37: Experimental method flowchart of the green-lipped mussel fermentation at laboratory scale. The experiment was started in November 2020. Design of the laboratory scale fermentation was described in Section 3.3.2.

7.2 Visual Observation of the Mussel Ferments

Based on overall observations, similar visual changes were observed in the fermentations at the same temperatures regardless of the type of salt used (Tables 30 and 31). The texture of the ferments, observed when handling samples, changed from firm meat with shell particles at the initial fermentation time to a combination of soft meat and shell particles with a liquid layer trapped in-between after Week 60 for ferments at 30 °C and appeared similarly at Week 68 and Week 71 for ferments fermented at 25 °C and 18 °C, respectively.

A distinct difference was noted in colour of the liquid layer trapped between the solid particles of the 30 °C ferments where it changed from brown colour with a yellow hue at Week 0 (similar to the colour of ground mussel) to dark brown by the end of the fermentation whereas lower temperature ferments changed to reddish-brown by Week 80.

At the start of the fermentation, the aroma of all mussel ferments was described as sweet and ocean-like. As the fermentation progressed, the ferments developed a salty, pungent, fishy and ammoniacal aroma with a hint of ocean-like aroma remaining by the end of the fermentation. Further investigations were conducted to determine the volatile fatty acids associated with mussel ferments and results obtained will be discussed in the following sections.

Table 30: Summary of the visual observations on the fish sauce ferments from green-shell mussels with stoved salt fermented at three different temperatures.

Fermentation temperature, (°C)	**Fermentation time (week)**	**Sample photo**	**Description**
N/A	0		The mixture of mussel and salt are firm with shell particles. The brown colour with yellow hue of the mixture is similar to that of the ground mussel. Sweet and ocean-like aroma were detected.
18	80		Reddish brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.
25	80		Reddish brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.
30	80		Dark brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.

Table 31: Summary of the visual observations on the fish sauce ferments from green-shell mussels with unstoved salt fermented at three different temperatures.

Fermentation temperature, (°C)	Fermentation time (week)	Sample photo	Description
N/A	0		The mixture of mussel and salt are firm with shell particles. The brown colour with yellow hue of the mixture is similar to that of the ground mussel. Sweet and ocean-like aroma were detected.
18	80		Reddish brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.
25	80		Reddish brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.
30	80		Dark brown liquid layer trapped in between dark brown solids observed. No gas bubble was observed in the airlock. Pungent, fishy smell with a hint of ocean-like aroma detected.

7.3 pH in Mussel Fermentations

The pH values of all mussel ferments were monitored throughout the fermentation process and are presented in Figure 38.

The ferments at Week 0 represents triplicate samples with either stoved or unstoved salts as no fermentation had taken place yet and samples at all temperatures should be identical. The pH values at Week 0 of all ferments was 7.1 ± 0.1. This implied that salt type had no significant effect on the starting pH. The pH of all ferments were steady at 7.0 – 7.2 during early fermentation (Weeks 2 – 10). Small spikes were observed in all ferments at different time-points depending on the fermentation temperatures: Weeks 44 – 60 for 30 °C stoved/unstoved ferments; later fermentation weeks for lower temperatures stoved/unstoved ferments (Figure 38). Then, the pH in all mussel ferments fluctuated between 7.2 and 7.3 for the remaining of the fermentation period.

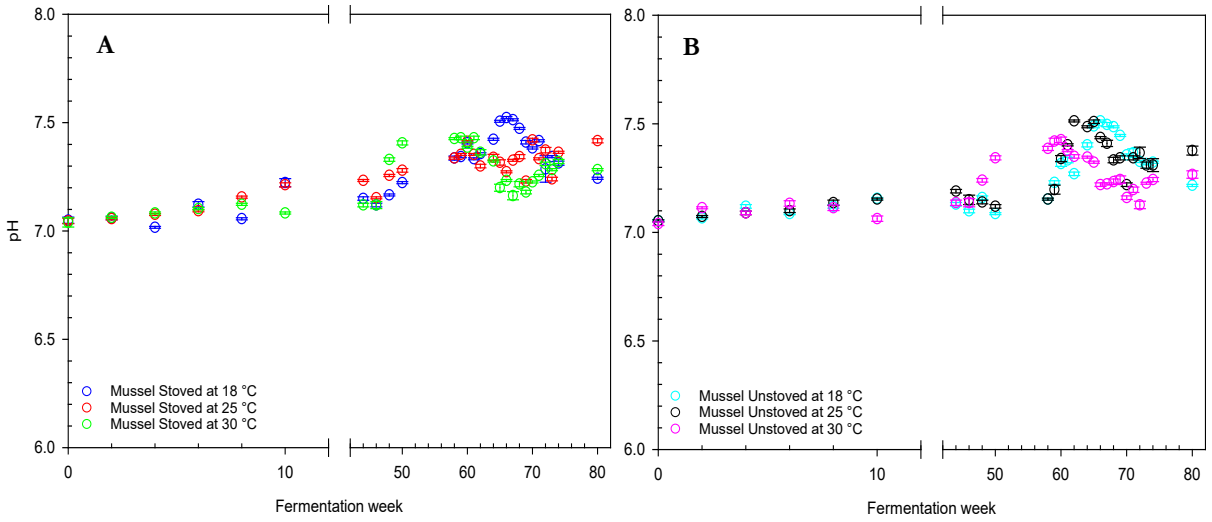


Figure 38: The pH of ferments from green-shell mussels fermented with stoved (A) and unstoved (B) salts at three different temperatures. Values represent means ± standard error of mean (n=3). n is the number of readings from the same sample bottle. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

The final pH values of all mussel ferments did not fall within the standard pH range of a traditionally fermented fish sauce underlined by CODEX as described in Section 2.6, where a traditional fish sauce should have a pH between 5 – 6.5 (FAO, 2013)

Statistical analysis was conducted to determine how significantly different all the datasets to each other were. Results obtained from Tukey *post-hoc* test revealed that there were no significant differences between all the pH values of all mussel ferments. The statistical analysis results may indicate that the different fermentation conditions did not influence the pH of the mussel ferments. Further analyses on other parameters used in assessing the quality and progress of the ferments in this current study will be discussed in the following sections.

7.4 Volatile Fatty Acid Formation in Mussel Fermentations

Four VFA were identified in mussel ferments: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Butanoic acid was present only in the unstoved ferments. Figure 39 shows that acetic acid was produced in the highest concentration in all mussel ferment followed by 3-methylbutanoic acid, butanoic acid (only for ferment liquors with unstoved salt) and, finally, propionic acid. These results are broadly consistent with Hoki and snapper fermentations (Chapters 5 and 6). For all VFAs tracked, all six fermentations showed a period of accumulation and a period of declining concentration. VFA l concentrations were generally highest in warmer fermentation temperatures than in cooler. This was not pronounced for acetic acid. Most of the fermentations exhibited a late recovery of higher VFA concentrations after about 70 weeks.

Acetic acid concentrations in mussel ferments at Week 0 in stoved and unstoved salts were measured at 17 ± 1 ppm and 21 ± 2 ppm, respectively. Sharp increases were observed in three mussel ferments between Weeks 2 – 10: in the 18 °C and 25 °C stoved ferments and 30 °C unstoved ferment. Acetic acid in the other three mussel ferments varied between 35 – 90 ppm during the same fermentation period. Increasing concentrations were observed during late the fermentation period (Weeks 60 – 80) in the ferments of higher temperatures: 25 °C and 30 °C stoved ferments and 30 °C unstoved ferment with values ranging from 130 to 150 ppm.

Meanwhile, acetic acid in ferments with either stoved or unstoved salt at lower temperatures fluctuated at lower values (30 – 100 ppm) during Weeks 44 – 80 without a hint of two phases of fermentation (Figure 3).

The concentrations of 3-methylbutanoic acid at Week 0 in the stoved and unstoved mussel ferments were 6 ± 1 ppm and 3 ± 1 ppm, respectively. There was evidence of two phases of fermentation in three mussel ferments: stoved and unstoved ferments at 25 °C and unstoved ferment at 30 °C where 3-methylbutanoic acid fluctuated over the range 40 – 80 ppm during Weeks 44 – 80 compared to lower concentrations (10 – 20 ppm) during Weeks 0 – 10 (Figure 39). For other mussel ferments, 3-methylbutanoic acid hovered between 10 – 40 ppm during the entire fermentation time course with no indication of the two phase fermentation.

Figure 39 shows that propionic acid at Week 0 in the mussel ferments with stoved and unstoved salt were 7 ± 1 and 16 ± 1 ppm, respectively. Propionic acid in all mussel ferments fluctuated between 10 – 40 ppm during the entire fermentation period with two noticeably independent observations made in the stoved and unstoved ferments at 18 °C – 1) propionic acid was not observed in the stoved ferment during Weeks 44 – 64 and 2) higher propionic acid concentrations were measured (25 – 50 ppm) in the unstoved ferment between Week 2 and 10.

Butanoic acid was observed only in unstoved mussel ferments and its concentration at Week 0 was 7 ± 1 ppm (Figure 39). Butanoic acid in the 18 °C and 25 °C unstoved ferments ranged between 10 – 40 ppm during the entire fermentation time course. For unstoved ferment at 30 °C, there was an increasing trend in the butanoic acid from 20 ppm at Weeks 66 to 60 ppm at Week 80.

Statistical analysis was conducted to determine any significant differences in VFA levels across all mussel ferments fermented at three different temperatures and the results obtained from the Tukey *post-hoc* test was presented in Figure 40.

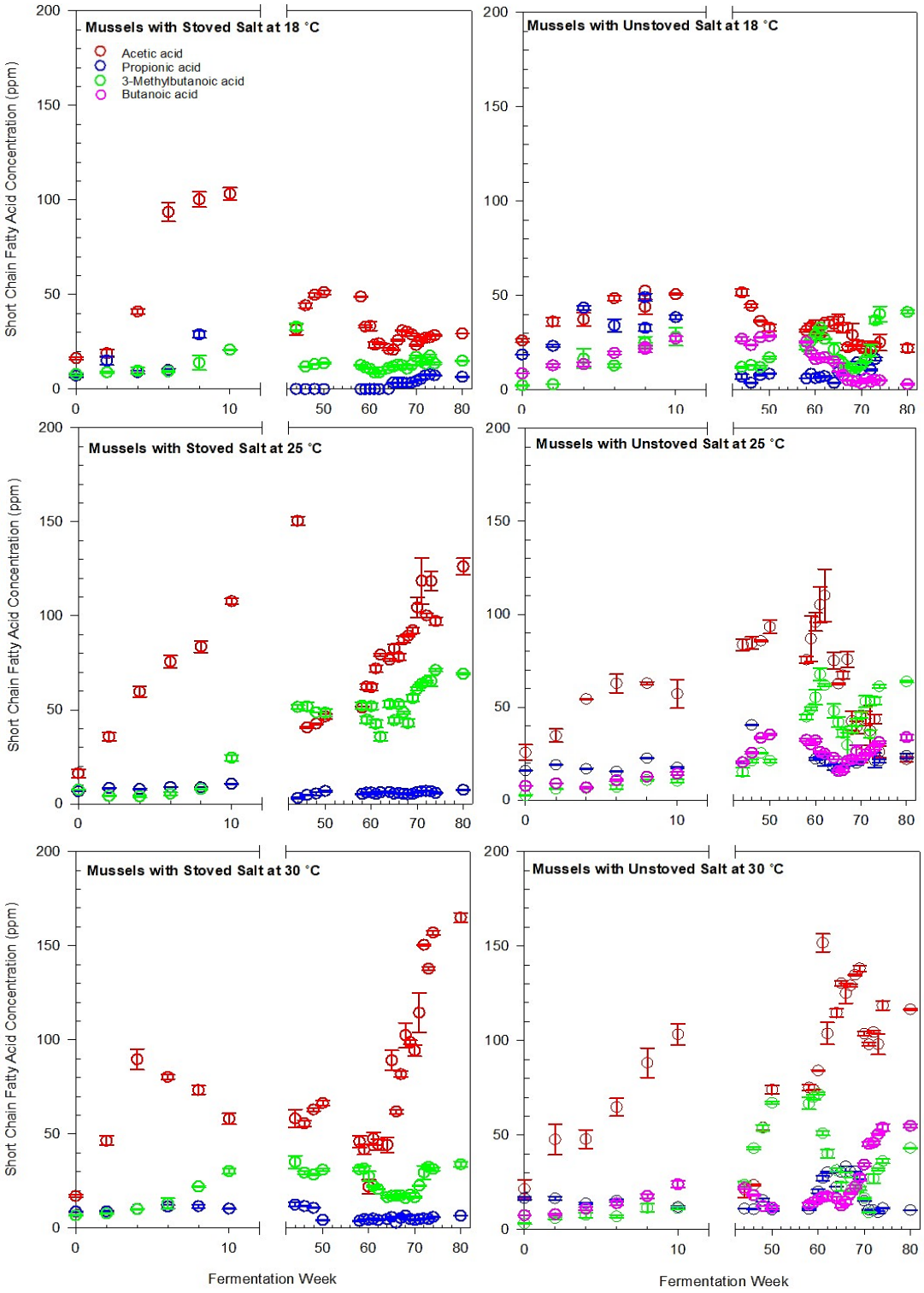


Figure 39: Short chain volatile fatty acids (VFA) in mussel ferments with stoved and unstoved salt fermented at three different temperatures. Values represent means ± standard error of mean(n=3). n is the number of injections from the same sample vial. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9.0 ppm, 2.2 ppm, 3.36 ppm and 2.0 ppm, respectively. The break in the plot indicates the missing data points due to the Covid-19 pandemic restrictions.

Figure 40(A) shows that acetic acid in the 18 °C stoved and unstoved ferments were significantly different than ferments at higher temperatures with stoved and/or unstoved salts. This shows that fermentation temperatures and salt types influenced the acetic acid production in mussel fermentation. Figure 40(B) shows that propionic acid concentrations in either stoved or unstoved ferments were significantly different than the ferments of different type of salt fermented at all three different temperatures. This may suggest that the behaviour of propionic acid was influenced by the type of salt.

Figure 40(C) shows that 3-methylbutanoic acid in the 18 °C stoved fermentation was significantly different than stoved and unstoved ferments fermented at higher temperatures. Also, 3-methylbutanoic acid concentration in the 18 °C unstoved ferments was significantly different that found in the 25 °C stoved ferments. The results obtained suggested that salt type influenced the 3-methylbutanoic acid behaviour in mussel fermentation.

Figure 40(D) revealed that butanoic acid in the 18 °C unstoved ferments was significantly different to the ferment at 25 °C.

Overall, the statistical analysis on the VFA identified in the mussel ferments may suggest that the combination of different factors of the fermentation conditions can influence the VFA formations in the samples. Also, the concentrations of all VFAs in almost all mussel ferments were higher by the end of the fermentation compared to that at Week 0, supporting the hypothesis outlined in Section 7.0 that the concentrations of VFAs produced will increase as the fermentation increases.

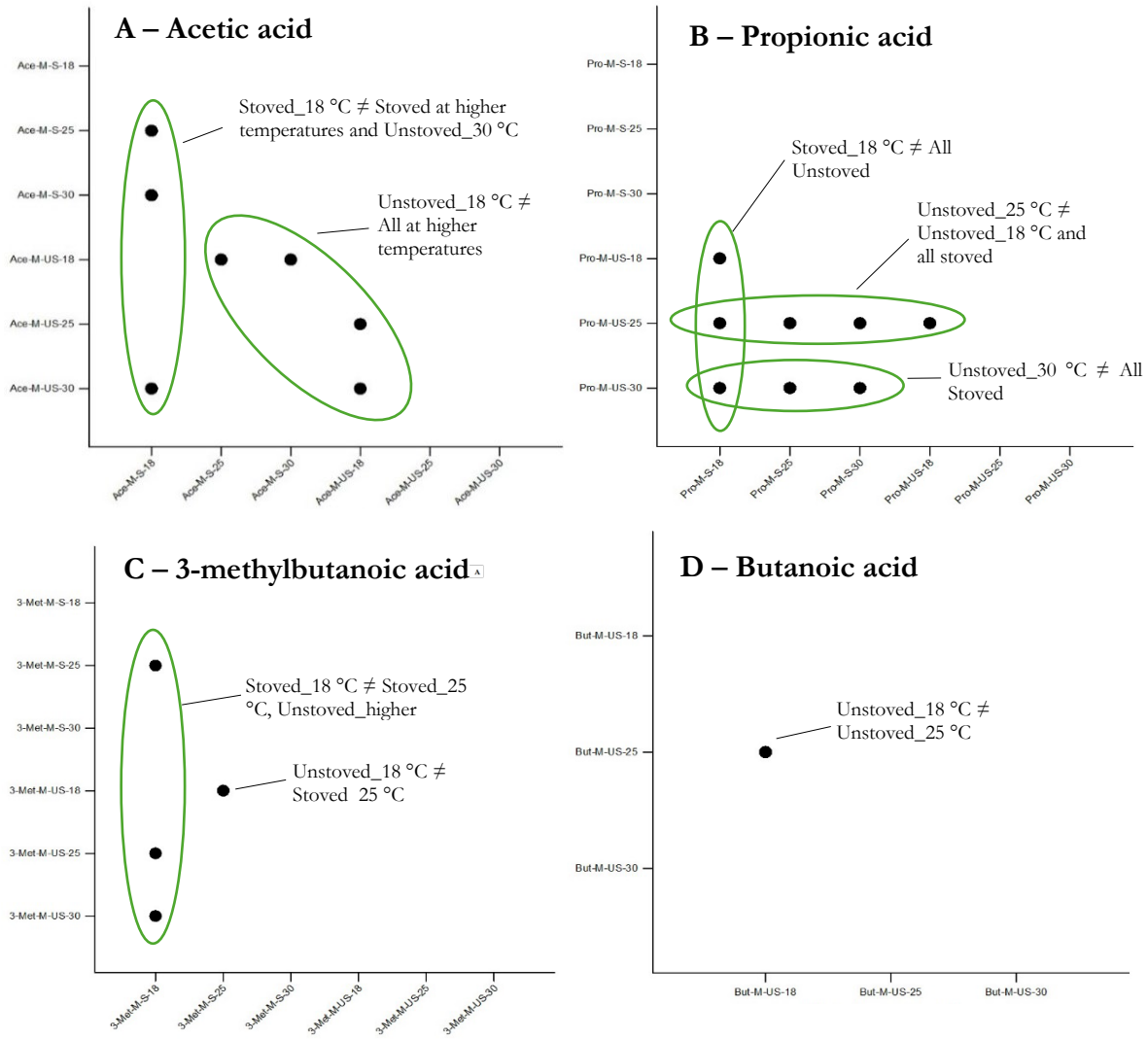


Figure 40: Pairwise comparison matrix based on Tukey test comparison of volatile fatty acid level in all green-lipped mussel ferments. Each graph represents one of four different volatile fatty acids. The three experiments (18, 25 and 30 °C) using stoved salt and the three using unstoved salts appear on both x- and y-axis. "Ace" = acetic acid; "Pro" = propionic acid; "3-Met" = 3-methylbutanoic acid, "But" = Butanoic acid, the "M" is abbreviated for Mussel, the "S" is abbreviated to stoved salt, the "US" is abbreviated to unstoved salt and the following number is the fermentation temperature used. It is to note that butanoic acid is only detected in mussel ferments with unstoved salt. The "•" symbol indicates that the two compared mussel ferments are significantly different to each other. The "•" symbols are clustered and annotated to visualise the statistical results obtained. The "≠" symbol is used to indicate "significantly different".

7.5 Bacterial Composition in Mussel Ferments

To understand the influence of different mussel fermentation conditions on bacterial compositions, metagenomic approaches were adopted as discussed in Section 3.5.8. Results are presented in Figure 41.

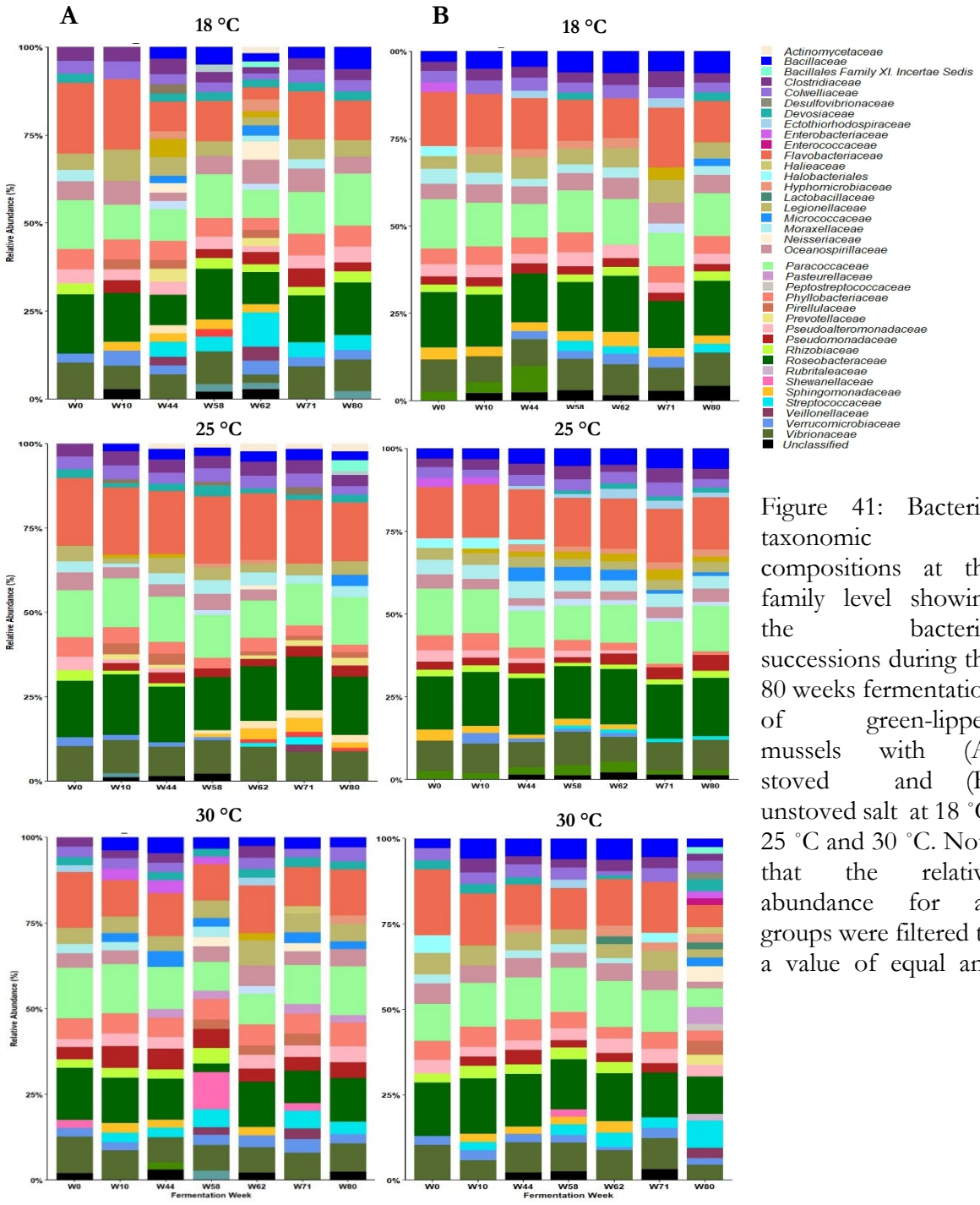


Figure 41: Bacterial taxonomic compositions at the family level showing the bacterial successions during the 80 weeks fermentation of green-lipped mussels with (A) stoved and (B) unstoved salt at 18 ˚C, 25 ˚C and 30 ˚C. Note that the relative abundance for all groups were filtered to a value of equal and

Figure 41 shows that there were 3 family groups that predominated across the 80 weeks fermentation in all mussel ferments fermented at three different temperatures: *Flavobacteriaceae, Paracoccaceae* and *Roseobacteriaceae.*

To reiterate, the ferments at Week 0 represents triplicate samples with either stoved or unstoved salts as no fermentation had taken place yet and samples at all temperatures should be identical. The relative abundances of *Flavobacteriaceae* family in the stoved and unstoved mussel ferments at Week 0 were 18.8 ± 1.8 % and 16.9 ± 1.9 %, respectively. *Flavobacteriaceae* remained as one of the predominant groups in all ferments across 80 weeks fermentation (Relative abundances: 11.0 – 20.0 %). It was observed in Figure 41(A) that *Flavobacteriaceae* significantly decreased to 3.4 % at Week 62 in the 18 °C stoved ferment before the value increased again to 13.6 % at Week 71. This fluctuation may be influenced by the complex interactions between successions of bacteria communities during the fermentation time course.

The relative abundances of *Paracoccaceae* at Week 0 in the stoved and unstoved ferments were 14.2 ± 0.5 % and 13.0 ± 1.5 %, respectively. *Paracoccaceae* remained as the predominant group throughout the entire fermentation time course where the relative abundance values fluctuated between 8.4 and 14.7 % - except in the 30 °C unstoved ferment where *Paracoccaceae* decreased to 5.4 % at the final fermentation week [Figure 41(B)].

Lastly, *Roseobacteriaceae* in the stoved and unstoved mussel ferments at the initial fermentation were 16.4 ± 0.7 % and 15.8 ± 0.1 %, respectively, and remained as the predominant group in all ferments across the 80 weeks fermentation. Interestingly, the relative abundance of *Roseobacteriaceae* in the 30 °C stoved fermentation decreased drastically from 12.0 % at Week 44 to 2.5 % at Week 58 before increased again to 13.2 % at Week 62 [Figure 41(A)] – similar to observation made on *Flavobacteriaceae* as discussed previously.

It was shown in Figure 41 that *Vibrionaceae,* a family group that potentially includes pathogenic strains, were present at steady values of 5.0 – 11.0 % abundance in all mussel ferments throughout the entire fermentation time course.

There were two differences observed between the stoved and unstoved mussel ferments shown in Figure 41. Firstly, *Halobacteriales* was present only in the unstoved ferments and the relative abundance at Week 0 was 3.6 ± 1.2 % [Figure 41(B)]. *Halobacteriales* disappeared at Week 10 in the 18 °C unstoved ferment and at Week 58 in the 25 °C unstoved ferment. Meanwhile, *Halobacteriales* in the 30 °C unstoved fermentation disappeared at Week 10 before it reappeared at Week 71 and disappeared again at the final fermentation week [Figure 4(B)]. The findings made on the presence of *Halobacteriales* exclusively in unstoved ferments in this current study were similar to the findings made on Hoki and snapper ferments discussed in Sections 5.5 and 6.5.

Secondly, Figure 41(A) shows that *Bacillaceae* was present after Weeks 10 or 44 and remained present until the final fermentation week in all stoved ferments (Relative abundances: 2.2 – 4.9 %). On the other hand, *Bacillaceae* was present throughout the entire fermentation time course in all unstoved ferments with higher overall relative abundances (Relative abundances: 3.0 – 6.3 %).

Overall, Figure 41 shows that the predominant family groups in all mussel ferments throughout the entire fermentation time course were similar. However, it was observed in Figure 41 that there were some differences in bacterial communities present between mussel ferments with stoved and unstoved salt. Figure 41 (A) shows that the bacterial diversities in all stoved ferments were higher during the mid fermentation period (between Week 58 and 62) compared to that during the initial and final fermentation periods. Meanwhile, the bacterial diversities in all unstoved ferments were higher during late fermentation period (between Week 71 and 80) compared to that during the initial and mid-fermentation periods [Figure 41(B)].

7.5.1 Heatmaps of the Mussel Ferments

The relative abundances of all bacterial family groups identified in all mussel ferments were further visualised on heatmaps presented in Figures 42 and 43.

Figures 42 and 43 show that the four predominant family groups throughout the entire fermentation time course in all mussel ferments with either stoved or unstoved salts were *Flavobacteriaceae, Roseobacteriaceae, Paracoccaceae* and *Vibrionaceae*. Figure 42 shows that the bacterial diversities in all mussel stoved ferments were higher during mid-fermentation compared to that in the initial and final fermentation period, supporting the findings made in Section 7.5. There was no noticeable difference in bacterial diversities between Week 0 and Week 80 in all snapper ferments however, it was noted that the bacteria family groups in the ferments at the initial and final weeks may differ.

Figure 43 shows that the bacterial diversities in all mussel unstoved ferments are greater during late fermentation compared to that in the earlier fermentation period which was also in agreement with the findings in Section 7.5.

Overall, Figures 42 and 43 also show that the mussel ferments were clustered based on fermentation temperatures regardless of the fermentation week suggesting that the bacterial communities were influenced by the fermentation temperatures. In addition, it was found that the bacterial compositions in all mussel ferments at the initial fermentation (Week 0) were similar and clustered close to each other which was predicted as no fermentation had taken place yet.

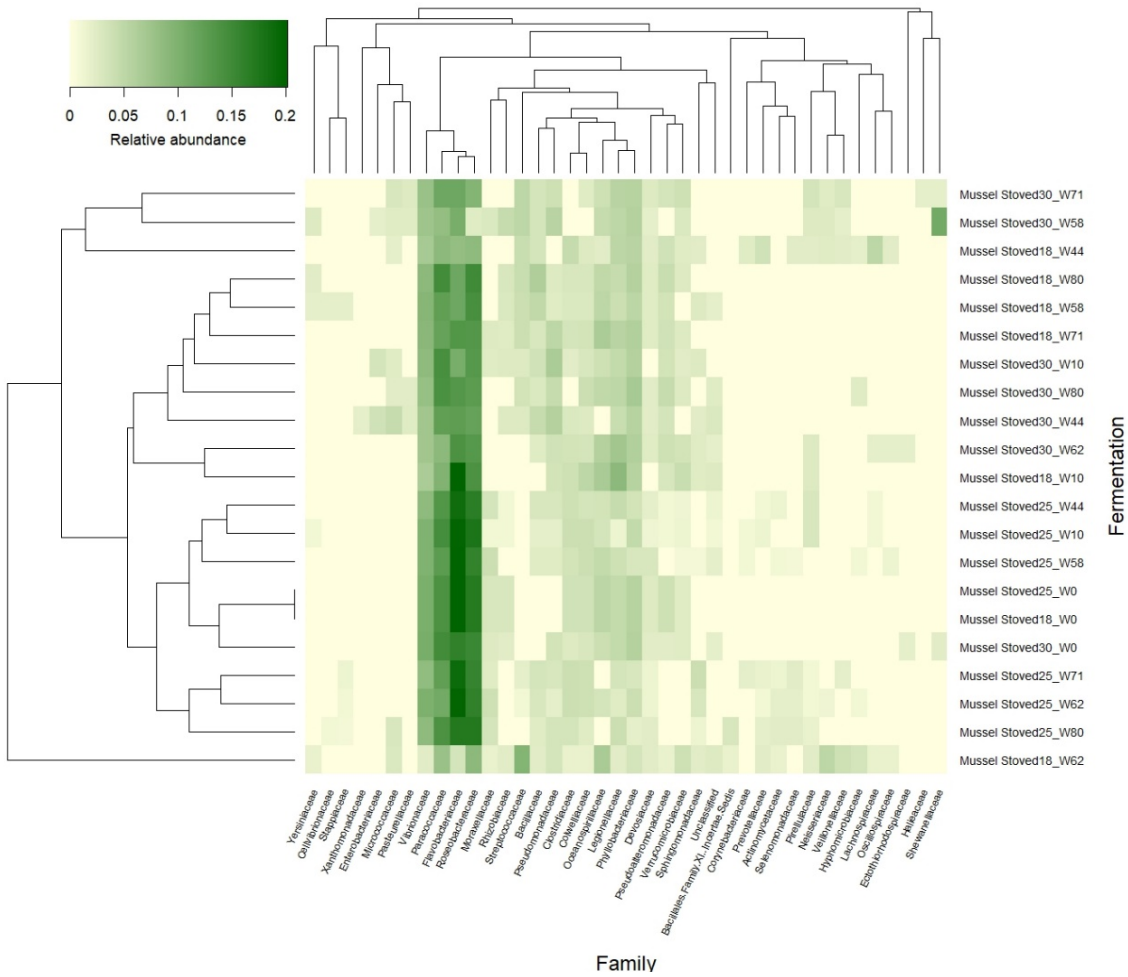


Figure 42: Heatmap of relative abundance at the family level across all green-lipped mussel ferments fermented with stoved salt at 18 °C, 25 °C and 30 °C for 80 weeks. Mussel ferments and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual mussel ferments where the number following the "Mussel Stoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

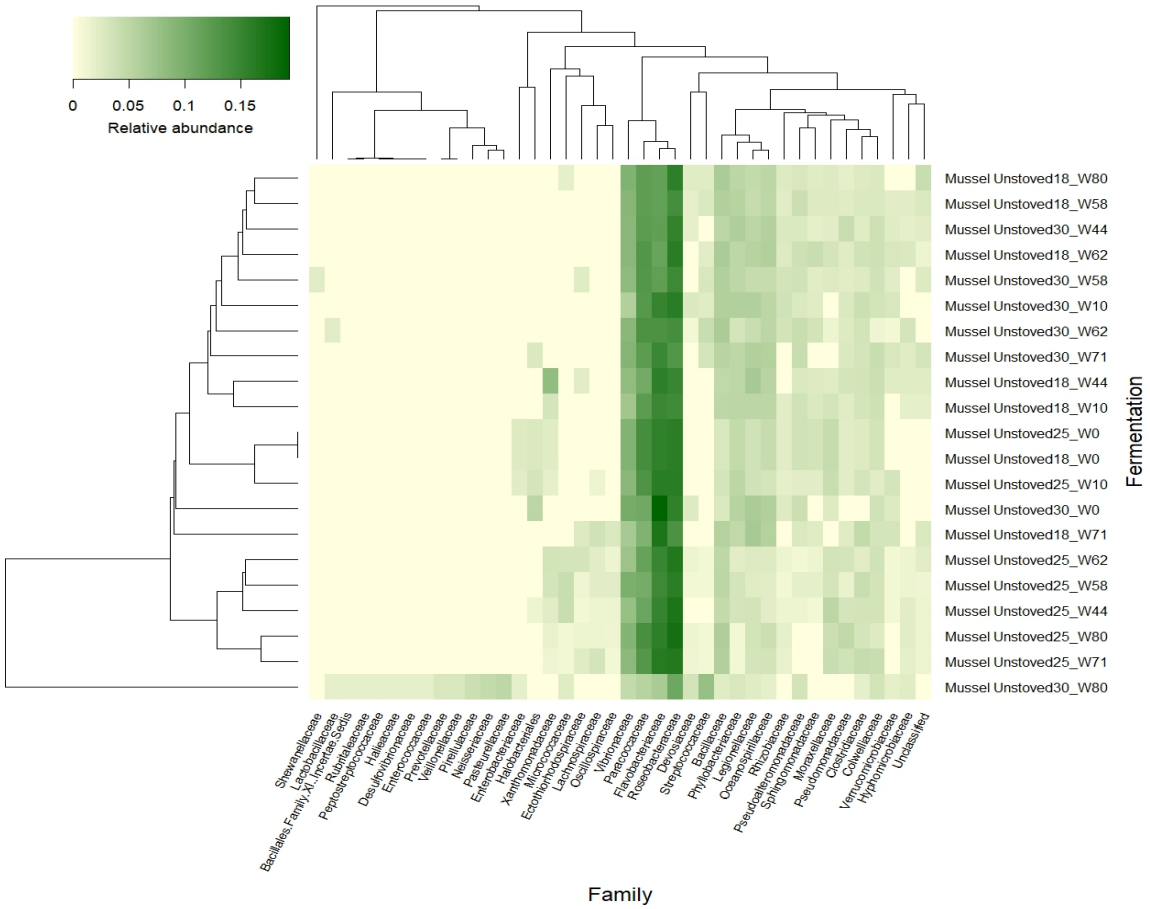


Figure 43: Heatmap of relative abundance at the family level across all green-lipped mussel ferments fermented with unstoved salt at 18 °C, 25 °C and 30 °C for 80 weeks. Mussel ferments and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual mussel ferments where the number following the "Mussel Unstoved" are the fermentation temperature, the "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

7.5.2 Binary Comparison of the Initial and Final Bacterial Composition in Mussel Fermentations

Binary comparison graphs were employed to identify differences in the bacterial communities between Week 0 and Week 80. It is important to reiterate that this approach is only providing a comparison between two time-points and overall bacterial compositions in all mussel ferments should be referred to Section 7.5 and Section 7.5.1. The binary comparisons between Week 0 and Week 80 of all mussel ferments are presented in Figure 44.

In Figure 44, each bacterial family identified in Section 7.5 is identified with a number. The key for the numbers are presented in the figure legend. Figure 44 shows that the *Roseobacteriaceae* (No. 94) was the predominant family group at both Weeks 0 and 80 in all mussel ferments as already apparent from Figures 41, 42 and 43. The relative abundances of *Roseobacteriaceae* were constant at the initial (Week 0) and final (Week 80) fermentation weeks in the 25 °C stoved and 18 °C unstoved ferments with the value of 16.0% (Figure 44). The relative abundance of *Roseobacteriaceae* in the 25 °C unstoved fermentation was lower at Week 0 compared to that at Week 80. In contrast, the relative abundances of *Roseobacteriaceae* in all other mussel ferments with either stoved or unstoved salts were higher at Week 0 compared to that at Week 80 (Figure 44).

Flavobacteriaceae (No. 36) and *Paracoccaceae* (No. 74) were the other predominant groups at both Weeks 0 and 80 in all mussel ferments except that in the 30 °C unstoved fermentation. Figure 44 shows that *Flavobacteriaceae* was one of the predominant groups in the 30 °C unstoved fermentation at the initial fermentation with the relative abundance of 19.0 % and the value decreased to 6.0 % by the final fermentation period while *Paracoccaceae* at Week 0 was 10.0 % and the value decreased to 6.0 % at Week 80.

It is shown in Figure 44 that the relative abundance of *Vibrionaceace* (No. 115) in the 30 °C unstoved fermentation decreased from 10.0 % at Week 0 to 4.0 % at Week 80. Meanwhile in all other mussel ferments, the relative abundances of *Vibrionaceae* were fairly constant consistent at the initial and final fermentation weeks – as revealed in Figure 41.

For stoved salt ferments, it is shown in Figure 44 that there were no apparent differences between the number of family groups present at Week 0 and at Week 80, indicating that the bacterial diversities between the two time-points were not very different. Despite this, the bacterial groups found only at Week 80 differed across the three different temperatures suggesting that the bacterial compositions during fermentation were influenced by the fermentation temperatures which supports findings shown in Figures 41 and 42.

With unstoved salt, there were more than two family groups that were present only at Week 0 while more than four family groups were present only at Week 80. Figure 44 shows that the number of family groups present exclusively at Week 80 were more than that present exclusively at Week 0 in all unstoved ferments indicating that the bacterial diversity in the final fermentation week was higher compared to that in the first week as shown in Figures 41 and 43. It was also found that the bacterial groups found only at Week 80 differed across three different temperatures suggesting that the bacterial compositions during fermentation were influenced by the fermentation temperatures.

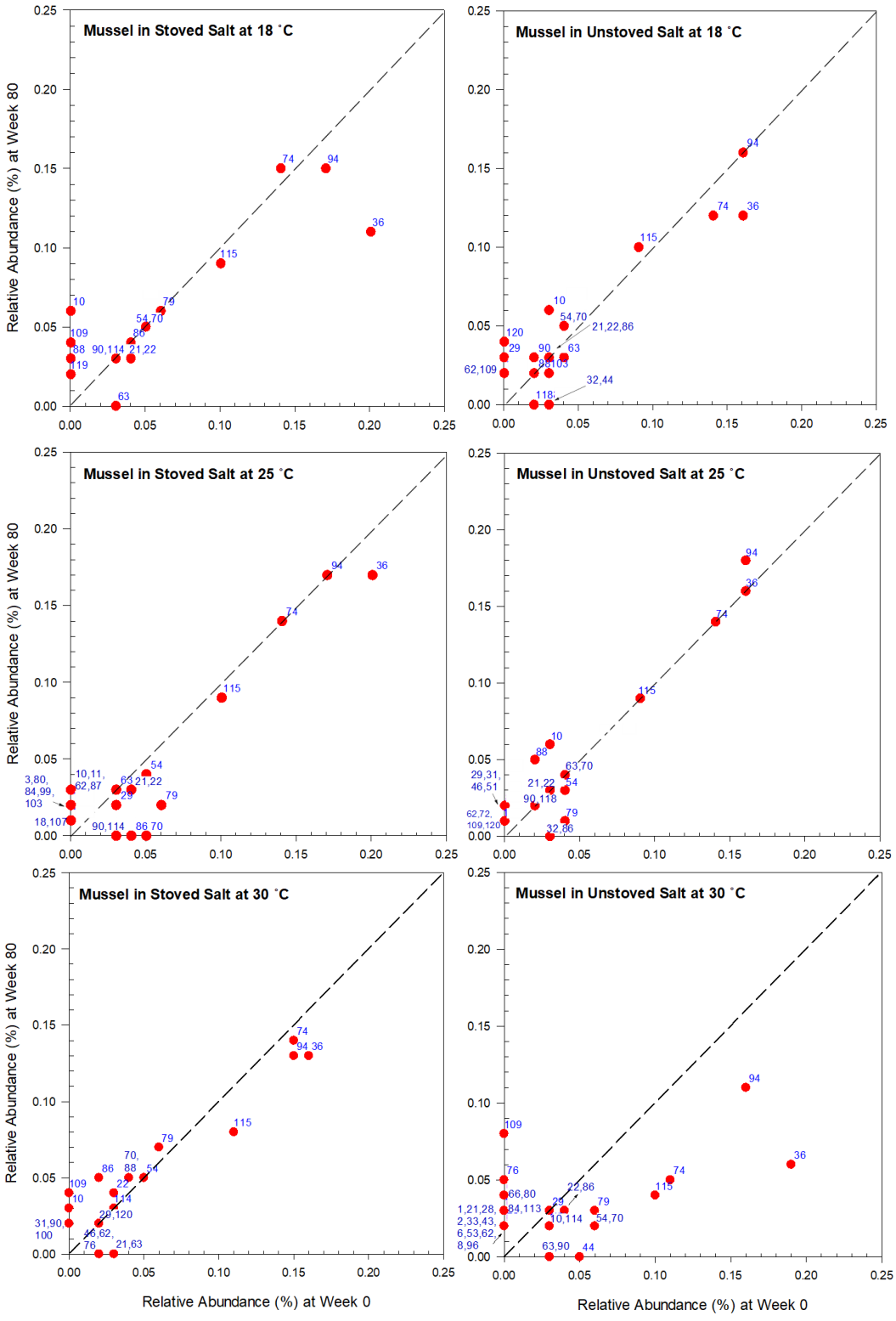


Figure 44: Binary comparison of relative abundance at family level between two fermentation weeks in green-shell mussel ferments with stoved and unstoved salts fermented at three different temperatures. A position below the 45˚ line indicates relative abundance is lower at Week 80 compared to Week 0 whereas a family positioned above the 45˚ line has relative abundance higher at Week 80 compared to Week 0. The relative abundance of the bacterial taxon at family level was filtered to a value of at least 0.01.

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

7.5.3 Alpha Diversity Analysis of Mussel Fermentations

Alpha diversity analysis was employed as described in Section 3.5.8 and Section 5.5.3 to further understand the bacterial diversities in all mussel ferments. Tables 32 and 33 summarize the sequencing data reads from mussel ferments post-software trimming procedures and alpha diversity indices calculated from the obtained sequence reads.

Based on the Chao1 indices in Table 32, there were small differences in the bacterial diversities in all mussel ferments with stoved salt between Week 0 and Week 80. The Chao1 indices show that the bacterial diversities in mussel fermentation with stoved salt fermented at 18 °C, 25 °C and 30 °C were highest at Week 62 (Chao1 = 382.11), Week 62 (Chao1 = 320.23) and Week 58 (Chao1 = 463.33), respectively.

Consequently, the Simpson's indices show that the highest values obtained in mussel ferments with stoved salt fermented at 18 °C, 25 °C and 30 °C were at Week 62 (H = 2.15), Week 62 (H = 2.05) and Week 58 (H = 2.14), respectively. The Chao1 and Simpson's indices obtained for all mussel stoved ferments indicated that the bacterial communities were more diverse during the mid fermentation period compared to the initial and final fermentation periods.

This contrasted markedly with the unstoved salt fermentations where Table 33 shows that the highest Chao1 indices for mussel ferments with unstoved salt fermented at 18 °C, 25 °C and 30 °C were obtained at Week 80 (Chao1 = 418.12), Week 71 (Chao1 = 382.45) and Week 80 (Chao1 = 412.21), respectively. Consequently, the Simpson's indices show that the highest values obtained in mussel ferments with unstoved salt fermented at 18 °C, 25 °C and 30 °C were at Week 80 (H = 2.10), Week 71 (H = 2.12) and Week 80 (H = 2.12), respectively. The Chao1 and Simpson's indices show more diversity later in the fermentation and supports the findings discussed previously in Section 7.5 and Section 7.5.1.

The Shannon-Wiener indices of all mussel ferments have values close to 1 (D values $\geq$ 0.97), indicating that the abundances of the bacterial species within each sample were similar.

Table 32: Summary of the sequencing data sets derived from ferments of green-shell mussel with stoved salt at three different temperatures for 80 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	41391	469	215	342.11	0.98	2.03
	10	40553	468	225	337.74	0.98	1.99
	44	44054	468	255	311.05	0.99	2.11
	58	41391	469	306	372.36	0.98	2.07
	62	40365	471	332	382.11	0.98	2.15
	71	41235	470	274	303.14	0.98	2.01
	80	39865	470	273	331.76	0.98	1.96
25 ± 1°C	0	39475	471	231	252.41	0.98	1.81
	10	39458	471	225	253.41	0.98	1.80
	44	38412	468	267	316.45	0.98	2.01
	58	37652	468	271	271.23	0.97	2.04
	62	40012	469	276	320.23	0.99	2.05
	71	41023	467	211	264.84	0.99	1.93
	80	41325	467	213	257.22	0.98	1.80
30 ± 1°C	0	42488	487	257	346.12	0.98	1.98
	10	48745	519	256	341.16	0.98	2.01
	44	53267	479	314	445.15	0.97	2.08
	58	42543	469	318	463.33	0.97	2.14
	62	39641	469	321	401.23	0.97	2.13
	71	33964	468	210	338.69	0.98	2.03
	80	34383	471	247	356.45	0.98	2.04

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

Table 33: Summary of the sequencing data sets derived from ferments of green-shell mussel with unstoved salt at three different temperatures for 80 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Fermentation temperature	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
18 ± 1°C	0	61873	468	222	286.36	0.98	1.96
	10	40502	469	301	290.12	0.98	1.95
	44	36806	469	273	316.32	0.97	1.89
	58	29118	468	274	280.12	0.98	1.96
	62	31254	466	217	289.87	0.98	1.94
	71	30510	469	314	343.11	0.98	2.09
	80	40840	469	332	418.12	0.97	2.10
25 ± 1°C	0	40123	471	224	276.14	0.98	1.80
	10	38754	472	225	275.25	0.96	1.78
	44	39745	472	231	280.32	0.98	1.81
	58	38425	471	233	281.33	0.97	1.83
	62	37415	469	234	283.84	0.97	1.84
	71	38794	469	332	382.45	0.98	2.12
	80	35661	467	326	377.15	0.98	2.07
30 ± 1°C	0	61873	468	222	286.11	0.98	2.06
	10	38325	468	204	245.12	0.98	1.81
	44	55840	475	259	222.25	0.99	1.95
	58	49559	468	221	321.01	0.97	1.96
	62	35784	470	240	188.62	0.97	1.93
	71	47718	470	320	397.01	0.98	2.10
	80	22484	468	330	412.21	0.97	2.12

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

7.6 Bacterial Composition in Raw Mussel

To understand the influence of the green-shell mussel paste used in this current study on the bacterial compositions during the fermentation process, 16S rRNA sequencing was employed to determine the bacterial composition in the paste prior to fermentation. The preparation of the mussel paste was described in Section 3.1.1. and the paste was subjected to the DNA extraction and subsequent sequencing analysis as described in Section 3.5.4 and Section 3.5.8. The bacterial composition in raw mussel paste is presented in Figure 45.

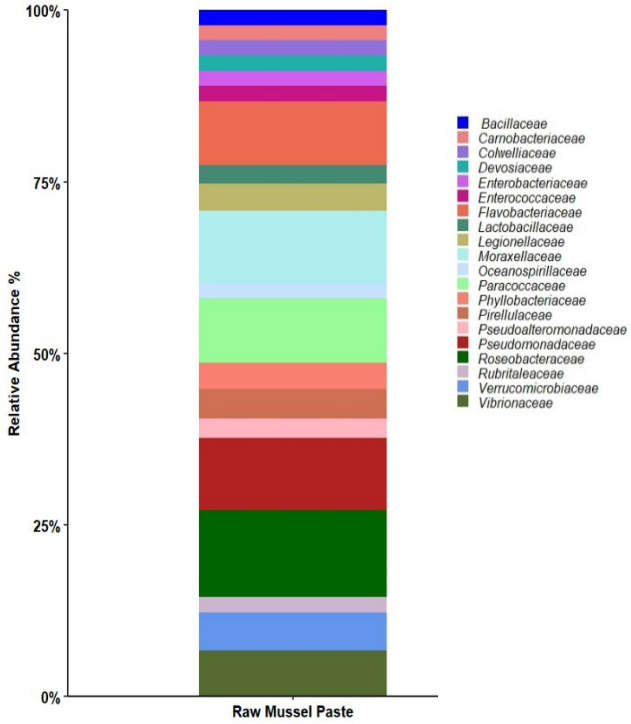


Figure 45: Bacterial taxonomic compositions at the family level showing the bacterial composition in raw material green-lipped mussel paste. Note that the relative abundance for all groups were filtered to at least 1%.

Figure 45 shows that the predominant family groups in raw mussel pastes were *Flavobacteriaceae, Moraxellaceae*, *Paracoccaceae, Pseudomonadaceae* and *Roseobacteriaceae* with the relative abundances of 9.3 %, 10.5 %, 9.4 %, 10.5 % and 12.7 %, respectively. The *Flavobacteriaceae, Paracoccaceae* and *Roseobacteriaceae* family groups were present as the predominant groups in all mussel ferments throughout the fermentation time course (Figure 41).

The family groups *Moraxellaceae* and *Pseudomonadaceae* were present either throughout or found at certain time points during the fermentation time course in all mussel ferments at low relative abundances (Relative abundance < 5.0 %). Figure 45 also shows that *Rubritaleaceae* was exclusively present in raw mussel paste but not in any of the mussel ferments.

Alpha diversity indices were determined and are presented in Table 34 to further understand the bacterial diversity in the mussel paste and to compare that to the overall bacterial diversities in all mussel ferments.

Table 34: Summary of the sequencing data sets derived from green-shell mussel paste and statistical analysis of bacterial diversity (alpha-diversity).

Sample	**High quality reads***	**Average read length, bp**	**OTU***	**Chao1**	**Shannon index, *H***	**Simpson's index, *D***
Mussel paste	40123	468	121	146.15	0.98	1.23

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

Table 34 shows that the Chao1 index of mussel paste was 146.15 which was much lower than that determined for all mussel ferments (refer to Table 32 and 33), this indicated that raw mussel paste was less diverse in bacterial composition than any of the mussel ferments. Consequently, the Simpson's index of mussel paste (1.23) was lower than that in all mussel ferments (refer Table 32 and 33). The Shannon-Wiener index, *H* of mussel paste was 0.98, which indicated that the abundances of the bacterial species within the sample were similar.

In addition, mussel paste had the highest bacterial diversity followed by Hoki paste (Chao1 = 53.6; Table 21), then snapper paste (Chao1 = 22.5; Table 28). The high bacteria diversity in mussel paste may be attributed to the nature of mussel as immobile, filter-feeding molluscs leading to the accumulation of biological particles including bacteria in their tissues (Nuñal et al., 2023).

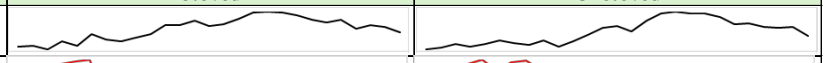
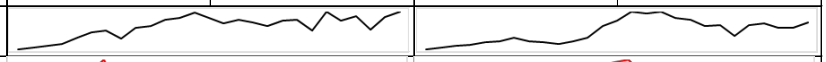
7.7 Discussion

Summary of the overall results from mussel fermentation is presented in Table 35. Figure 38 showed that the pH values of all mussel ferments with either stoved or unstoved salt ranged between 7.0 to 7.4 which were higher than the standard pH range of a traditionally fermented fish sauce outlined by CODEX (2011) as described in Section 2.6.

In a study on the effect of oyster shell powder on the extension of the shelf life of Kimchi, it was reported that a higher percentage of shell powder increased the pH of the final product (Choi et al., 2006). It was explained that the shell powder reacted with lactic acid produced during the Kimchi fermentation, producing calcium lactate which then acted as a buffer and a soluble calcium fortifier (Choi et al., 2017). In addition, it was reported that traditionally Korean fermented salted shrimp (*saeu-jeot)* with different salt concentrations [20 - 32 % (w/v)] had final pH ranging between 6.8 – 7.5 after 150 days of fermentation (Lee et al., 2014). In another study on the fermentation of shrimp sauces from shrimp by-products (heads, shells, and tails) the pH values of the final products after 6 months of fermentation ranged between 7.3 and 7.5 (Kim et al., 2003). The minerals in shrimp shells are composed of 20 – 50 % calcium carbonate ($CaCO_3$) which is similar to the main component of oyster and mussel shells (Kim et al., 2003; Mititelu et al., 2022; Zhao et al., 2019). Therefore, the presence of mussel shell with $CaCO_3$ present acted as a buffer to maintain pH.

As discussed in Section 7.2, it was observed that the texture of the mussel meat in all ferments changed from firm to soft over the fermentation period (after Week 60), with a noticeable small volume of liquid layer formed by the end of the fermentation. The slow rate of the mussel meat degradation compared to previously reported Hoki and snapper (refer to Chapters 5 and 6) may be influenced by the high pH values during fermentation which may be linked to the presence of the mussel shells. The muscle protein within mussel meat can be divided into three categories; sarcoplasmic, myofibrillar and stroma proteins (Chen et al., 2023).

Table 35: Summary of the overall results from green-shell mussel fermentation.

Temperature	Parameter	Mussel			
		Stoved		Unstoved	
18 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Flavobacteriaceae *Oceanospirillaceae* *Paracoccaceae* *Phyllobacteriaceae* *Roseobacteriaceae* *Vibrionaceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Phyllobacteriaceae* *Roseobacteraceae* *Vibrionaceae*	*Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*
25 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Flavobacteriaceae *Oceanospirillaceae* *Paracoccaceae* *Phyllobacteriaceae* *Roseobacteraceae* *Vibrionaceae*	*Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*	*Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*
30 °C	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 80	Week 0	Week 80
		Devosiaceae *Paracoccaceae* *Pirellulaceae* *Roseobacteraceae* *Verrucomicrobiaceae*	*Devosiaceae* *Paracoccaceae* *Pirellulaceae* *Roseobacteraceae* *Verrucomicrobiaceae*	*Flavobacteriaceae* *Legionellaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*	*Flavobacteriaceae* *Roseobacteraceae* *Streptococcaceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

The myofibrillar protein accounts for approximately 55 to 60 % of the total muscle protein in a mussel and is the main functional protein that determines the physiochemical properties of the meat products (Yu et al., 2022). The myofibrillar proteins are composed of large size proteins (50 -95 kDa) including myosin and actin; and small size proteins (28 – 32 KDa) including tropomyosin, troponin and actinin (Chen et al., 2023; Yates et al., 1983).

In a study on the effect of temperature and pH on the degradation of myofibrillar protein, it was determined that pH 5.4 lead to a higher rate of myofibrillar protein degradation compared to that at pH 7.0 (Yates et al., 1983). It was found at pH 5.4, there was a high percentage of small sized proteins (troponin and actinin) and a low percentage of large sized proteins (myosin and actin) after 12 hours incubation at 37 °C (Yates et al., 1983). Conversely, at pH 7.0, there was a low percentage of small sized proteins and a high percentage of large sized proteins (Yates et al., 1983). It was suggested that the proteolytic enzyme activities decreased at high pH and low temperature thus decreasing the rate of myofibrillar degradation and consequently, causing the meat to be less tender (Yates et al., 1983). The study by Yates et al. (1983) concluded that the combination of 37 °C and pH 5.4 was the most favourable condition for myofibrillar degradation contributing to meat tenderization.

The pH may also influence the bacterial compositions in an environment (Mueda, 2015). Figure 41 shows that *Flavobacteriaceae* was one of the predominant family groups in all mussel ferments throughout the fermentation time course. The abundance of *Flavobacteriaceae* in mussel ferments during the fermentation time-course may be explained by the neutral to alkaline pH of the ferments. It was reported that the genus *Actibacter sediminis* belonging to the *Flavobacteriaceae* family grows at pH 5 – 8 and in the presence of up to 15 % (w/v) sea salt (Kim et al., 2008). *Paracoccaceae* was also one of the predominant family groups in all mussel ferments throughout the fermentation time course (Figure 41). *Paracoccaceae* are aquatic bacteria and some genera of this family require salt for growth (Shivaji et al., 2014).

The *Rhodothalassium* genus can tolerate up to 20 % salts and requires glutamate to grow (Shivaji et al., 2014). Another genus belonging to the *Paracoccaceae* family, *Paracoccus* – isolated from a Korean shrimp sauce (*saeu-jeot*) grow between pH 6.0 and 10.0 (Kim et al., 2019). Li et al. (2013) reported that the genus *Albimonas pacifica* (Family: *Paracoccaceae*) isolated from the Pacific Ocean seawater has an optimum growth at pH 7.5.

As discussed in Section 6.7, the microbial communities in a fish sauce can influence the formation and concentration of VFAs which consequently influence the overall sensory profile and quality of the final product (Ma et al., 2022). Figure 39 shows that the concentration of acetic acid was the highest of the VFAs in all mussel ferments using either stoved or unstoved salt at all three temperatures and the observations may be explained by the high relative abundances of *Flavobacteriaceae* in all ferments throughout the fermentation time course. It has been reported that *Actibacter sedimis (*Family: *Flavobacteriaceae)* can utilize L-glutamate leading to the production of acetic acid (Kim et al., 2008).

The fluctuations in the concentrations of propionic acid in the mussel ferments during the fermentation time course may be explained by the presence of *Paracoccaceae.* According to Kim et al. (2019), *Paracoccus* genus (family: *Paracoccaceae*) has the ability to utilize propionic acid. In the 30 °C unstoved ferment, there were low values of propionic acid (9 – 10 ppm) observed during the late fermentation period (Weeks 71 – 80) (Figure 39). Consequently, there was a decreased in the relative abundances of *Paracoccaceae* from 12.2 % at Week 71 to 5.8 % at Week 80 in the 30 °C unstoved ferment (Figure 41). Similar observations were made in the 18 °C unstoved ferment where there was an increase of *Paracoccaceae* from 9.3 % (Week 71) to 12.2 % (Week 80) that may explain the slight increase of propionic acid from Week 72 until Week 80 (Figures 39 and 41). The relative abundances of *Bacillaceae* may also explain the significant difference in the concentrations of propionic acid between the stoved and unstoved ferments as presented in (Figures 39 and 41).

Overall, the concentrations of propionic acid were higher in all unstoved ferments compared to that in stoved ferments which may be associated with the higher relative abundances of *Bacillaceae* in the unstoved compared to the stoved ferments (Figures 39 and 41). Two or more bacterial family groups may collectively affect the VFAs formation in the fish sauce ferments. It was shown in Figure 39 that the propionic acid was not observed in the 18 °C stoved fermentation between Week 44 and 64 which may be explained by the decrease in the relative abundances of both *Paracoccaceae* and *Bacillaceae* at Week 44 and Week 62 (Figures 39 and 41).

Results reported in Section 7.5 showed that there were differences in microbial compositions between mussel ferments using stoved and unstoved salts. Figure 39 shows that butanoic acid was only present in mussel ferments with unstoved salt but not in ferments with stoved salt. Similar to findings made on the snapper unstoved ferments as discussed in Section 6.7, the presence of butanoic acid in all mussel unstoved ferments salt may be explained by the higher relative abundances of *Bacillaceae* in the respective ferments compared to that in all stoved ferments. In all mussel stoved ferments, *Bacillaceae* was present at noticeably lower relative abundances at either selected time-points or throughout the 80 weeks fermentation period and coherently, no formation of butanoic acid was detected in these samples (Figure 39; Section 7.5).

Figure 39 shows that the concentrations of acetic acid in almost all mussel ferments showed a steady increase over time supporting the hypothesis that the concentration of VFA increases as the fermentation time increases. It was noted that the concentrations of other VFA identified in all mussel ferments fluctuated during the fermentation time. However, it is important to point out that in most ferments, the concentrations of acetic, propionic and butanoic acids were higher by the end of the fermentation time compared to their values at Week 0.

7.8 Conclusion

In conclusion, the different fermentation conditions (type of salts and temperatures) influenced the bacterial diversity and compositions as well as the VFA produced in the mussel ferments during the fermentation process. The findings from this current study also imply that the presence of mussel shell may influence the pH which consequently influenced the product's texture, bacterial compositions and the VFA produced. Further investigations should be conducted to further understand the impact of the shell on the mussel fermentation.

Chapter 8: Fish Sauce Fermentation from Snapper (*Pagrus auratus*) Heads and Frames at Pilot Scale

8.0 Introduction

Traditionally, fish sauce fermentation takes place in fermentation tanks, wooden barrels or large earthen jars where the mixture of fish and salt are left to ferment in ambient temperature for up to 18 months (Salampessy et al., 2010). In commercial manufacturing of fish sauce, the fermentation will take place in large concrete tanks, sometimes circular with a dimension of ~0.9 m diameter × 1.0 m deep (Lopetcharat et al., 2001). The pilot scale fermentation was conducted to simulate the industrial manufacturing process. The aim of this experiment was to investigate the influence of sampling depth on snapper fermentation. Near the top surface of the barrel, oxygen content is quite high making it a more aerobic environment (Lopetcharat et al., 2001). Meanwhile, the oxygen level is limited under the liquid surface and extremely low at the bottom of the barrel thus creating a more anaerobic environment (Lopetcharat et al., 2001). In addition, near the bottom layer, more solid components will settle over time such as bones and scales. Higher pressure and carbon dioxide solubility may also result from deeper levels of the barrel. These conditions may impact a complex fermentation.

Two 30 L barrels were set-up using two types of salt: stoved and unstoved salt. The fermentation temperature of 30 °C was chosen to represent the average temperature commonly used in commercially produced Thai fish sauce. Ten millimetres of ferment liquor was taken from three different sampling depths from each fermentation set to provide triplicate data at Week 0. Based on the visual observations from the laboratory scale fermentation set-up, it was decided that the next sampling point for the ferment liquor would be at12 weeks to allow the fish frames and muscles to break down.

Unlike laboratory scale fermentation set-up, it was noted that the pilot scale fermentation design cannot sample the whole ferment but will provide sample dominant by ferment liquor. Hence, it may undercover bacteria involved in biofilms and favour planktonic bacteria. In addition, sample withdrawals may also disturb the fermentation process. The pilot fermentation set-up was described in detail in Section 3.3.2. The physical appearances, pH values, bacterial compositions and the formation of volatile fatty acids (VFA) of the ferment liquors were monitored throughout the fermentation time-course.

Hypotheses that will be tested in experiment were:

1. The microbiological composition and diversity present during the fermentation period are affected by the different fermentation conditions including type of salt and sampling depth in the fermentor, for snapper ferment liquors.
2. The concentration of the volatile fatty acids will increase as the fermentation time increases.

8.1 Experimental method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in a Figure 46.

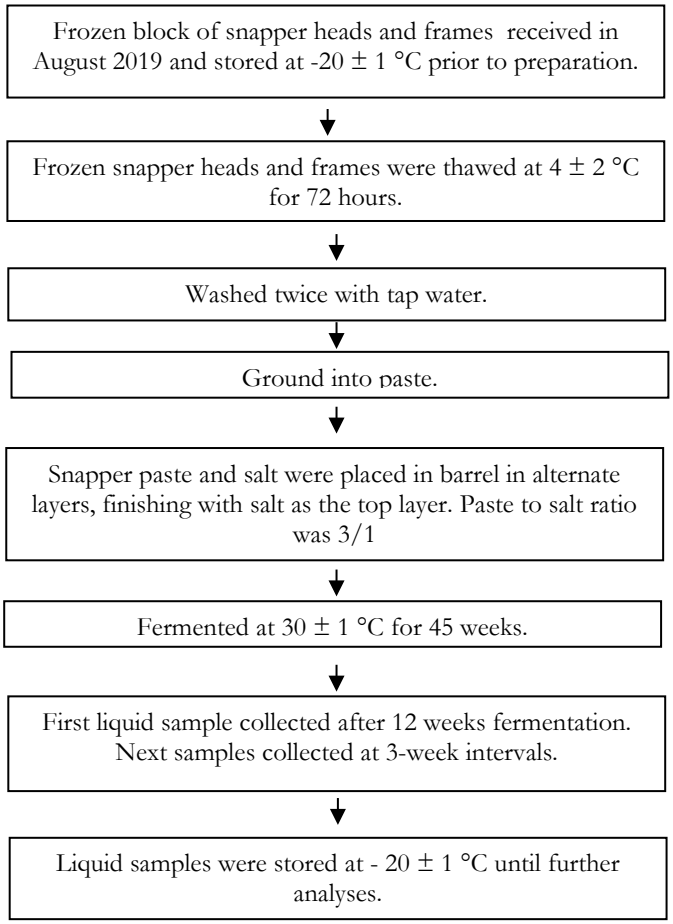


Figure 46: Experimental method flowchart of the snapper heads and frames fermentation at pilot scale. Note that the experiment was started in December 2021. Design of the pilot scale fermentation was described in Section 3.3.2.

8.1 Experimental method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in a Figure 46.

8.2 Visual Observation of the Snapper Fermentations

Based on overall observations, similar visual changes were observed in all ferment liquors sampled from three different sampling depths by the end of the fermentation. The colour of the ferment liquor changed from light brown with a grey hue, similar to the snapper paste at Week 0, to light brown colour by the end of the fermentation (Week 45).

A light brown colour with pinkish hue appeared at around Week 24 for all ferment liquor samples before it disappeared at around Week 30 until it reached the final colour recorded in Tables 36 and 37.

It was observed that small particulates were also collected when the ferment liquor was sampled. The sampling system was intended to sample the liquid from the snapper ferments. However, particulates including fish muscle and small bone/scales that were able to pass through the sampling port valves were also collected during the sampling process.

At the start of the fermentation, the aroma of all liquor ferments was described as marine-like and fishy. As the fermentation progressed, the samples developed a salty, pungent, fishy and ammoniacal aroma.

Table 36: Summary of the visual observations on the ferment liquors from snapper heads and frames with stoved salt fermented at 30 °C and sampled from three different depths of the fermentation barrel.

Sampling depth from the barrel's headspace (mm)	**Fermentation time (week)**	**Sample photo**	**Description**
N/A	0		A light brown with a hint of grey hue similar to the snapper paste observed.
Bottom (470 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.
Middle (370 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.
Top (270 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.

Table 37: Summary of the visual observations on the ferment liquors from snapper heads and frames with unstoved salt fermented at 30 °C and sampled from three different depths of the fermentation barrel.

Sampling depth from the barrel's headspace (mm)	**Fermentation time (week)**	**Sample photo**	**Description**
N/A	0		A light brown with a hint of grey hue similar to the snapper paste observed.
Bottom (470 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.
Middle (370 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.
Top (270 mm)	45		A light brown liquid with small solid materials observed. Pungent aroma and strong fishy smell was detected.

8.3 pH in Snapper Fermentations

The pH values of all snapper fish ferments were monitored throughout the fermentation process and presented in Figure 47.

The liquor ferments at Week 0 represents triplicate samples with either stoved or unstoved salts as no fermentation had yet taken place. Triplicate values at Week 0 allowed the calculation of mean standard errors that estimate the accuracy and reproducibility of the data point.

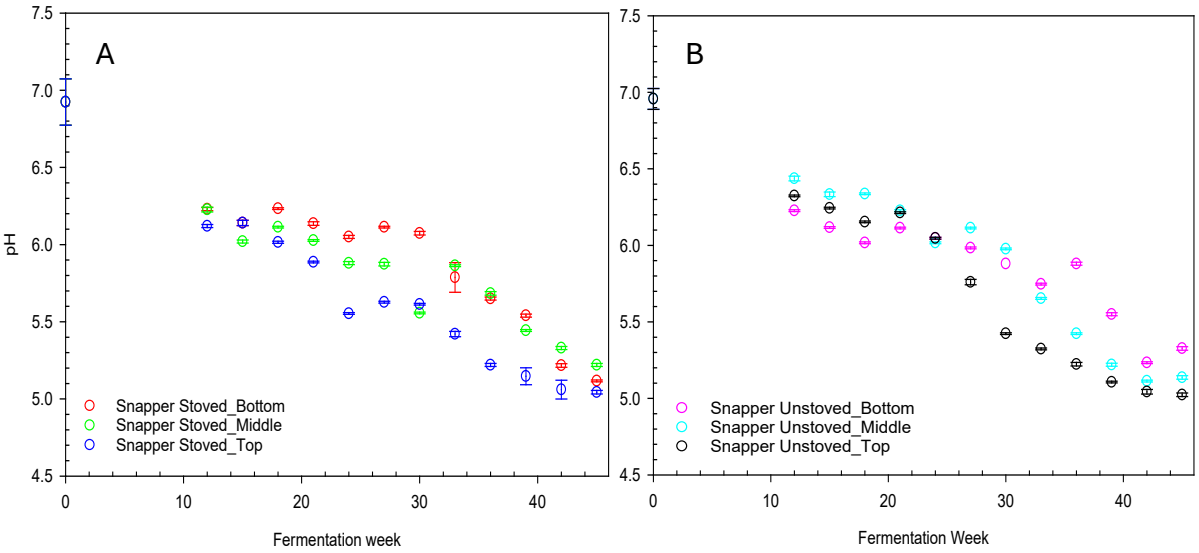


Figure 47: pH in snapper ferment liquors with stoved [Figure 1(A)] and unstoved [Figure 1(B)] salts fermented at 30 °C, sampled at different depths (Bottom: 470 mm from the headspace; Middle: 370 mm from the headspace; Top: 270 mm from the headspace). Values represent means ± standard error of mean (n=3). n is the number of readings from the same sample bottle.

The pH at Week 0 of stoved ferment liquors was 6.9 ± 0.2 and unstoved ferment liquors was 7.0 ± 0.1. The pH was found to decrease from Week 10 until Week 45 with the pH values recorded at the final week ranging between 5.0 and 5.3 (Figure 47). The final pH of all samples were within the standard pH range of traditionally fermented fish sauce underlined by CODEX (FAO, 2011). The pH values also agreed with small fermentations in Chapter 6.

Statistical analysis was performed to determine whether or not the pH values of all ferment liquors were significantly different to one another and the results obtained are presented in Figure 48. The Tukey post-hoc test revealed that the pH of stoved ferment liquor from the bottom layer was significantly different to the stoved samples from the top layer.

All other ferment liquors were not significantly different to one another. Overall, the statistical results indicate that the pH values between all snapper ferment liquors were not significantly different to one another.

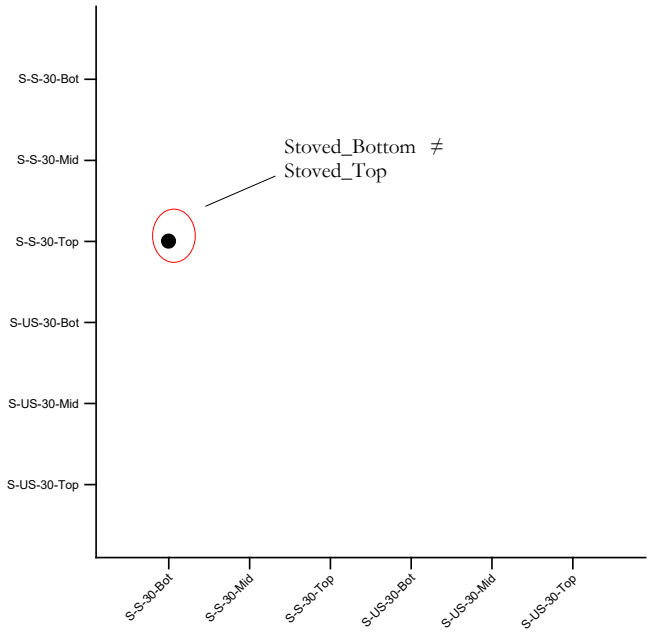


Figure 48: All pairwise comparisons based on Tukey test comparing pH values in all snapper fish ferment samples. The x- and y-axis labels represent each sample using stoved and unstoved salts fermented at 30 °C where the first "S" is abbreviated for snapper, the second "S" is abbreviated for stoved salt, the "US" is abbreviated for unstoved salt, the following number is the fermentation temperature used and the next abbreviations represents the different sampling depths where "Bot" is 470 mm from the headspace; "Mid" is 370 mm from the headspace; "Top" is 270 mm from the headspace. The "•" symbol indicates that the two compared snapper samples are significantly different to each other (p-value < 0.05). The "≠" symbol is used to indicate "significantly different".

8.4 Volatile Fatty Acid Formation in Snapper Fermentations

Four VFA were identified in snapper ferment liquors: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Butanoic acid was only present in ferment liquors with unstoved salt. Figure 49 shows that acetic acid had the highest concentration in all snapper ferment liquors followed by 3-methylbutanoic acid, butanoic acid (only for f unstoved salt) and propionic acid.

The concentration of acetic acid in ferment liquors at Week 0 with stoved and unstoved salts were 23 ± 2 and 22 ± 1 ppm, respectively. After 12 weeks of fermentation, acetic acid in either stoved or unstoved salt samples increased markedly to values ranging between 80 ppm and 200 ppm. Acetic acid in all stoved samples fluctuated between 100 and 180 ppm during Week 15 until Week 36 then higher values were observed during the remainder of the fermentation time (280 – 380 ppm) (Figure 49). Meanwhile, acetic acid in all unstoved samples remained between 160 to 300 ppm throughout the entire fermentation period.

Figure 49 shows that the concentration of 3-methylbutanoic acid in ferment liquors at Week 0 with stoved and unstoved salts were 10 ± 1 ppm and 11 ± 0, respectively. The 3-methylbutanoic acid in all stoved samples were between 10 – 20 ppm during mid-fermentation (Weeks 12 – 27) . For the remaining fermentation time, different trends were observed for 3-methylbutanoic acid in the stoved samples: its concentration decreased in the bottom layer; remained relatively constant in the middle layer and increased in the top layer (Figure 49). On the other hand, the 3-methylbutanoic acid concentration in all unstoved samples increased during the first 12 weeks of fermentation to values ranging between 26 – 34 ppm. Then, the 3-methylbutanoic acid fluctuated between 20 ppm and 38 ppm, gradually declining for the remainder of the fermentation time-course (Figure 49).

The concentrations of propionic acid in all ferment liquors with either stoved or unstoved salts taken from all three depths ranged between 3 ppm and 7 pm throughout the entire fermentation time course.

Figure 49 shows that butanoic acid was only present in unstoved ferment liquors and the concentration at Week 0 was 5 ± 1 ppm. The butanoic acid in all samples increased after 12 weeks to values of 14 – 18 ppm. The concentrations of butanoic acid in all unstoved samples remained relatively constant until the end of the fermentation.

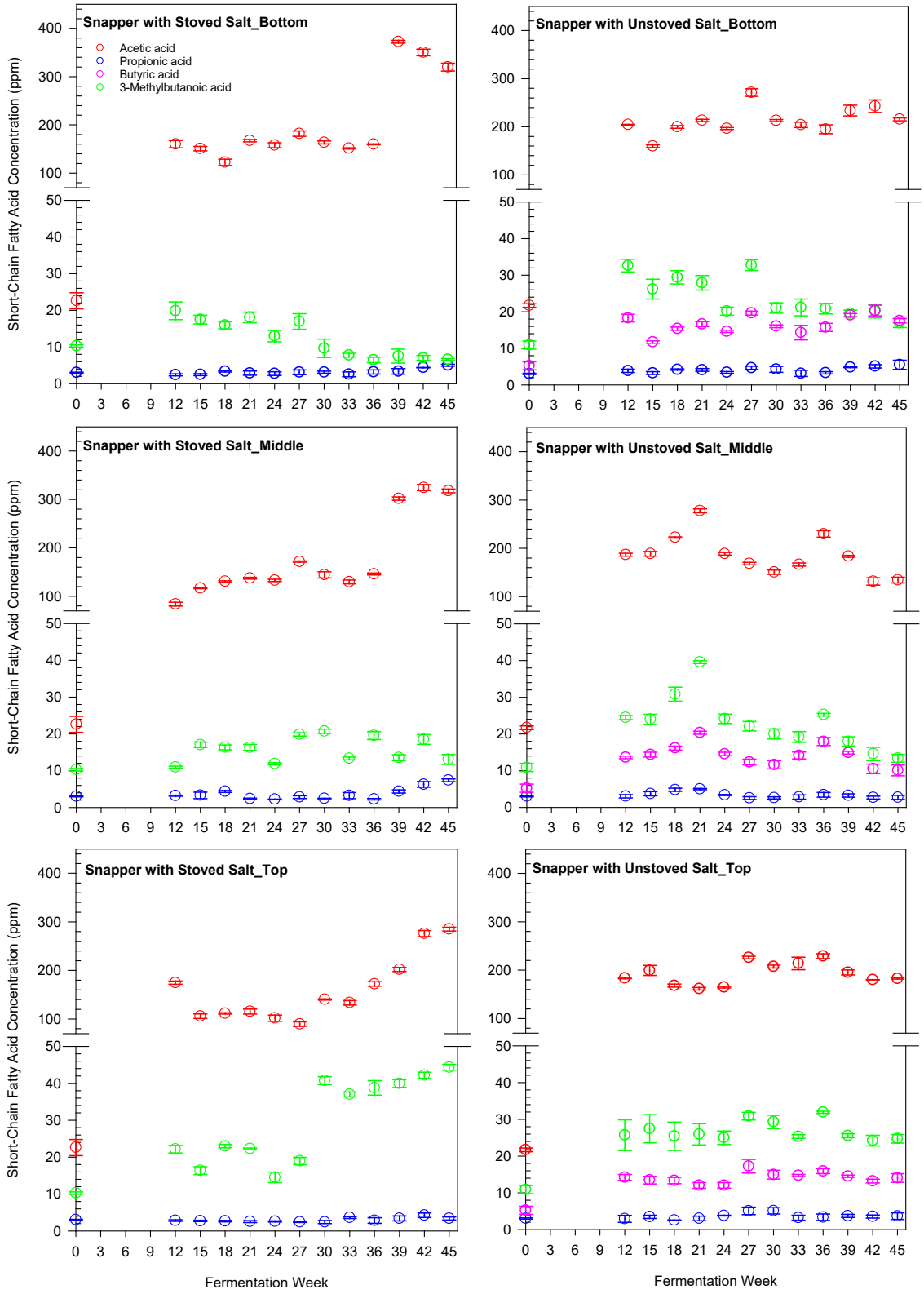


Figure 49: Short-chain volatile fatty acids (VFA) in ferment liquors from snapper heads and frames with stoved and unstoved salt fermented at 30 ˚C, sampled at different depths (Bottom: 470 mm; Middle: 370 mm; Top: 270 mm from the headspace). Values represent means ± standard error of means (n=3). n is the number of injections from the same sample vial except at Week 0 where n represents triplicate of the biological samples taken from three different sampling depths. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9.0 ppm, 2.2 ppm, 3.36 ppm and 2.0 ppm, respectively.

Statistical analysis was conducted to determine if there were any significant differences in any of the VFA identified in all snapper ferment liquors sampled from the three different depths. The Kruskal-Wallis test revealed no significant difference in the concentrations of acetic acid and butanoic acid between all the ferment liquors. Tukey post-hoc test was conducted only for propionic and 3-methylbutanoic acid and the results obtained are presented in Figure 50.

Figure 50(A) shows that propionic acid in the unstoved ferment liquors sampled from the bottom was significantly different to that in the stoved ferment liquors sampled from the top layer. No significant differences in propionic acid values were apparent between other samples.

Figure 50(B) shows that 3-methylbutanoic acid in stoved samples collected from the bottom layer was significantly different to all other samples except for the stoved sample collected from the middle layer. The 3-methylbutanoic acid in the stoved sample from the middle layer was significantly different to samples from either stoved or unstoved salts from the top layers.

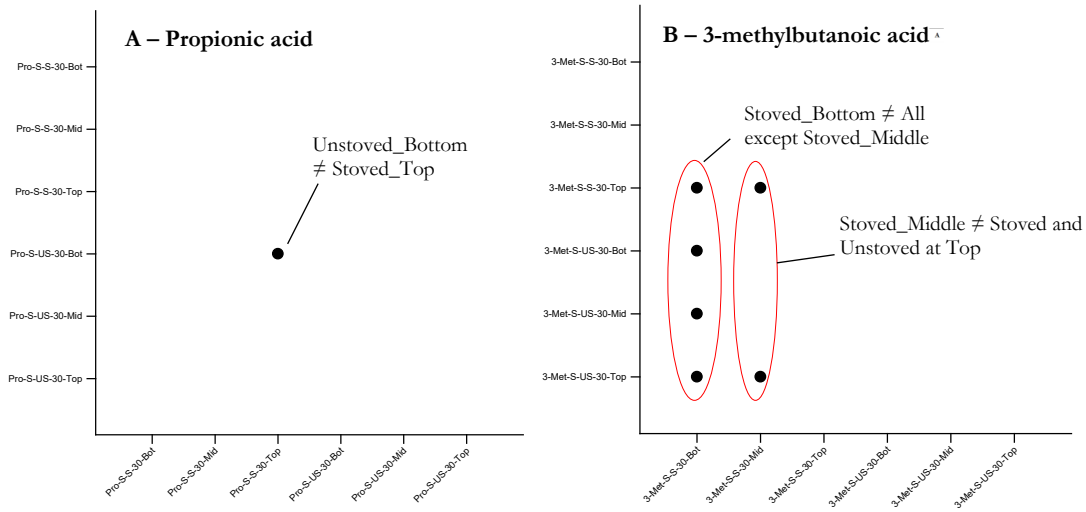


Figure 50: All pairwise comparisons based on Tukey test comparing volatile fatty acids identified in all Snapper fish ferment samples. Each graph represents two of four volatile fatty acids identified in Snapper ferment liquors and the x- and y-axis labels represent each sample using stoved and unstoved salts fermented at 30 °C, sampled at three different depths, where "Pro" = propionic acid; "3-Met" = 3-methylbutanoic acid, the first "S" is abbreviated for Snapper, the second "S" is abbreviated to stoved salt, the "US" is abbreviated to unstoved salt and the following abbreviations refers to the different sampling depths where "Bot" = 470 mm, "Mid" = 370 mm, and "Top" = 470 mm from the headspace. The "•" symbol indicates that the two compared samples are significantly different to each other (p-value < 0.05). The "≠" symbol is used to indicate "significantly different".

Overall, statistical analysis revealed that only the concentrations of two out of four VFAs identified were significantly different between all snapper ferment liquors but dependent on depth in the fermentor. This may imply that not all VFA had their accumulation influenced by the fermentation conditions. It is shown in Figure 49 that the concentrations of all VFA increased after 12 weeks of fermentation consistent with the hypothesis that the concentration of VFA will increase as the fermentation time increases. The concentrations of the majority of the VFA identified fluctuated from Week 15 until the final fermentation period could imply influence by complex interactions between bacterial communities during the fermentation process which will be further discussed in the following sections.

8.5 Bacterial Composition in Snapper Fermentations

To help understand the influence of depth on bacterial compositions in snapper ferment liquors during the 45 weeks fermentation period, a metagenomic approach was employed as discussed in Section 3.5.8.

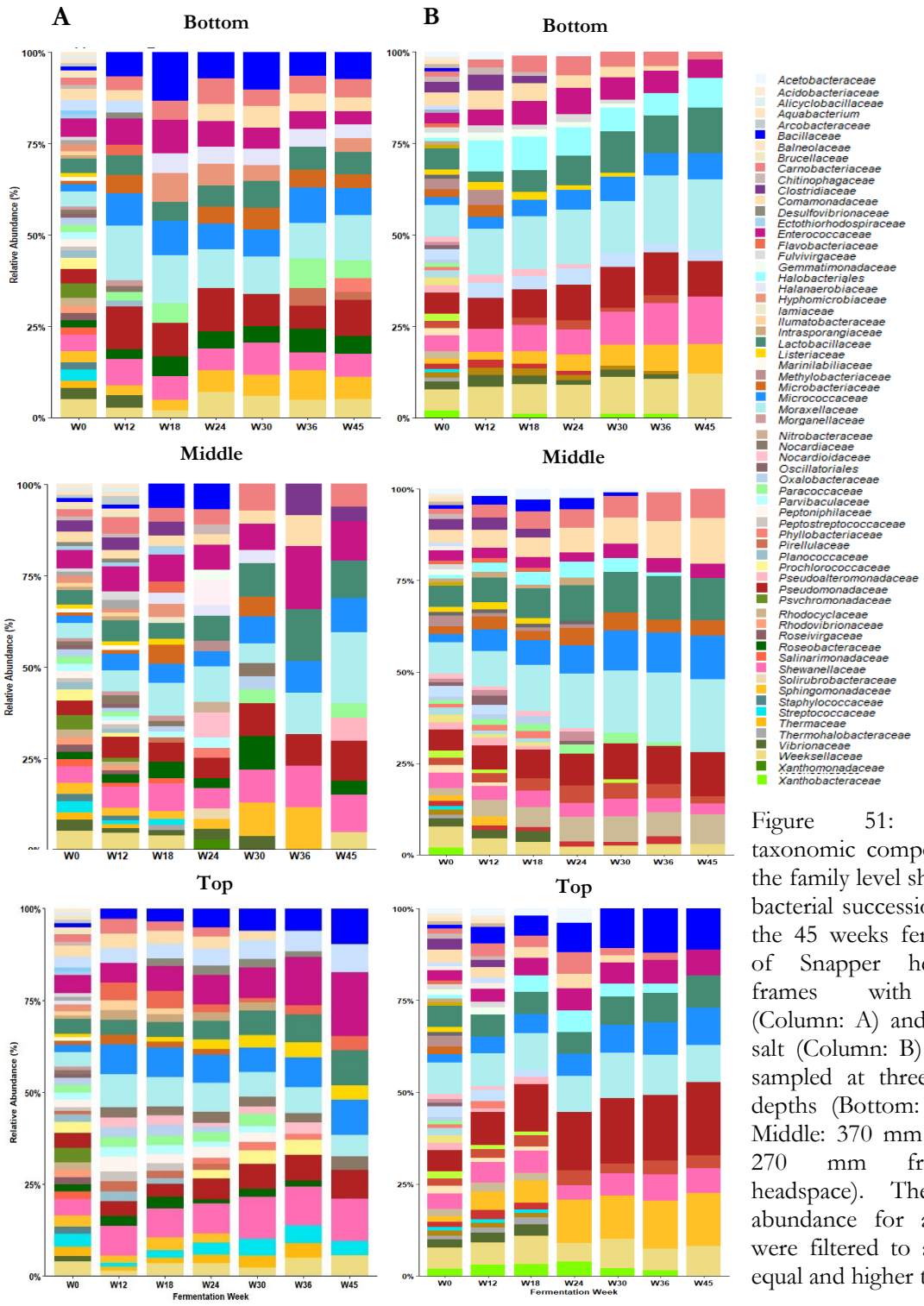


Figure 51: Bacterial taxonomic compositions at the family level showing the bacterial successions during the 45 weeks fermentation of Snapper heads and frames with stoved (Column: A) and unstoved salt (Column: B) at 30 °C, sampled at three different depths (Bottom: 470 mm, Middle: 370 mm and Top: 270 mm from the headspace). The relative abundance for all groups were filtered to a value of equal and higher than 1%.

The bacterial compositions in all snapper ferment liquors sampled from different depths are presented in Figure 51. No family group predominated at the initial fermentation period (Week 0) in any ferment liquors with stoved salt, meanwhile, the *Moraxellaceae* family was the predominant group in all unstoved samples at Week 0 showing a relative abundance of 8.6 ± 0.4 %. Figure 51(A) shows that the relative abundances of *Moraxellaceae* increased after 12 weeks of fermentation to 16.6 %, 5.6% and 9.0 % relative abundance in stoved ferment liquors from the bottom, middle and top layers, respectively. *Moraxellaceae* became one of the predominant family groups at the final fermentation week (Week 45) in unstoved ferment liquors from bottom (relative abundance = 12.2 %) and middle (relative abundance: 19.5 %) layers, while *Moraxellaceae* decreased to 5.7 % in the top layer (Figure 51(B)). The relative abundances of *Moraxellaceae* in all unstoved ferment liquors increased after 12 weeks of fermentation. *Moraxellaceae* increased gradually over the fermentation period in the bottom and middle layers in unstoved ferment liquors and eventually became the predominant group at the final fermentation period (Week 45) with the relative abundances of 19.1 % (bottom layer) and 20.0 % (middle layer). *Moraxellaceae* fluctuated between 10.0 % and 12.5 % from Week 18 until Week 45 in the unstoved ferment liquors from the top layer (Figure 51(B)).

Figure 51 also shows that the relative abundances of the *Enterococcaceae* family increased during the first 12 weeks of fermentation in all ferment liquors. Figure 51(A) shows that *Enterococcaceae* in the stoved ferment liquors from the bottom layer had decreasing abundance from Week 18 until the final fermentation period with the lowest relative abundance recorded at Week 45 (3.7 %). Meanwhile, the opposite pattern was observed in the stoved samples taken from the top layer where an increasing abundance of *Enterococcaceae* was observed during Week 18 until Week 45, making *Enterococcaceae* one of the predominant groups during the later stages of the fermentation period (Figure 51(A)). There was a noticeable increase in *Enterococcaceae* in stoved samples from the middle layer at Week 36 at 17.1 % before it decreased to 10.9 % by the end of the fermentation time.

In all unstoved ferment liquors (Figure 51(B)) the relative abundances of *Enterococcaceae* fluctuated between 2.9 % and 7.1 % from Week 12 until the final fermentation period, these relative abundances were lower than for samples from the middle layer.

Overall, Figure 51 shows that the relative abundances of *Moraxellaceae* decreased when that of *Enterococcaceae* increased during the fermentation period and vice versa. The observations could be explained by the inhibition of the growth of *Moraxellaceae* by the bacteriocins produced by *Enterococcaceae.* A study on Korean fermented soybean (*doenjang*) reported that the relative abundances of *Enterococcus* (family: *Enterococcaceae*) and *Bacillus* (family: *Bacillaceae;* the genus is associated with food spoilage) were influenced by one another (Kim et al., 2016). The study found that the relative abundances of *Bacillus* decreased as the amount of *Enterococcus* increased (Kim et al., 2016). This occurrence was ascribed to bacteriocins produced by *Enterococcus* acting as growth inhibitors to *Bacillus* in the ferment liquors (Kim et al., 2016). No similar relationship between *Enterococcaceae* and *Bacillaceae* was observed in Figure 51 and no attempt was made in the current study to identify and quantify bacteriocin levels in ferments.

Figure 51(A) shows that *Pseudomonadaceae* became one of the predominant groups in ferment liquors collected from the bottom and middle layers by the end of fermentation. *Pseudomonadaceae* in the stoved sample from the top layer fluctuated between 4 - 8 % during the entire fermentation time course(Figure 51 (A)). Figure 51(B) shows that there were increments in the relative abundances of *Pseudomonadaceae* in all unstoved ferment liquors throughout the fermentation period with the highest in samples collected from the top layer. *Pseudomonadaceae* became one of the predominant groups in unstoved samples by the end of the fermentation period.

The relative abundance of *Lactobacillaceae* increased after 12 weeks of fermentation in all ferment liquors (Figure 51). Figure 51(A) shows *Lactobacillaceae* increased as the fermentation progressed in the stoved ferment liquors for middle and top layers. *Lactobacillaceae* in the stoved samples from

the bottom layer did not change staying between 6 – 7 % relative abundance throughout the entire fermentation time (Figure 51(A)).

Figure 51(B) shows that there were increments in the relative abundances of *Lactobacillaceae* in all unstoved ferment liquors from Week 18 – 45 where the highest value of 13 % was found at Week 45 in the bottom layer.

Figure 51 shows that the relative abundances of *Shewanellaceae* increased after 12 weeks of fermentation in all ferment liquors except for in the unstoved sample from the middle layer. Figure 51(A) shows that the overall relative abundances of *Shewanellaceae* were highest in stoved ferment liquors from the top layer followed by the middle and then bottom layers. Figure 51(B) shows that the overall relative abundances of *Shewanellaceae* were highest in the unstoved samples from the bottom layer and there were no noticeable differences between the overall values of the relative abundances in samples collected from the middle and top layers. The *Shewanellaceae* family consisted of the sole genus *Shewanella* and the family group can be found in proteinaceous foods as well as marine environment and organisms (Satomi, 2014). The family *Shewanellaceae* can reduce trimethylamine oxide (TMAO) to trimethylamine (TMA), a compound commonly associated with fishy odour (Satomi, 2014).

Figure 51 shows that *Halobacteriales* was present only in ferment liquors with unstoved salt. The relative abundance of *Halobacteriales* increased after 12 weeks of fermentation (Figure 51(B)). *Halobacteriales* had the highest overall value range (6 – 9 %) in the unstoved sample in the bottom layer during Weeks 15 – 45. Meanwhile, *Halobacteriales* in the unstoved samples from the middle and top layers were fluctuating at lower ranges of 1 – 6 % during the same fermentation period (Figure 51(B)). It was observed that *Halobacteriales* disappeared at Week 45 in samples collected from the middle and top layers (Figure 51(B)). A study on a Korean anchovy fish sauce (*Meyolchi-Aekjeot*), reported that *Halobacteriales* grew well at the initial fermentation period and reached the

highest number of bacteria/gram at about Day 35 before rapidly decreasing as the fermentation progressed (Lopetcharat et al., 2001).

Overall, Figure 51 shows that the bacterial compositions at Week 0 in all snapper ferment liquors were more diverse compared to that at later fermentation weeks. The bacterial diversities in all samples decreased as the fermentation progressed. There were several family groups that increased in relative abundances as the fermentation progressed leading them to become the predominant groups by the end of the fermentation. In addition, it was determined that the bacterial compositions in snapper ferment liquors from the pilot scale fermentation were influenced by the type of salt used and the depth in the fermenter.

8.5.1 Heatmaps of the Snapper Fermentations

To further understand the bacterial compositions in all snapper ferment liquors during the fermentation process, the relative abundances of all the bacterial family groups present in the ferment liquors are visualised in heatmaps presented in Figures 52 and 53.

Figure 52 shows that there were some differences in the predominant family groups in all ferment liquors by the end of the fermentation period (Week 45). *Moraxellaceae* and *Pseudomonadaceae* were the predominant groups at Week 45 in samples collected from the bottom layer; *Enterococcaceae, Moraxellaceae, Pseudomonadaceae* and *Shewanellaceae* were the predominant groups in samples collected from the middle layer; and *Enterococcaceae* and *Shewanellaceae* were the predominant groups in samples collected from the top layer. Based on the relative abundances of the bacterial compositions, almost all stoved ferment liquors sampled at the same depth were clustered together regardless of the fermentation period (Figure 52).

Figure 53 shows that the relative abundances of *Moraxellaceae* in the unstoved ferment liquors from the middle and bottom layer were increasing as the fermentation progressed with this family group as the predominant group from Week 18 until Week 45. *Pseudomonadaceae* and *Sphingomonadaceae*

were the predominant family groups from Week 18 until the final fermentation period in the unstoved samples from the top layer.

There were increments in relative abundances of some family groups in ferment liquors as the fermentation progressed where most of these families became the predominant groups by the end of the fermentation period.

These occurrences were observed in the relative abundances of *Sphingomonadaceae, Shewanellaceae* and *Lactobacillaceae* in ferment liquors collected from the bottom layer; *Lactobacillaceae, Microbacteriaceae* and *Pseudomonadaceae* in ferment liquors collected from the middle layer; *Pseudomonadaceae* and *Sphingomonadaceae* in samples collected from the top layer (Figure 53). Based on the relative abundances of the bacterial family groups, all unstoved ferment liquors were clustered based on the sampling depths regardless of the fermentation week (Figure 53).

Overall, Figures 52 and 53 show that at Week 0 the bacterial compositions in all ferment liquors with either stoved or unstoved salts sampled from three different depths were similar and more diverse compared to all other samples of the same type of salt at later fermentation times. Figures 52 and 53 also show that there were differences in the bacterial compositions particularly the predominant family groups between stoved and unstoved salt, indicating that the type of salt influenced the bacterial compositions. In addition, all samples were clustered based on the sampling depths, suggesting that the sampling depth influence the bacterial composition within the samples fermented with the same type of salt. The results highlighted in this current section support the findings discussed previously in Section 8.5 and the hypothesis outlined in Section 8.0 that the microbiological compositions are affected by the salt and the sampling depth. .

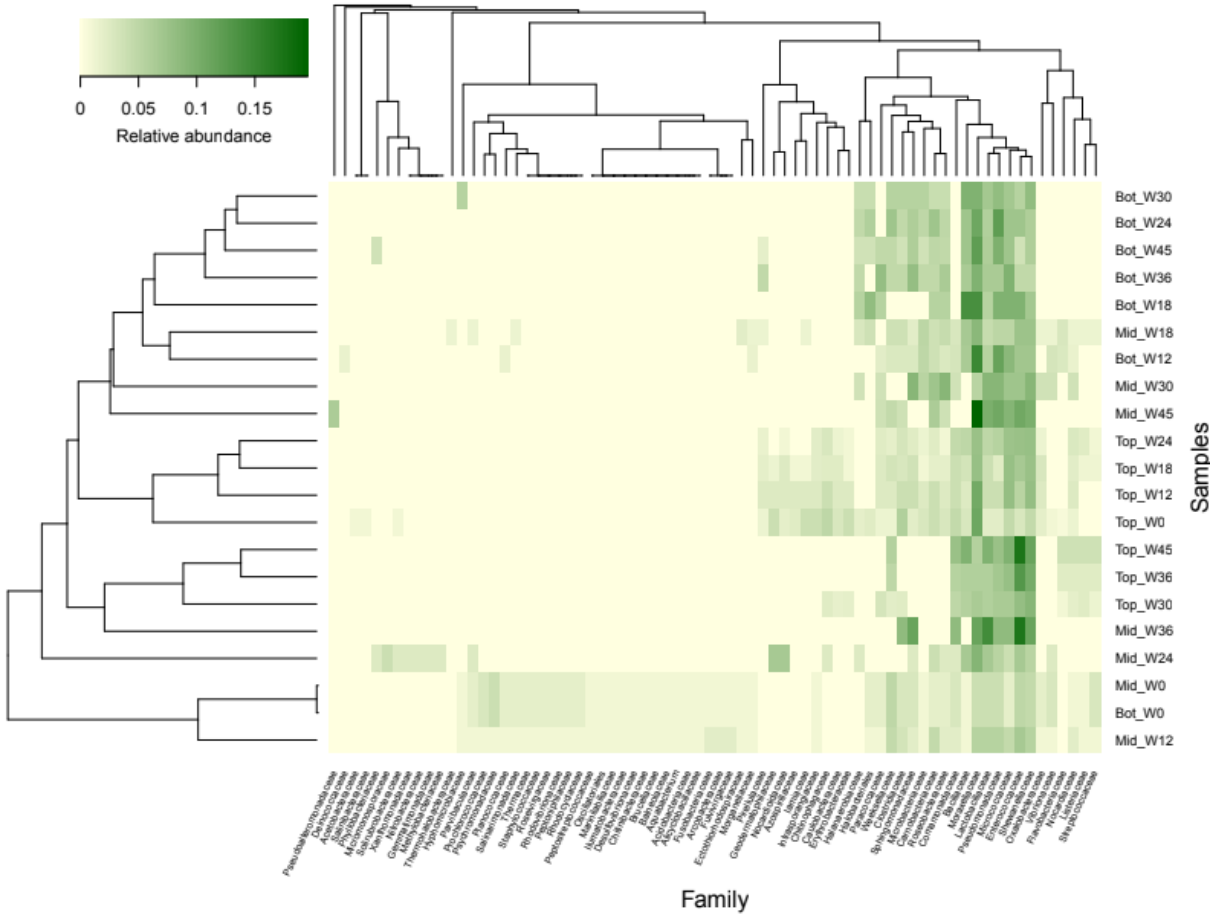


Figure 52: Heatmap of relative abundance at the family level across all snapper samples fermented with stoved salt at 30 °C for 45 weeks, sampled at three different depths from the headspace. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual snapper ferment liquors where "Bot", "Mid" and "Top" represent samples taken from 470 mm, 370 mm and 270 mm from the headspace, respectively. The "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

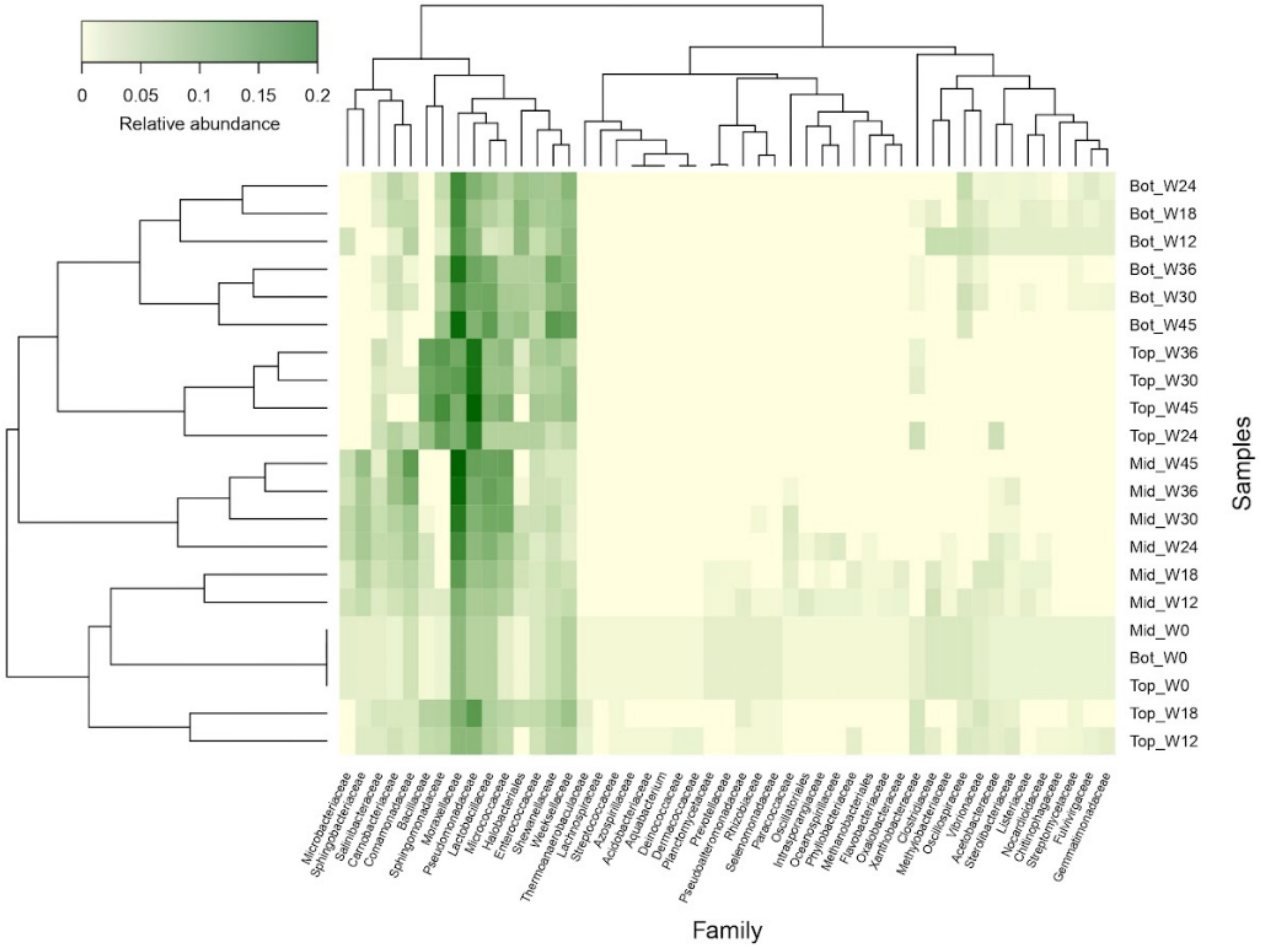


Figure 53: Heatmap of relative abundance at the family level across all snapper samples fermented with unstoved salt at 30 ˚C for 45 weeks, sampled at three different depths from the headspace. Samples and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual snapper ferment liquors where "Bot", "Mid" and "Top" represent samples taken from 470 mm, 370 mm and 270 mm from the headspace, respectively. The "W" is abbreviated for the fermentation week and the following number is referring to the timepoint of the fermentation week in which the sauce was sampled. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

8.5.2 Binary Comparison of Initial and Final the Bacterial Composition in the Snapper Fermentations

As discussed in Sections 8.5 and 8.5.1, it was found that the bacterial compositions in all snapper ferment liquors were more diverse at Week 0 compared to that at later fermentation weeks; as the fermentation progressed the bacterial diversity in all ferment liquors decreased. Binary comparison graphs are employed to help understand the differences in bacterial compositions in all samples at the beginning (Week 0) and end (Week 45) of fermentation. The binary comparison graphs will also indicate any noteworthy changes in the relative abundances of bacterial compositions between the two time-points. The binary comparisons between Week 0 and Week 45 in all ferment liquors with stoved and unstoved salt sampled from three different depths are presented in Figure 54.

Figure 54 shows that in the top layer, the relative abundances of *Moraxellaceae* (no. 63) were higher at Week 45 compared to that at Week 0 in all samples except in the unstoved sample. Figure 54 shows that *Moraxellaceae* was the predominant group by the end of the fermentation week in ferment liquors collected from the bottom and middle layers. From the top layer the relative abundances of *Enterococcaceae* (no. 33) and *Shewanella* (no. 100) were noticeably higher at Week 45 in the stoved samples and the relative abundances of *Pseudomonadaceae* (no. 88) and *Sphingomonadaceae* (no. 103) were also noticeably higher at Week 45 in the unstoved sample collected from the same sampling depths (Figure 54) making them the predominant groups at the final fermentation week. It was also noticed that most of the predominant groups at Week 45 were not dominant at Week 0 for all ferment liquors which was in agreement with the results discussed in Section 8.5 and Section 8.5.1 where there were increments in relative abundances in some family groups as the fermentation progressed.

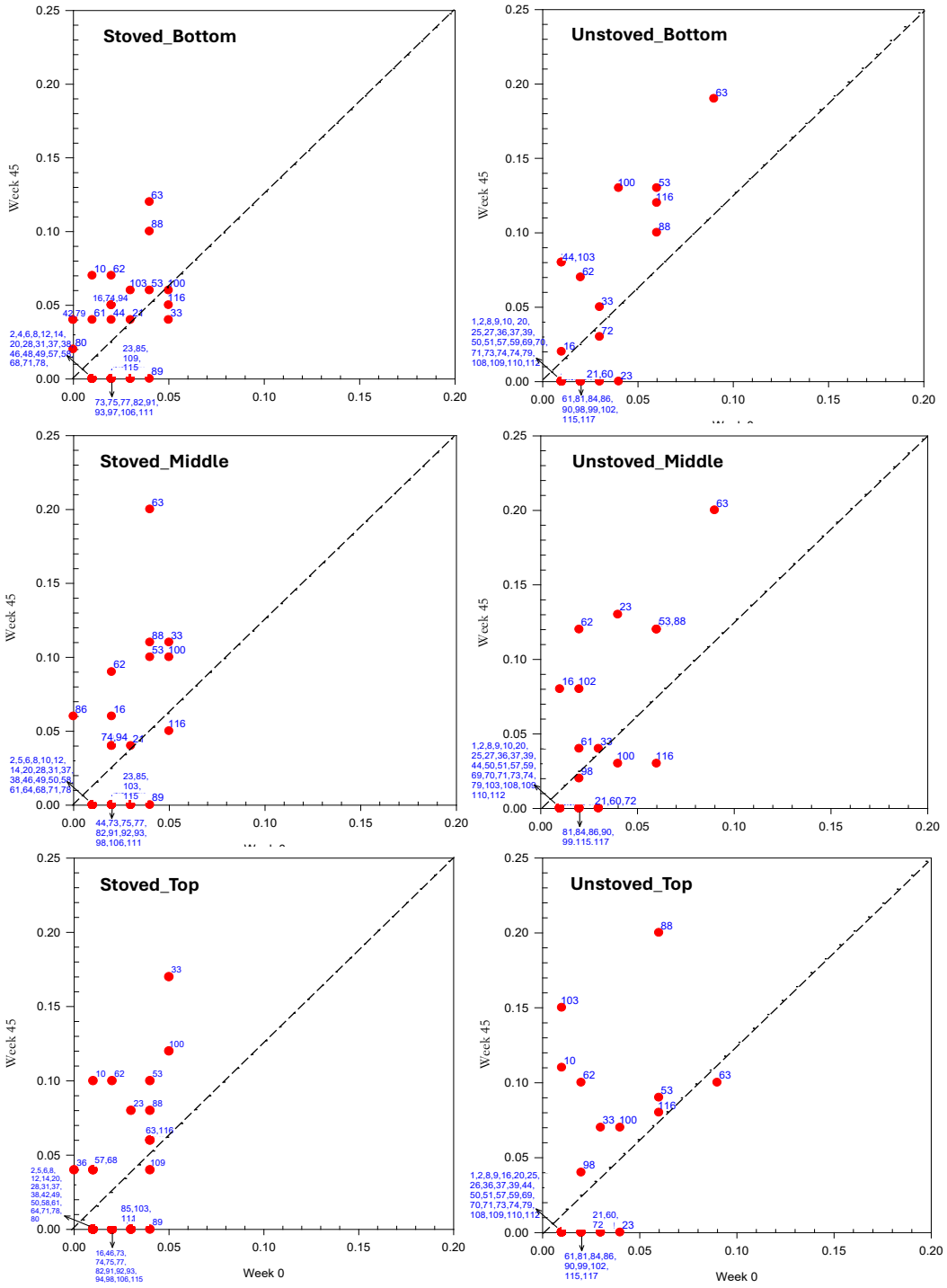


Figure 54: Binary comparison of relative abundance at the family level between two fermentation weeks in snapper frames and heads with stoved and unstoved salts at 30˚C, sampled at three different depths (Bottom: 470 mm from the headspace; Middle: 370 mm from the headspace; Top: 270 mm from the headspace). Family that are positioned below the 45˚ line indicate that the percentage of relative abundance is lower at Week 45 compared to that at Week 0 whereas family that are positioned above the 45˚ line indicate that the percentage of relative abundance is higher at Week 45 compared to that at Week 0. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01.

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

It is shown in Figure 54 that there were more than 34 family groups that were present exclusively at the initial fermentation period (Week 0) in all snapper ferment liquors. These findings support the results obtained in Section 8.5 and Section 8.5.1 that the bacterial compositions were more diverse at the initial fermentation period compared to that at the final fermentation week. Interestingly, there were three family groups that were present only at Week 45 in all stoved ferment liquors sampled from all three depths which were *Flavobacteriaceae* (no. 36), *Pirellulaceae* (no. 80) and *Pseudoalteromonadaceae* (no. 86) (relative abundances: 4 – 6 %).

Overall, Figure 54 revealed that the bacterial family groups present in the stoved ferment liquors were slightly different than the groups present in the unstoved samples indicating that the type of salt influenced the bacterial compositions. It was also found that the family groups present in the ferment liquors with the same type of salt differ between the three different sampling depths indicating that the sampling depths also influence the bacterial compositions during fermentation.

8.5.3 Alpha Diversity Analysis of the Snapper Fish Sauce

Alpha diversity analysis was employed as described in Section 3.5.8 and Section 5.5.3 to further understand the bacterial diversities in all snapper ferment liquors. Tables 38 and 39 summarize the sequencing data reads from snapper ferment liquors post-software trimming procedures and alpha diversity indices calculated from the obtained sequence reads.

Table 38 shows that the Chao1 indices in snapper ferment liquors with stoved salt were highest at Week 0 with the values of 219.56, 218.21 and 221.40 for samples collected from the bottom, middle and top layer, respectively. The Chao1 indices in all snapper ferment liquors showed a decreasing trend as the fermentation progressed with the lowest values found at Week 45 (Table 38). Consequently, the Simpson's indices were highest at Week 0 in all ferment liquors with stoved salt, with the values of 2.07, 2.07 and 2.08 for samples collected from the bottom, middle and top layers, respectively.

The Chao1 and Simpson's indices indicate that the bacterial diversity was the most diverse at Week 0 in all snapper ferment liquors with stoved salt, at all three sampling depths, with bacteria diversity decreasing as the fermentation progressed. Similar observations were observed in Table 39 where Chao1 indices in snapper ferment liquors with unstoved salt were highest at Week 0 with the values of 217.45, 219.45 and 220.58 for samples collected from the bottom, middle and top layers, respectively. The Chao1 indices in all ferment liquors decreased as the fermentation progressed (Table 39). Consequently, the Simpson's indices in all snapper ferment liquors with unstoved salt sampled from different sampling depths were the highest at Week 0 and decreased as the fermentation progressed. Table 38 and Table 39 shows that the Shannon indices of all samples had values close to 1 (D values ≥ 0.97), indicating that the abundances of the bacterial species within each sample were similar. The results obtained from the alpha diversity analysis support the findings in Section 8.5, 8.5.1 and 8.5.2 that the bacterial diversity was more diverse at the initial fermentation period compared to that at other fermentation weeks.

Table 38: Summary of the sequencing data sets derived from ferment liquors fermented from snapper frames and heads with stoved salt at 30 °C sampled from three different heights of the fermentation barrel for 45 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Layer	Depth of the sampling port from the headspace, mm	Fermentation week	High quality reads*	Average read length, bp*	OTU*	Chao1	Simpson's index, H	Shannon index, *D*
Bottom	470	0	22145	469	151	219.56	2.07	0.98
		12	21693	467	79	165.13	1.81	0.99
		18	19264	467	65	142.35	1.72	0.98
		24	18798	468	58	96.54	1.68	0.98
		30	21056	467	55	95.12	1.51	0.98
		36	22137	469	40	93.10	1.42	0.98
		45	25935	467	37	92.20	1.34	0.96
Middle	370	0	20159	467	148	218.21	2.06	0.98
		12	21711	467	109	202.13	1.90	0.98
		18	28810	467	98	181.08	1.83	0.98
		24	19452	467	88	130.50	1.78	0.99
		30	19586	468	84	121.00	1.67	0.98
		36	15967	467	75	117.50	1.54	0.99
		45	34763	471	79	120.44	1.46	0.97
Top	270	0	22135	467	154	221.40	2.08	0.99
		12	21493	468	101	158.21	1.74	0.97
		18	32410	467	100	141.07	1.66	0.98
		24	31027	467	99	132.54	1.60	0.98
		30	18715	467	75	125.63	1.56	0.98
		36	16589	467	68	113.11	1.54	0.99
		45	21964	487	67	105.58	1.51	0.98

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*bp = base pair
*OTU = operational taxonomic unit.

Table 39: Summary of the sequencing data sets derived from ferment liquors fermented from snapper frames and heads with unstoved salt at 30 °C sampled from three different heights of the fermentation barrel for 45 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Layer	Depth of the sampling port from the headspace, mm	Fermentation week	High quality reads*	Average read length, bp*	OTU*	Chao1	Shannon index, *H*	Simpson's index, *D*
Bottom	470	0	20369	477	150.24	217.45	2.08	0.98
		12	21465	469	80	200.98	1.73	0.98
		18	20147	467	75	190.78	1.68	0.99
		24	19036	476	68	164.86	1.74	0.98
		30	22784	467	55	154.32	1.60	0.98
		36	18978	468	46	95.12	1.46	0.97
		45	21485	468	35	92.12	1.38	0.97
Middle	370	0	20165	480	153.12	219.45	2.06	0.99
		12	19876	467	75	121.41	1.73	0.98
		18	18456	469	65	111.37	1.68	0.98
		24	20138	471	57	101.23	1.58	0.97
		30	21465	469	45	96.58	1.55	0.98
		36	19784	469	43	96.12	1.48	0.99
		45	18987	471	32	90.01	1.42	0.97
Top	270	0	21254	468	151.32	220.58	2.07	0.98
		12	24369	467	70	168.27	1.69	0.98
		18	20185	467	62	115.11	1.65	0.97
		24	19847	468	50	105.61	1.71	0.98
		30	21456	469	45	92.36	1.64	0.99
		36	20147	469	41	91.23	1.58	0.98
		45	21478	467	33	89.90	1.43	0.98

*High quality reads are the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*bp = base pair
*OTU = operational taxonomic unit.

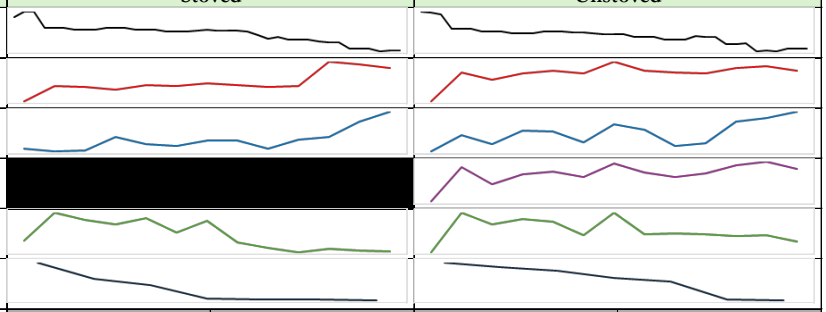
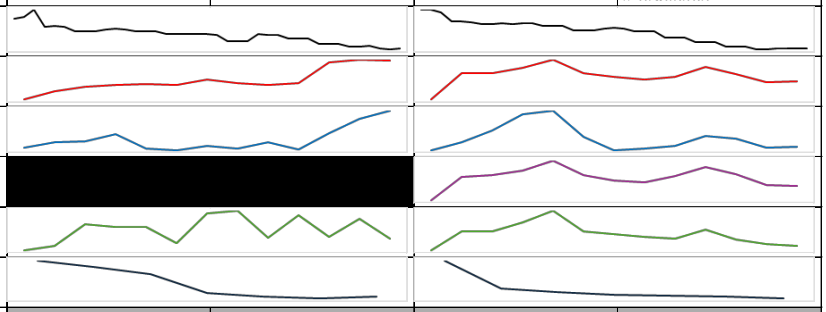
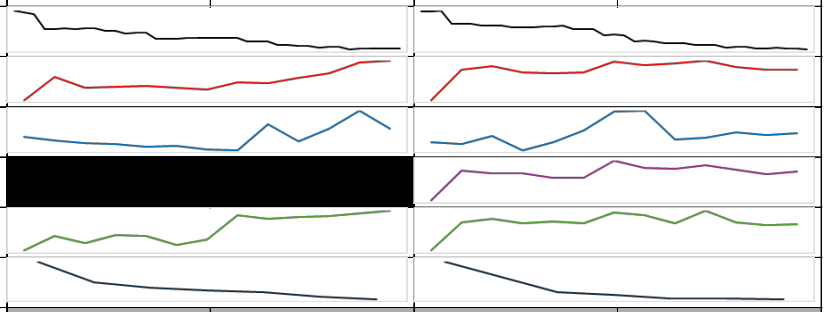
8.6 Discussion

Summary of the overall results from pilot scale snapper fermentation is presented in Table 40. Figure Section 8.5 reports on differences in microbial compositions between snapper ferment liquors using stoved and unstoved salts. The microbial communities in a fish sauce can influence the formation and concentration of volatile fatty acids (VFA) which consequently influence the overall sensory profile and quality of the final product (Ma et al., 2022). Figure 49 shows that butanoic acid was only present in the unstoved ferment liquors but not in samples with stoved salt. A difference was already evident at Week 0. A study by Amiri et al. (2016) revealed that *Nesterenkonia* sp. strain F, a halophile bacterium of the *Micrococcaceae* family had the ability to produce acetic and butanoic acids under aerobic and anaerobic conditions. The presence of butanoic acid in all unstoved ferment liquors may be explained by the high relative abundances of *Micrococcaceae* over the fermentation time course. In contrast, *Micrococcaceae* had lower relative abundances in all stoved ferment liquors where no formation of butanoic acid was detected in these samples (Figures 49 and 51).

The production of 3-methylbutanoic acid can be associated with the presence of lactic acid bacteria (LAB) as discussed in Section 6.7. Figure 51 showed that the aggregate relative abundance of LAB in stoved ferment liquors sampled from the bottom layer decreased from 20 % at Week 24 to 15 % at Week 45 which may have influenced the correlated decrease of 3-methylbutanoic acid between Week 27 and Week 45 (Figure 49). Similar occurrences were observed for unstoved ferment liquors sampled from the bottom and top layers but not the middle layer (Figures 49 and 51). In contrast, the cumulative relative abundance of LAB in the stoved ferment liquors from the top layer increased from 16 % at Week 24 to 301 % at Week 45, and in parallel the concentrations of 3-methylbutanoic acid in the sample increased from Weeks 27 until 45 (Figures 49 and 51).

The relationship between bacterial metabolic activities and pH during fermentation are reversible to an extent (Ratzke and Gore, 2018).

Table 40: Summary of the results from pilot scale snapper fermentation.

Sampling depth	Parameter	Snapper			
		Stoved		Unstoved	
Bot	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 45	Week 0	Week 45
		Nil	*Bacillaceae* *Lactobacillaceae* *Micrococcaceae* *Moraxellaceae* *Pseudomonadaceae* *Shewanellaceae* *Sphingomonadaceae*	*Moraxellaceae*	*Halobacteriales* *Lactobacillaceae* *Micrococcaceae* *Moraxellaceae* *Pseudomonadaceae* *Shewanellaceae* *Sphingomonadaceae* *Weeksellaceae*
Mid	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 45	Week 0	Week 45
		Nil	*Carnobacteriaceae* *Enterococcaceae* *Lactobacillaceae* *Micrococcaceae* *Moraxellaceae* *Pseudoalteromonadaceae* *Pseudomonadaceae*	*Moraxellaceae*	*Carnobacteriaceae* *Comamonadaceae* *Lactobacillaceae* *Micrococcaceae* *Pseudomonadaceae* *Sphingobacteriaceae*
Top	pH				
	Acetic acid				
	Propionic acid				
	Butanoic acid				
	3-methylbutanoic acid				
	Chao1				
	Predominant family groups	Week 0	Week 45	Week 0	Week 45
		Nil	*Bacillaceae* *Comamonadaceae* *Enterococcaceae* *Lactobacillaceae* *Micrococcaceae* *Pseudomonadaceae* *Shewanellaceae*	*Moraxellaceae*	*Bacillaceae* *Enterococcaceae* *Lactobacillaceae* *Moraxellaceae* *Shewanellaceae* *Sphingomonadaceae* *Weeksellaceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

Bacterial metabolic activities can influence pH by producing acids or bases as by-products which result in the alteration of the environment pH (Ratzke and Gore, 2018). Changes in pH can also influence bacterial metabolic pathways through feedback mechanisms, which in turn influence the bacterial communities in the environment, thus creating a dynamic relationship between the two factors (Ratzke and Gore, 2018). Figure 47 shows that overall, the pH values in all ferment liquors decreased to values ranging between 6.1 – 6.4 after 12 weeks of fermentation from the initial pH of 6.9 ± 0.2 (stoved ferment liquors) and 7.0 ± 0.1 (unstoved ferment liquors) at Week 0. One of the explanations could be due to the increased in the cumulative relative abundances of LAB which are known to produce organic acids (Ding et al., 2023). In a study on the diversity and dynamics of microbial populations during the fermentation of a Chinese fermented tofu (*gray sufu*), the genera *Lactobacillus* and *Pediococcus,* both belonging to the family *Lactobacillaceae* were found to be the predominant group during the fermentation which was associated with the decrease in pH (Ding et al., 2023). While the relationship between bacterial metabolic activities and pH during fermentation is dynamic and reversible, there are limits to this reversibility (Ratzke and Gore, 2018). Extreme pH conditions can inhibit bacterial growth or even cell death (Ratzke and Gore, 2018).

Tables 38 and 39 shows that the bacteria diversity in all ferment liquors decreased as the fermentation progressed. During fermentation processes, the initial stages often see a diverse array of bacteria present and as the fermentation progresses, certain bacteria dominate due to several factors such as nutrient depletion, competition and selective conditions which results in the decrease of bacterial diversity (Lestari et al., 2023). In a study on the fermentation of Korean anchovy sauce (*Meyolchi-Aekjeot*), the bacterial diversity was the highest at the initial fermentation period and gradually decreased with *Lentibacillus* and *Alkalibacillus* dominating by the end of the fermentation period (Jung et al., 2015).

In another study on Malaysian fish sauce (*budu*), it was reported that the bacterial diversity decreased as the fermentation progressed with the Chao1 indices recorded at Month 1 (initial fermentation) and 12 (final fermentation) were 82 and 46, respectively (Lestari et al., 2023).

8.7 Conclusion

In conclusion, the different sampling depths influenced the bacterial diversity and compositions as well as the VFA produced in the snapper fish sauce sample during the fermentation process. The findings from this current study also showed that sampling depth may influence the growth of some bacterial families to become the predominant groups by the end of the fermentation.

Chapter 9: The Effect of Shell on the Green-shell Mussel (*Perna canaliculus*) Fermentation

9.0 Introduction

As discussed in Section 7.7, the presence of the shell in the meat-shell mixture of the mussel ferments may influence the pH and consequently, influence the bacterial compositions. Mussel's shells are composed of calcium carbonate and organic material created by the mussels through biomineralization (Fitzer et al., 2015). During biomineralization process, mussels use bicarbonate ions from seawater along with proteins in their bodies to create calcium carbonate crystals, forming their protective shells (Fitzer et al., 2015). The shells are composed of two calcium carbonate polymorphs: calcite (outer layer) and aragonite (inner layer) (Fitzer et al., 2015).

The aim of this chapter is to understand the effect of different meat to shell ratios on the overall production of mussel fermentations using unstoved salt at 30 °C for 40 weeks. The fermentation set-up was described in detail in Section 3.3.3. The physical appearance, pH, bacterial composition and the formation of volatile fatty acids (VFA) of and in the mussel ferments were monitored throughout the fermentation time course.

Hypotheses that will be tested in this chapter are:

1. The microbiological composition and diversity present during the fermentation period are affected by the different ratios of meat to shell for the mussel fermentations.
2. The concentration of volatile fatty acids produced will increase as the fermentation time increases.
3. The influence of volatile fatty acid on pH decline will be muted by the presence of added shell.

9.1 Experimental Method

The experimental method to investigate the hypotheses outline for this current chapter is presented in Figure 55.

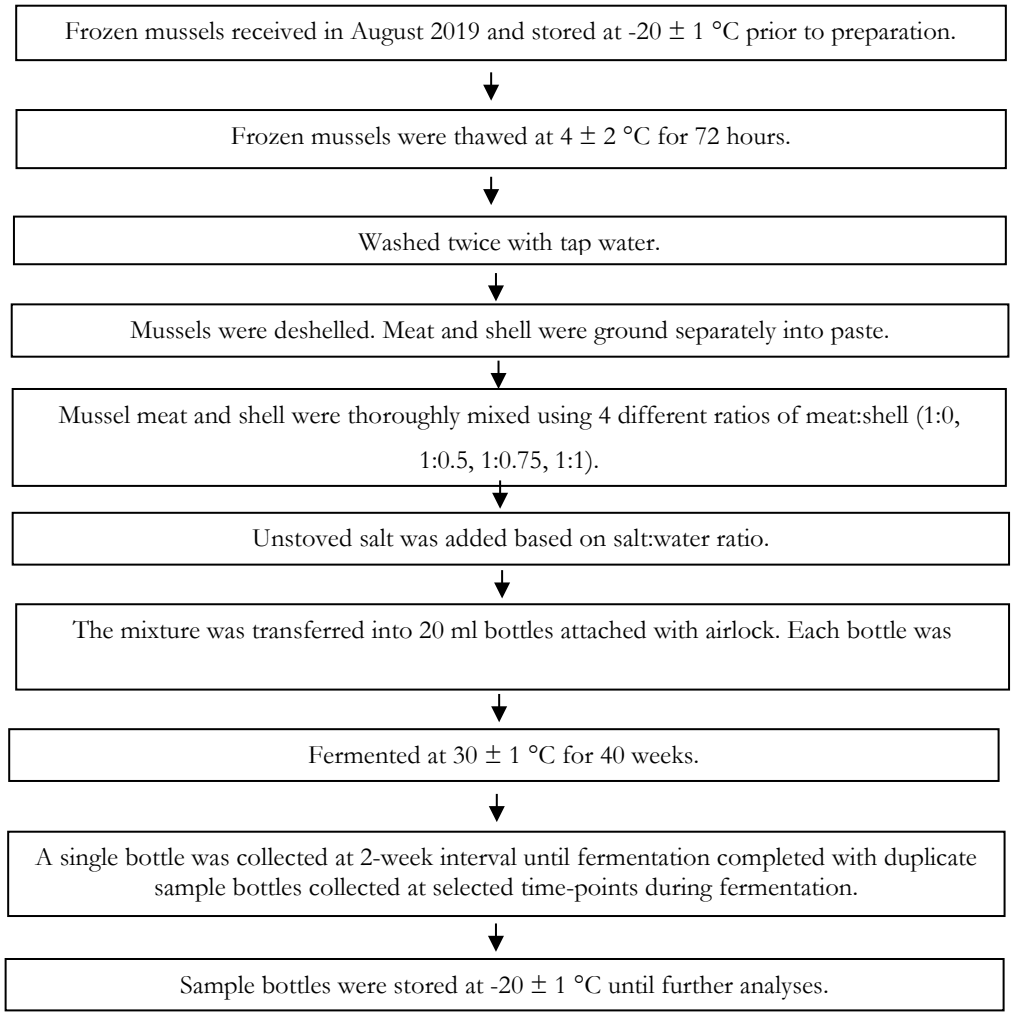


Figure 55: Experimental method flowchart of the green-lipped mussel ferments with different meat/shell ratios. The experiment was started in September 2022. Design of the fermentation set-up was described in Section 3.3.2.

The abbreviations for all fermentations with different meat:shell ratios are presented in Table 41.

Table 41: Mussel ferments abbreviations

Mussel meat content	Mussel shell content	Relevance	Abbreviation
1.0	0	No-shell	1/0 M/S
1.0	0.5	Half shell	1/.5 M/S
1.0	0.75	¾ shell	1/.75 M/S
1.0	1.0	Normal shell	1/1 M/S

*Content of mussel meat or shell is on a wet weight basis. M/S refers to mussel meat:shell ratio.

9.2 Visual Observation of the Mussel Fermentations

Visual observations on mussel ferments with different meat/shell ratios are presented in Table 42. For the 1/0 M/S ferment, it was observed that the texture of the ferments changed from firm at the initial fermentation time to soft solids after Week 12. For ferments with higher shell contents, it was observed that the texture of the ferments changed from firm meat with shell particles at the initial fermentation time to a combination of soft meat and shell particles after Week 16 for 1/.5 M/S ferment and appeared similarly at Week 18 and Week 22 for ferments with 1/.75 M/S and 1/1 M/S. The reddish hue appeared at Week 22 for the 1/0 M/S ferment and at Week 24, Week 26 and Week 30 for 1/.5 M/S, 1/.75 M/S and 1/1 M/S ferments, respectively. The colour of the ferments intensified as the fermentation progressed until it reached the final colour recorded in Tables 42.

Gas bubbles were observed in the Durham tubes in all ferments regardless of shell content from Week 2 until the final fermentation time – presumably carbon dioxide was the fermentation gas by-product, indicating that fermentation process was taking place. No gas bubble was observed in the Durham tube in any ferments at Week 0 indicating that fermentation had not yet taken place. No further investigation was carried out to determine the type and volume of gas produced.

At the start of the fermentation, the aroma of all mussel ferments was sweet and marine-like. As the fermentation progressed, the ferments developed a salty, pungent, fishy and ammoniacal aroma with a hint of marine-like aroma remaining by the end of the fermentation. Volatile fatty acids associated with mussel ferments were measured and results obtained will be discussed in the following sections.

Table 42: Summary of the visual observations on the green-shell ferments of four different meat:shell ratios with unstoved salt fermented at 30 ± 1 °C.

Ferment	Fermentation time (week)	Sample photo	Description
1/0 M/S	0		The mixture of mussel meat and salt are firm. The light brown colour with yellow hue of the mixture was similar to that of the ground mussel. No gas bubble observed in the Durham tube. Sweet and ocean-like aroma were detected.
	40		A reddish brown liquid layer on top of solid sediment observed. Gas bubble was observed in the Durham tube. Pungent, fishy smell with a hint of marine-like aroma detected.
1/.5 M/S	0		The mixture of mussel and salt are firm with noticeable hard texture from the shells. The brown colour with yellow hue of the mixture was similar to that of the ground mussel. No gas bubble observed in the Durham tube. Sweet and marine-like aroma were detected.
	40		A reddish brown liquid layer on top of solid sediment observed. Gas bubble was observed in the Durham tube. Pungent, fishy smell with a hint of marine-like aroma detected.
1/.75 M/S	0		The mixture of mussel and salt are firm with noticeable hard texture from the shells. The brown colour with yellow hue of the mixture was similar to that of the ground mussel. No gas bubble observed in the Durham tube. Sweet and marine-like aroma were detected.
	40		A reddish brown liquid layer on top of solid sediment observed. Gas bubble was observed in the Durham tube. Pungent, fishy smell with a hint of marine-like aroma detected.
1/1 M/S	0		The mixture of mussel and salt are firm with noticeable hard texture from the shells. The brown colour with yellow hue of the mixture was similar to that of the ground mussel. No gas bubble observed in the Durham tube. Sweet and marine-like aroma were detected.
	40		A reddish brown liquid layer on top of solid sediment observed. Gas bubble was observed in the Durham tube. Pungent, fishy smell with a hint of marine-like aroma detected.

9.3 pH in Mussel Fermentations

The pH values of all mussel ferments were monitored throughout the fermentation process and are presented in Figure 56.

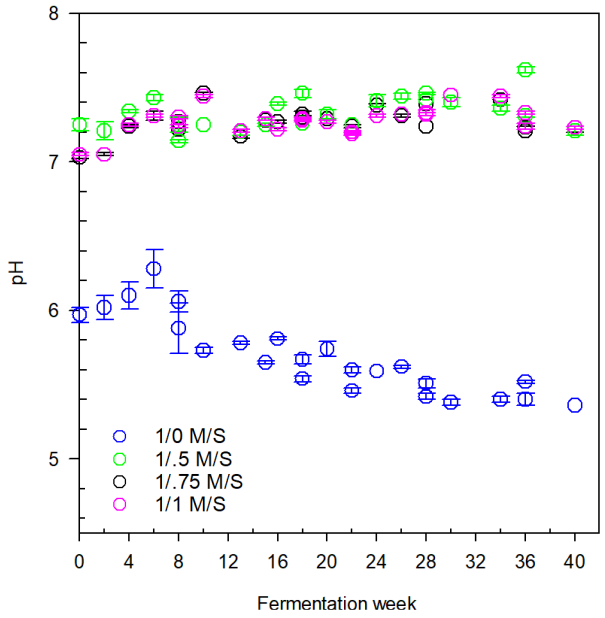


Figure 56: The pH of ferments from green-lipped mussels fermented using different meat/shell ratios. Values represent means ± standard error of mean (n=3). n is the number of readings from the same sample bottle.

The pH of the 1/0 M/S ferment at Week 0 was the lowest (pH: 5.9) while the pH of ferments with higher shell contents at the initial week ranged from 7.0 to 7.3 (Figure 2). The no-shell ferment showed a slight increase in pH from Week 2 – 6, then a decreasing trend over the remaining fermentation time course to a value recorded at Week 40 of 5.4. The pH values of all mussel ferments with added shell fluctuated between 7.1 – 7.6 throughout the fermentation time course.

Two sample bottles were drawn at selected time-points during the fermentation time course serving as duplicate biological samples. Duplicate values allow the calculation of variance that estimate the accuracy and reproducibility of the experimental method.

The final pH value of the no-shell ferment fell within the standard pH range of a traditionally fermented fish sauce underlined by CODEX and described in Section 2.6 while all ferments with added shell did not.

Statistical analysis was conducted to determine how significantly different all the datasets were from each other and the results are presented in Figure 57. The pH of no-shell ferment was significantly different to all fermentations with added shell. No significant difference was observed in the pH values between any mussel fermentations with added shell. Figure 57 revealed that the presence of shell influenced the pH of the mussel ferments, however, the amount of shell added did not. Further analyses on other parameters used in assessing the quality and progress of the ferments in this current study will be discussed in the following sections.

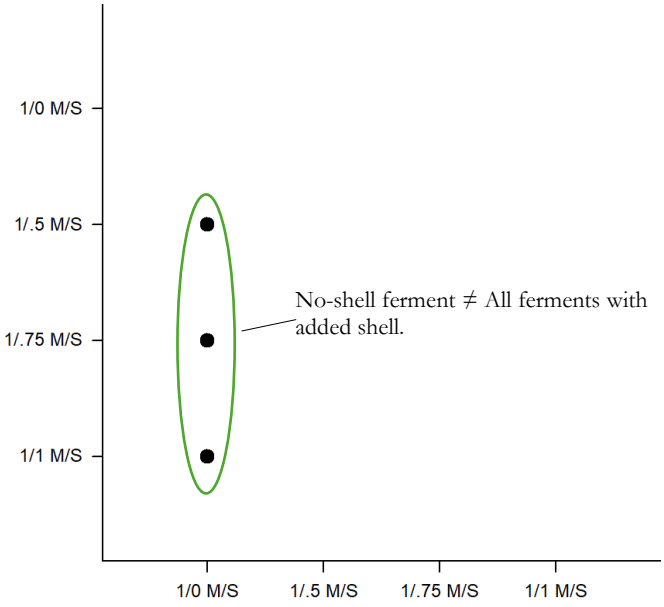


Figure 57: All pairwise comparisons based on Tukey test comparing pH values in all mussel ferments. The x- and y-axis labels represent each sample using different meat/shell ratios. The "•" symbol indicates that the two compared ferments are significantly different to each other (p-value < 0.05). The "≠" symbol is used to indicate "significantly different".

9.4 Volatile Fatty Acid Formation in Mussel Fermentations

Four VFAs were assayed in mussel ferments: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Figure 58 shows that acetic acid had the highest concentration in all mussel ferment followed by either propionic or 3-methylbutanoic acid and finally, butanoic acid.

Figure 58 shows that the 1/.75 M/S ferment had the highest overall acetic acid with values ranging between 65 – 160 ppm throughout the entire fermentation time course. Meanwhile, acetic acid in other ferments with different shell contents were fluctuating at a lower range of 11 – 120 ppm throughout the fermentation time course (Figure 58).

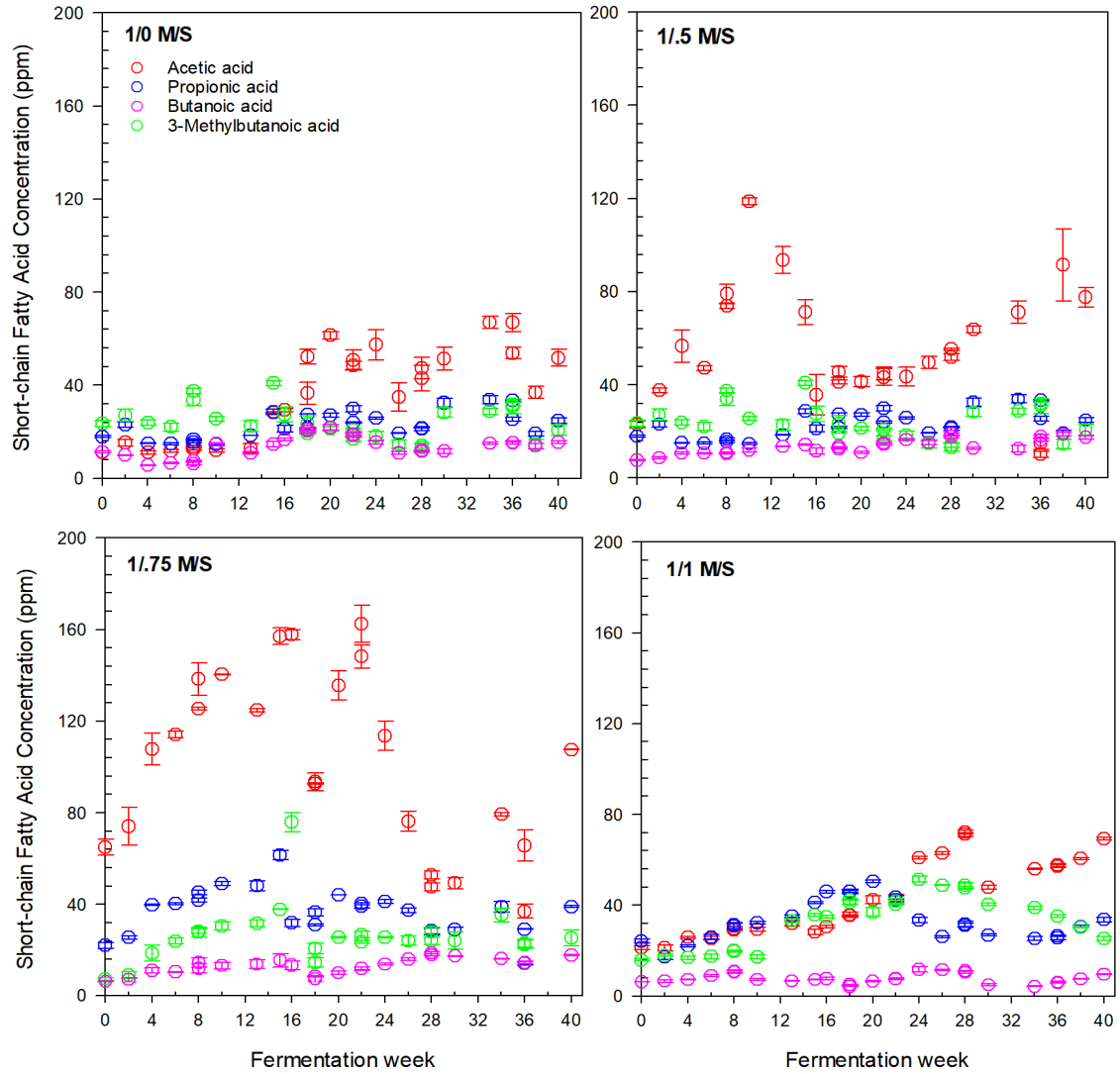


Figure 58: Short chain volatile fatty acids (VFA) in mussel ferments with different meat/shell ratios fermented with unstoved salt at 30 °C. Values represent means ± standard deviation(n=3). n is the number of injections from the same sample vial. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9 ppm, 2 ppm, 3 ppm and 2 ppm, respectively.

There were also hints of two phases of fermentation in the no-shell and normal shell fermentations where the acetic acid values were higher during the second half of the fermentation period compared to the earlier weeks. The acetic acid in the half and ¾ shell ferments both showed periods of acetic acid accumulation then loss of their accumulation.

Propionic acid and 3-methylbutanoic acid in no-shell and half shell ferments were hovering between 8 – 40 ppm throughout the entire fermentation time-course while the two VFAs were fluctuating at higher ranges between 8 – 64 ppm in ferments with higher shell contents (Figure 58). The concentrations of butanoic acid in all mussel ferments with four different meat/shell ratios were fairly consistent throughout the entire fermentation period between 12 – 41 ppm. Overall, Figure 58 shows that the values of all VFAs in almost all mussel ferments with different meat/shell ratios were higher by the end of the fermentation compared to that at the initial period (Week 0), supporting the hypothesis that VFA are produced as fermentation products.

Statistical analysis was conducted to evaluate whether or not the VFAs are significantly different between the mussel ferments with different shell contents and the results are presented in Figure 59. Figure 59(A) shows that acetic acid in the 1/.75 M/S ferment was significantly different than that in other mussel ferments with different shell contents. Figure 59(B) shows that propionic acid in mussel ferments with higher shell (¾ and normal shell) were significantly different than that in the ferments with lower shell contents (no-shell and half shell).

Based on Figure 59(C), it was shown that butanoic acid in normal shell ferment was significantly different than all other mussel ferments with lower shell contents. Similar results were obtained for 3-metylbutanoic acid as shown in Figure 59(D) – except no significant difference between normal and ¾ shell ferments.

Overall, the statistical analysis revealed that ferments with higher shell contents stood out as being different in VFA behaviour from the ferments with lower shell contents.

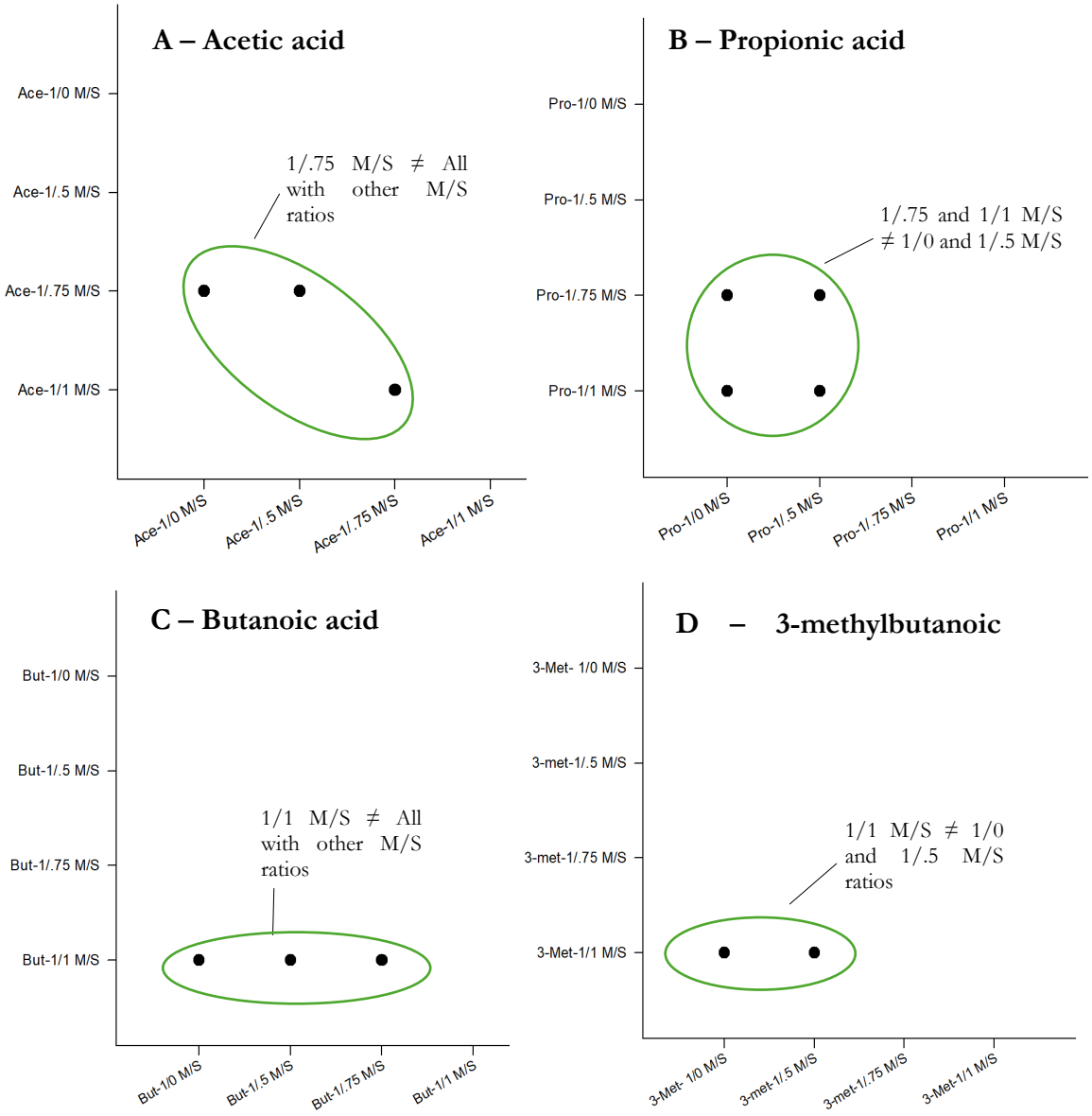


Figure 59: Pairwise comparison matrix based on Tukey test comparison of volatile fatty acid level in all green-shell mussel ferments. Each graph represents one of four different volatile fatty acids. The four experiments with different meat:shell ratios appear on both x- and y-axis. "Ace" = acetic acid; "Pro" = propionic acid; "3-Met" = 3-methylbutanoic acid, "But" = Butanoic acid. The following numbers are the meat/shell ratios. The "•" symbol indicates that the two compared mussel ferments are significantly different to each other. The "•" symbols are clustered and annotated to visualise the statistical results obtained. The "≠" symbol is used to indicate "significantly different".

9.5 Bacterial Composition in Mussel Fermentations

To understand the influence of different mussel fermentation conditions on the bacterial communities, metagenomic approaches were adopted as discussed in Section 3.3.9. Bacterial composition in the mussel ferments during the fermentation process is shown in Figure 6.

Figure 60 shows that four family groups predominated in all mussel ferments regardless of meat/shell ratio and throughout almost the entire fermentation time course: *Flavobacteriaceae* [Relative abundance(RA): 7.0 – 13.6 %], *Paracoccaceae* (RA: 5.4 – 14.7 %), *Roseobacteriaceae* (RA: 7.3 – 16.9 %) and *Vibrionaceae* (RA: 5.4 – 12.8 %). *Flavobacteriaceae* in the ¾ shell ferment became the predominant group after Week 12.

Several family groups predominated in only certain mussel ferments and/or at certain time-points during the fermentation period. Figure 60 shows that *Bacillaceae* was one of the predominant groups in the 1/0, 1/.5 and 1/.75 M/S ferments throughout the entire fermentation time-course but not in the 1/1 M/S ferment. *Legionellaceae* was one of the predominant groups only at certain time-points (Weeks 0, 6 and 30) during the no-shell fermentation. Meanwhile, *Legionellaceae* fluctuated at 3.0 – 7.0 % across the fermentation time course in mussel ferments with higher shell contents (Figure 60). Interestingly, *Pseudomonadaceae* was one of the predominant groups at Week 0 (RA: 12.0 %) and at Week 18 (RA: 13.7 %) in the normal shell ferment while the values were only 3 – 5 % most of the time. *Pseudomonadaceae* ranged from 2 – 4 % with lower shell contents and the group was not present at certain time-points in the no-shell and half shell ferments (Figure 60). Results shown in Figure 60 suggest that *Pseudomonadaceae* may prosper better with higher shell content. Conversely, *Clostridiaceae* ranged between 4.1 and 7.0 % throughout no-shell fermentation but were present only at certain time-points in ferments with higher shell contents and only at lower relative abundances of 2 – 5 %.

There were two family groups that belong to the *Haloarchaea* class present at either all or some mussel ferments with different shell contents – they were *Halobacteriales* and *Haloferacales*. Figure 60 shows that *Halobacteriales* was present at almost all fermentation weeks in the ferments with ¾ shell and normal shell (RA: 2.0 – 4.7 %) while the group was present at fewer time-points in the ferments with lower shell contents. *Haloferacales* was present at almost all fermentation weeks in the normal shell ferment with values ranging from 2.0 % to 3.9 % (Figure 60).

It was observed that *Haloferacales* was not present at all in the no-shell and ¾ shell ferments and only present during mid-fermentation in the half shell ferment. Based on Figure 60, *Haloarchaea* prospered better at ferments with higher shell contents.

Overall, Figure 60 revealed that there were similarities in the predominant family groups across mussel ferment regardless of meat/shell ratio. Some groups tended to predominate either at high or at low shell content supporting the hypothesis outlined in Section 9.0.

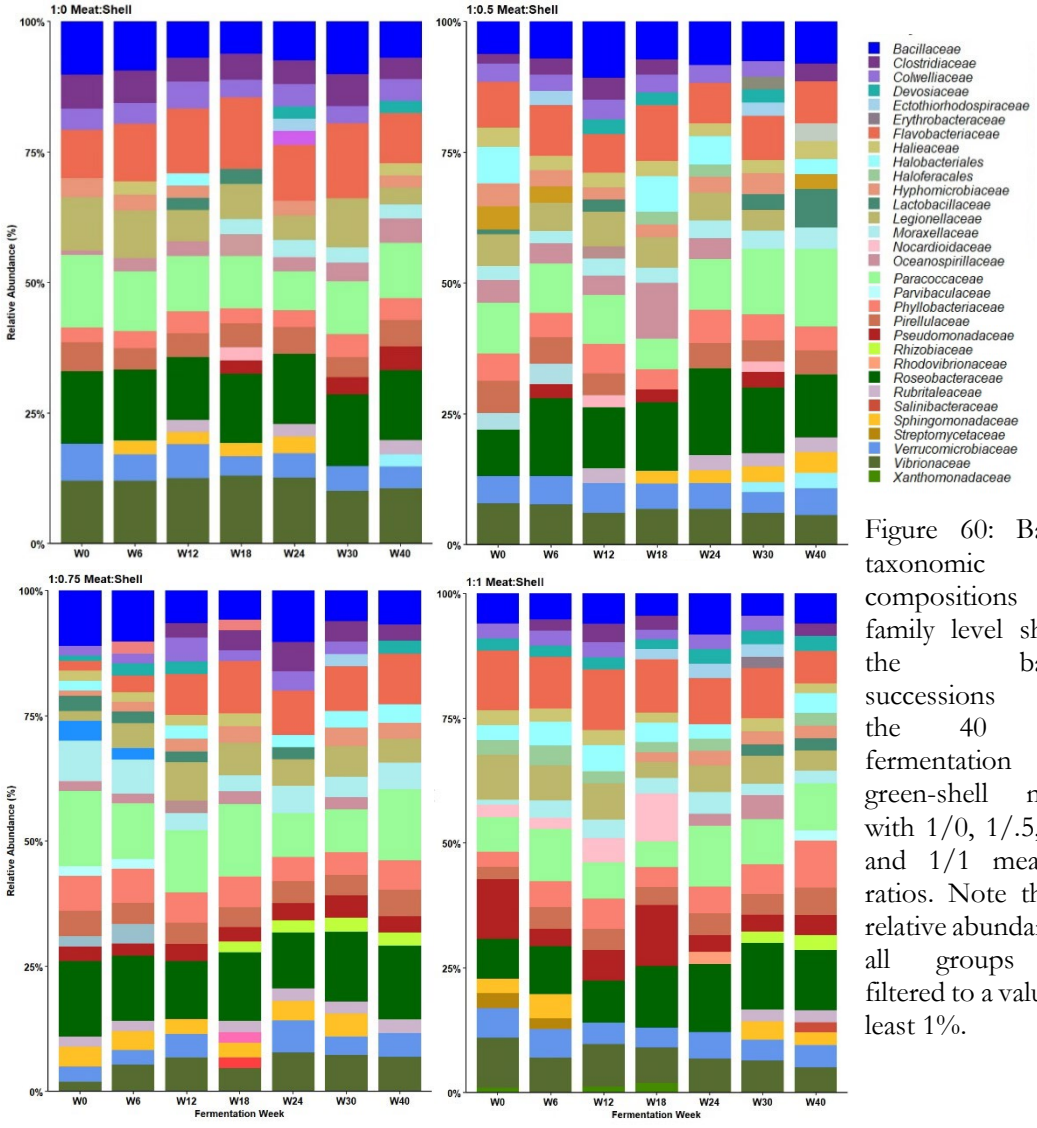


Figure 60: Bacterial taxonomic compositions at the family level showing the bacterial successions during the 40 weeks fermentation of green-shell mussels with 1/0, 1/.5, 1/.75 and 1/1 meat/shell ratios. Note that the relative abundance for all groups were filtered to a value of at least 1%.

9.5.1 Heatmaps of the Mussel Fermentations

The relative abundances of all bacterial family groups identified in all mussel ferments were further visualised on heatmaps presented in Figures 61. There were four predominant family groups throughout the entire fermentation time course in all mussel ferments with different meat/shell ratios were *Flavobacteriaceae, Roseobacteriaceae, Paracoccaceae* and *Vibrionaceae* (Figure 61). The mussel ferments were clustered based on meat/shell ratios regardless of the fermentation week suggesting that the bacterial communities were influenced by the shell contents.

Figure 61 shows that the bacterial diversities in no-shell and normal shell mussel ferments were higher during late fermentation compared to that in the earlier period while the opposite observations were made for half and ¾ shell ferments. Further analysis was conducted to verify the observations made in this current section.

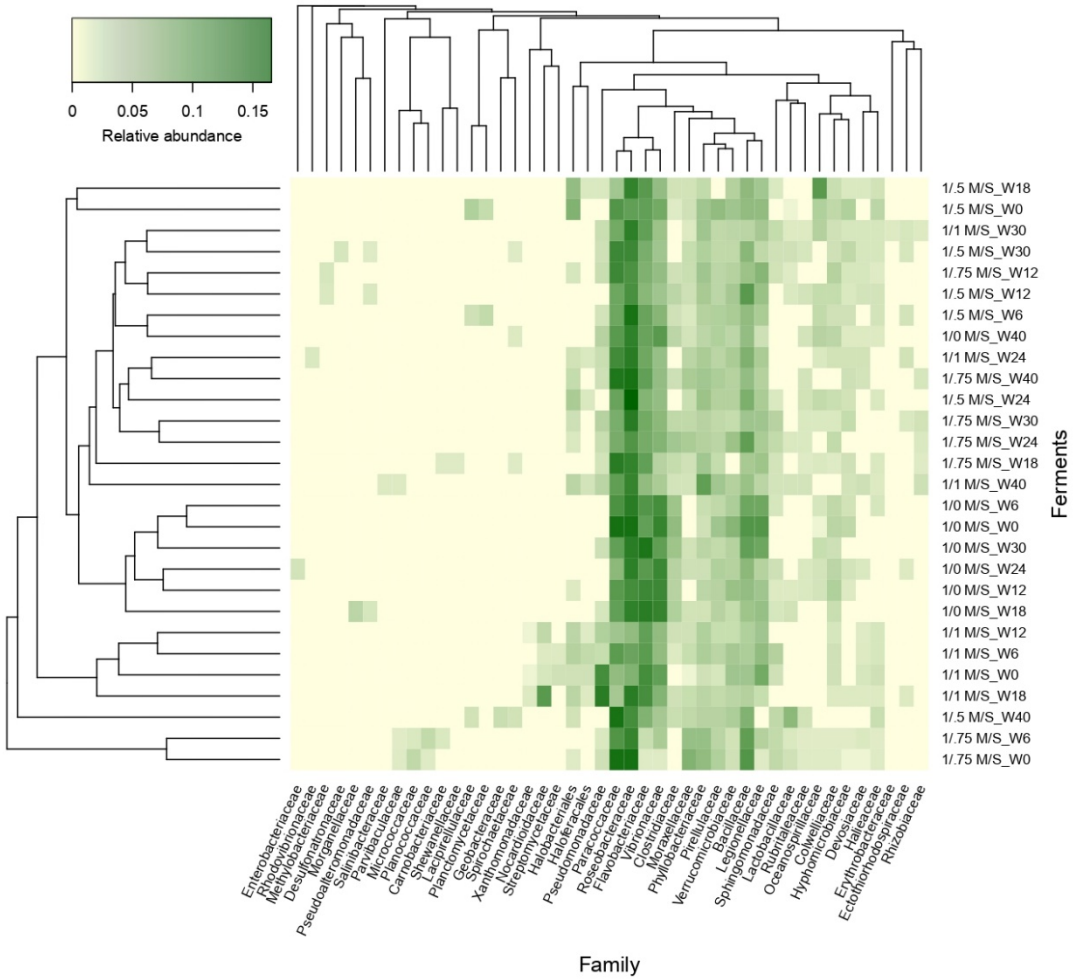


Figure 61: Heatmap of relative abundance at the family level across all mussel samples with four different meat/shell ratios fermented with unstoved salt at 30 °C for 40 weeks. Ferments and bacterial family groups are clustered hierarchically based on relative abundance profiles. The relative abundance of the bacterial taxonomy at the family level was filtered to a value of equal and higher than 0.01. The right y-axis labels represent individual mussel ferment specified with meat/shell ratios. The "W" is abbreviated for the fermentation week and the following number is referring to the sampling timepoint. The family groups are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from light yellow to dark green.

9.5.2 Binary Comparison of the Initial and Final Bacterial Composition in Mussel Fermentations

Binary comparison graphs were employed to spotlight differences in the bacterial communities between Week 0 and Week 40. It is important to reiterate that this approach is only providing a comparison between two time-points and overall bacterial compositions in all mussel ferments should be referred to Section 9.5 and Section 9.5.1. The binary comparisons between Week 0 and Week 40 of all mussel ferments are presented in Figure 62.

Figure 62 shows that the *Roseobacteriaceae* (No. 94) and *Paracoccaceae* (No. 74) were the predominant family groups at both Weeks 0 and 40 in all mussel ferments as revealed in Figures 60 and 61. The relative abundances of *Roseobacteriaceae* were consistent at the initial (Week 0) and final (Week 40) fermentation weeks in the ¾ shell ferment with the value of 15.0% (Figure 62). The relative abundance of *Roseobacteriaceae* (only in no-shell ferment) and *Paracoccaceae* (in both no-shell and ¾ shell ferments) were higher at Week 0 compared to that at Week 40. In contrast, the relative abundances of *Roseobacteriaceae* and *Paracoccaceae* in the other 2 ferments were lower at Week 0 compared to that at Week 40 (positioned above the 45° line).

Despite Figure 61 revealing that *Flavobacteriaceae* (No. 36) and *Vibrionaceae* (No. 115) were among the predominant groups throughout the entire fermentation period in all mussel ferments, Figure 62 has highlighted that there were noteworthy differences in the relative abundances of *Flavobacteriaceae* and *Vibrionaceae* between Week 0 and Week 40 in some of the ferments. Figure 62 shows that *Flavobacteriaceae* and *Vibrionaceae* in the ¾ shell ferment had low relative abundances (RA: 2.0 %) at Week 0, and by the end of the fermentation, both groups increased to 10.0 % (*Flavobacteriaceae)* and 7.0 % (*Vibrionaceae)*. Conversely, the relative abundances of *Flavobacteriaceae* and *Vibrionaceae* had an apparent decrease by the end of the fermentation when compared to that in Week 0 for normal shell ferment. *Flavobacteriaceae* and *Vibrionaceae* in the other two ferments with lower shell contents had small differences when comparing the relative abundances between the two time-points (positioned either on/close to the 45° line).

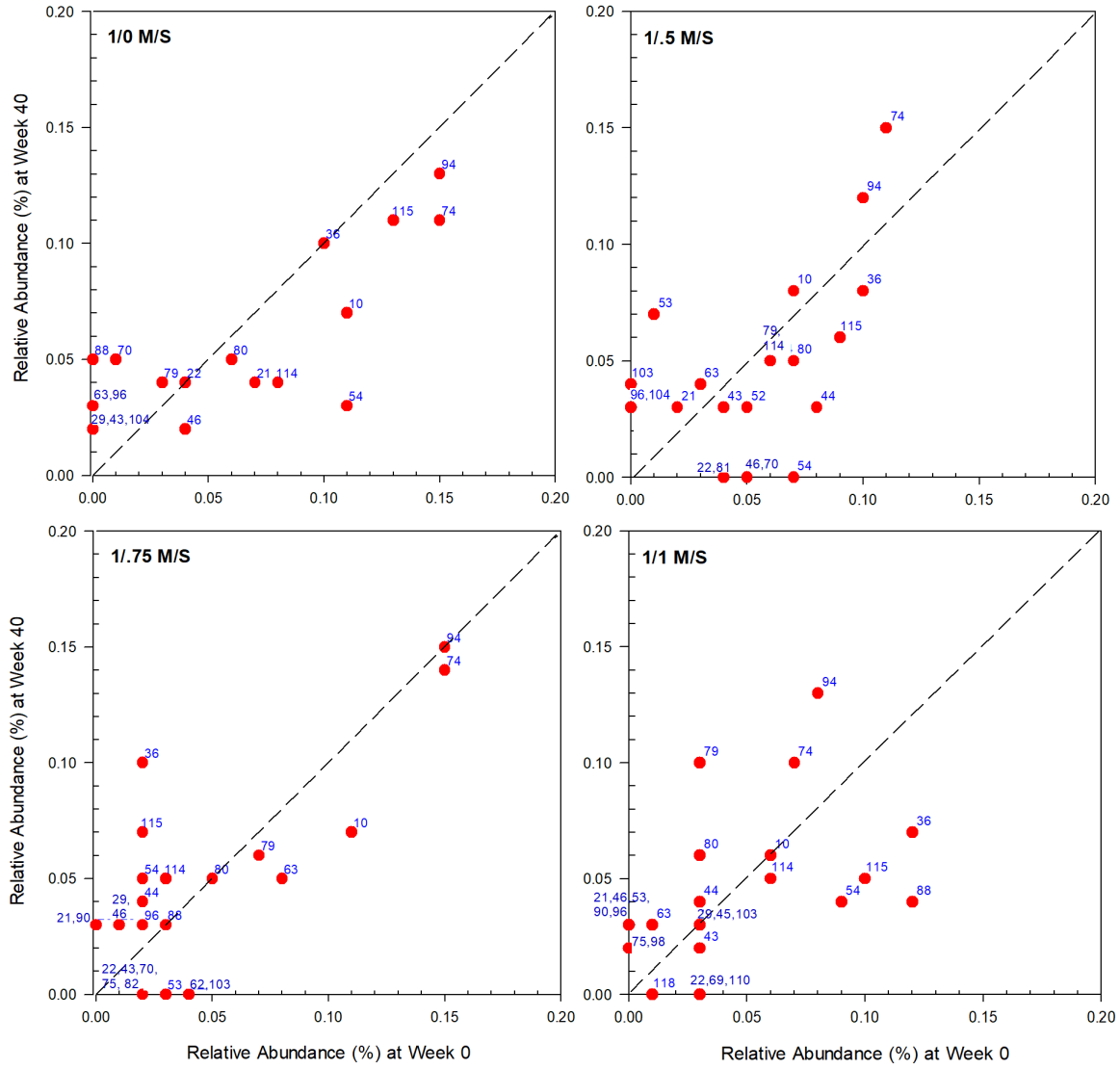


Figure 62: Binary comparison of relative abundance at family level between two fermentation weeks in green-shell mussel ferments with different meat/shell ratios fermented with unstoved salt at 30 °C. A position below the 45° line indicates relative abundance is lower at Week 40 compared to Week 0 whereas a family positioned above the 45° line has relative abundance higher at Week 40 compared to Week 0. The relative abundance of the bacterial taxon at family level was filtered to a value of at least 0.01.

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

Legionellaceae (No. 54) was one of the predominant groups at Week 0 (RA: 7.0 – 11.0 %) in all ferments except in ¾ shell ferment. Figure 62 shows that the relative abundance of *Legionellaceae* significantly decreased to 3.0 – 4.0 % in no-shell and normal shell ferments at Week 40, and the respective group was not present above the reporting threshold for half shell ferment at the same time-point.

Figure 62 shows that in no-shell and normal shell ferments, the number of groups present only at Week 40 were higher compared to that present only at Week 0 implying the bacterial diversity at the final fermentation week was greater compared to that at the initial week. For ferments with half and ¾ shells, opposite observations were made implying the bacterial diversities at the final fermentation week was lower than that at the initial week – already apparent from Figure 61. The bacterial groups present exclusively at either Week 0 or 40 differed depending on the shell contents suggesting that the meat/shell ratios influenced bacterial compositions during mussel fermentations which was already revealed in Figures 60 and 61.

9.5.3 Alpha Diversity Analysis of Mussel Ferments

Alpha diversity analysis was employed as described in Section 3.5.8 and Section 5.5.3 to further understand the bacterial diversities in all mussel ferments. Tables 43 summarizes the sequencing data reads from mussel ferments post-software trimming procedures and alpha diversity indices calculated from the sequence reads obtained.

Based on the Chao1 indices in Table 43, the bacterial diversities in 1/0, 1/.5, 1/.75 and 1/1 M/S ferments peaked at Week 30 (Chao1 = 343.14), Week 0 (Chao1 = 460.03), Week 0 (Chao1 = 461.03) and Week 40 (Chao1 = 413.90), respectively. Consequently, the Simpson's indices show that the highest values obtained in mussel ferments with 1/0, 1/.5, 1/.75 and 1/1 M/S were at Week 30 (D = 2.03), Week 0 (D = 2.07), Week 0 (D = 2.07) and Week 40 (D = 2.05), respectively. Table 43 revealed that the bacterial diversities in the no-shell and normal shell were higher at the final (Week 40) compared to that at initial (Week 0). Contrary, for half and ¾ shell ferments, the

bacterial diversities were higher at the initial (Week 0) compared to that at final (Week 80). These results support the observations made in Sections 9.5.1 and 9.5.2.

The Shannon-Wiener indices of all mussel ferments have values close to 1 (D values ≥ 0.97), indicating that the abundances of the bacterial species within each sample were similar.

Table 43: Summary of the sequencing data sets derived from ferments of green-shell mussel with different meat/shell ratios fermented with unstoved salt at 30 °C for 40 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Meat:shell ratio	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon index, H	Simpson's index, D
1/0	0	21085	467	262	265.03	0.98	1.96
	6	20975	469	227	330.68	0.98	2.01
	12	22372	487	183	326.68	0.98	2.00
	18	14603	468	201	323.45	0.98	2.00
	24	22811	468	183	317.69	0.98	1.99
	30	24027	469	223	348.72	0.98	2.03
	40	16416	468	170	343.14	0.98	2.02
1/.5	0	21080	467	263	460.03	0.98	2.07
	6	17035	468	179	318.22	0.98	2.03
	12	16557	468	171	302.32	0.99	2.00
	18	21851	468	168	267.49	0.98	1.99
	24	22480	469	178	326.78	0.98	2.00
	30	23242	514	163	353.38	0.98	2.01
	40	16147	468	172	298.67	0.99	2.03
1/.75	0	21090	467	260	461.03	0.98	2.07
	6	17035	468	149	332.80	0.98	2.02
	12	16557	468	193	329.88	0.99	2.04
	18	21851	468	210	366.02	0.98	2.03
	24	22480	469	181	318.52	0.99	2.03
	30	23242	514	209	370.57	0.99	2.05
	40	16147	468	181	312.80	0.98	2.01

1/1	0	21789	467	259	361.02	0.99	2.00
	6	18573	468	179	365.39	0.98	2.02
	12	17665	468	181	360.25	0.99	1.99
	18	18539	469	189	352.50	0.98	1.98
	24	16557	468	172	363.29	0.99	2.00
	30	14595	468	205	375.00	0.98	2.03
	40	22299	497	161	413.90	0.99	2.05

*High quality reads is the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

9.6 Discussion

The overall results from mussel fermentation with different meat/shell ratios are presented in Table. Figure 56 shows that 1/0 M/S ferment had pH between 5.4 – 5.9 while all ferments with added shell fluctuated at 7.1 – 7.6 throughout the fermentation time course. Statistical analysis presented in Figure 57 revealed that the pH of mussel ferments was influenced by the presence of shell but not quantitatively with the amount of shell added. Findings made in this chapter support the discussions made previously in Section 7.7 stating that high pH values may be associated with the presence of mussel shells, although the relationships is not simple.

Despite there being no significant difference in pH between ferments with different amount of added shell, there were differences in microbial composition between mussel ferments which apparently influenced the VFA profiles. *Bacillaceae* is associated with propionic acid production via isoleucine and valine degradations (Conrad et al., 1974). A "bump" in propionic acid was observed between Weeks 30 – 38 in no-shell ferment which was concurrent with the increase of *Bacillaceae* at Week 30 (Figures 58 and 60). A similar phenomenon was observed in normal shell where the propionic acid "bump" between Weeks 16 – 24 may be related to the increase of *Bacillaceae* at Week 24. Inversely, in ¾ shell ferment, an apparent decrease of propionic acid at Week 16 may be associated with the decrease of *Bacillaceae* at the same week (Figures 58 and 60). Figure 58 also shows that propionic acid in ferments with higher shell contents (¾ shell and normal shell) were fluctuating across a higher range of 8 – 64 ppm compared to ferments with lower shell contents (8 – 40 ppm). These observations may be explained by the higher overall relative abundance of *Pseudomonadaceae* in both ¾ and normal shells ferments compared to that in the ferments with lower shell contents. As discussed in Section 7.7, two or more bacterial family groups may collectively affect the VFA behaviours during fermentation.

Another difference observed between ferments with high shell contents (¾ shell and normal shell) and ferments with low shell contents (half shell and no-shell), was the presence of *Halobacteriales*. A study on the metabolism of halophilic archaea found that under anaerobic conditions, genus *Halobacterium salinarum* (Family: *Halobacteriales*) can convert pyruvate to alanine as the primary product, and to an extent to acetic acid and lactic acid (Falb et al., 2008). Figure 60 shows that *Halobacteriales* was present at almost all fermentation weeks in ferments with high shell contents – with ¾ shell ferment had the highest overall relative abundance, while *Halobacteriales* was present at fewer time-points in ferments with low shell contents. Concurrently, it was revealed in Figure 58 that ¾ shell ferment had the highest overall acetic acid concentration (65 – 160 ppm) compared to other ferments with different shell contents (11 – 120 ppm).

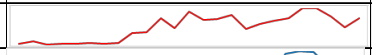
Figure 58 shows that the values of all VFAs in almost all mussel ferments were higher by the end of the fermentation compared to that at the initial period supporting the hypothesis that the concentration of VFA increases as fermentation progresses. Interestingly, there were differences in microbial composition and VFA concentrations at Week 0 in all ferments with different meat/shell ratios implying that shell contents influenced these parameters even before the fermentation was initiated.

It may be assumed that the presence of shell in the fermentation could:

1. Introduce inocula of different organisms or,
2. Provide growth surfaces favouring particular organisms or,
3. Offer fermentable substrate or,
4. Add buffering capacity

This complex mix of potential mechanisms made it unsurprising that the relationship between fermentation behaviour and meat/shell ratio was complex.

Table 44: Summary of the overall results from mussel fermentation with different meat/shell ratio.

Meat/Shell Ratio	Parameter	Mussel with Unstoved Salt		Meat/Shell Ratio	Parameter	Mussel with Unstoved Salt	
1M/0S	pH			1M/.75S	pH		
	Acetic acid				Acetic acid		
	Propionic acid				Propionic acid		
	Butanoic acid				Butanoic acid		
	3-methylbutanoic acid				3-methylbutanoic acid		
	Chao1				Chao1		
	Predominant family groups	Week 0	Week 40		Predominant family groups	Week 0	Week 40
		Bacillaceae *Flavobacteriaceae* *Legionellaceae* *Paracoccaceae* *Pirellulaceae* *Roseobacteraceae* *Verrucomicrobiaceae* *Vibrionaceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*			*Bacillaceae* *Moraxellaceae* *Paracoccaceae* *Phyllobacteriaceae* *Roseobacteraceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Phyllobacteriaceae* *Roseobacteraceae* *Vibrionaceae*
1M/.5S	pH			1M/1S	pH		
	Acetic acid				Acetic acid		
	Propionic acid				Propionic acid		
	Butanoic acid				Butanoic acid		
	3-methylbutanoic acid				3-methylbutanoic acid		
	Chao1				Chao1		
	Predominant family groups	Week 0	Week 40		Predominant family groups	Week 0	Week 40
		Bacillaceae *Flavobacteriaceae* *Halobacteriales* *Legionellaceae* *Paracoccaceae* *Phyllobacteriaceae* *Pirellulaceae* *Roseobacteraceae* Vibrionaceae	*Bacillaceae* *Flavobacteriaceae* *Lactobacillaceae* *Paracoccaceae* *Roseobacteraceae* *Vibrionaceae*			*Bacillaceae* *Flavobacteriaceae* *Legionellaceae* *Paracoccaceae* *Pseudomonadaceae* *Roseobacteraceae* *Verrucomicrobiaceae* *Vibrionaceae*	*Bacillaceae* *Flavobacteriaceae* *Paracoccaceae* *Phyllobacteriaceae* *Pirellulaceae* *Roseobacteraceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

9.7 Conclusion

In conclusion, the different meat/shell ratios influence the bacterial diversity and composition as well as the VFA behaviours in the mussel ferments during the fermentation process. The high pH was associated with the presence shell in the ferments, however, no significant difference in pH was found in ferments with different shell contents. Further investigation should be conducted to further understand the impact of shell on fermentation with other fish type.

Chapter 10: The Effect of Shell on Snapper (*Pagrus auratus*) Heads and Frames Fermentation

10.0 Introduction

Chapter 9 revealed that the amount of shell present influences mussel fermentation, potentially through buffering pH. The aim of this chapter is to test whether this observation can be corroborated with other fish fermentations via the presence of mussel shell in snapper paste fermented at 30 °C with unstoved salt for 40 weeks. Data already reported in Chapter 6 for snapper heads and frames were used as a control. Data from Week 0 – 50 of the control snapper ferment will be reproduced for this chapter – note that there are missing data from Week 12 – 42 because the samples were unable to be collected due to Covid-19 pandemic restrictions.

The fermentation set-up was described in detail in Section 3.3.3. The physical appearance, pH, bacterial composition and the formation of volatile fatty acids (VFA) of and in the snapper ferment will be monitored throughout the fermentation time course.

Hypotheses that will be tested in this chapter are:

1. The microbiological composition and diversity present during the fermentation period are affected by the presence of shell for the snapper fermentation.
2. The concentration of volatile fatty acids produced will increase as the fermentation time increases.
3. The presence of mussel shell in snapper fermentation will have a similar influence on the fish sauce as that observed in mussel fermentation with added shell.

10.1 Experimental Method

The experimental method to investigate the hypotheses outlined for this current chapter is presented in Figure 63.

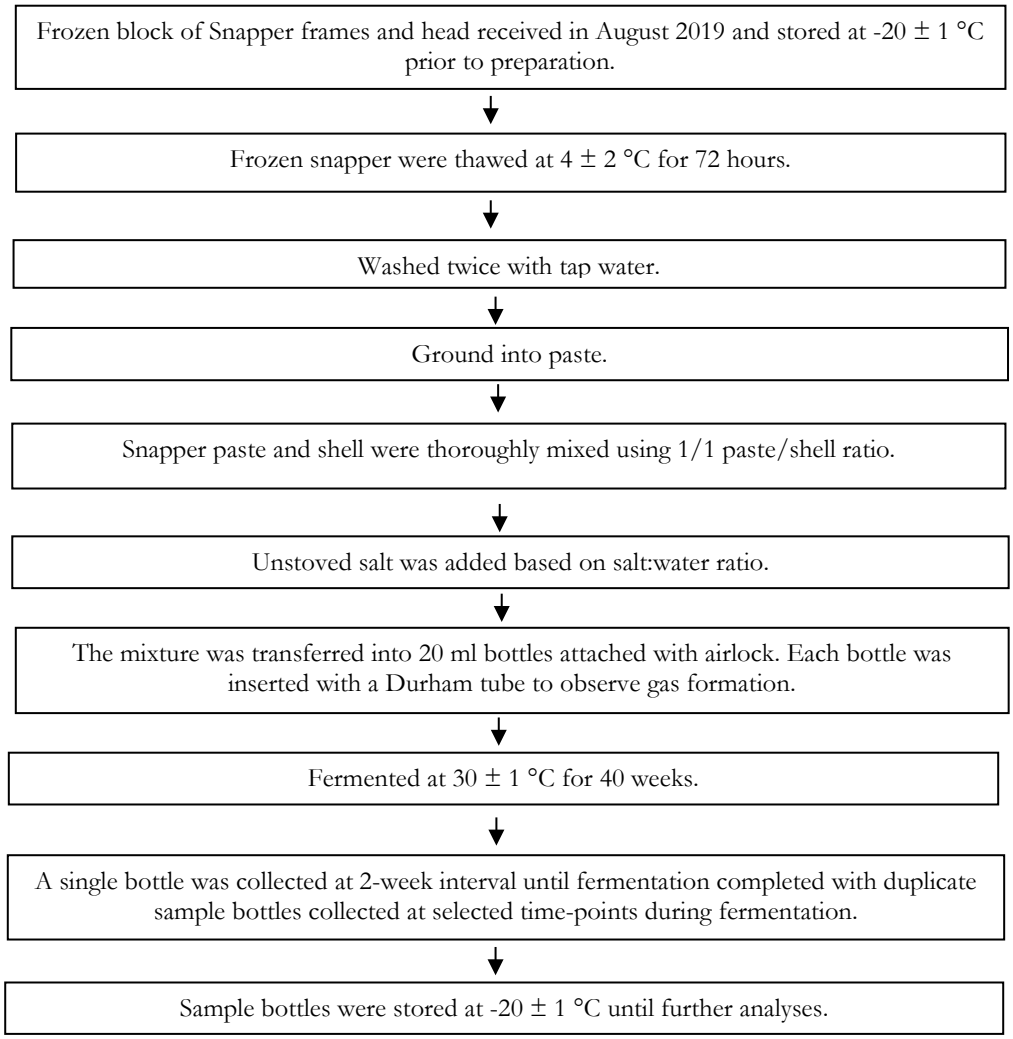


Figure 63: Experimental method flowchart of Snapper heads and frames with shell from green-shell mussel. The experiment was started in September 2022. Design of the fermentation set-up was described in Section 3.3.3.

The abbreviation for snapper fermentations is presented in Table 45.

Table 45: Snapper ferment abbreviations

Snapper paste ratio	Mussel shell ratio	Relevance	Abbreviation
1	1	Shell	1/1 P/S
1	0	No-shell/Control	1/0 P/S

*Content of snapper paste or shell is on a wet weight basis. P/S refers to snapper paste:shell ratio.

Note that the experimental method for control snapper ferment was presented in Chapter 6. The 1/1 P/S and 1/0 P/S were conducted at different times but using identical apparatus, preparation methods and raw material (stored at -20 °C in the interim).

10.2 Visual Observation of Snapper Fermentation

Visual observations on snapper ferments are presented in Table 46.

Table 46: Visual observations on Snapper ferment with shell from green-shell mussel with unstoved salt fermented at 30 ± 1 °C.

Ferment	Fermentation time (week)	Sample photo	Description
1/1 P/S	0		The mixture of snapper paste, shell and salt are firm. The grey colour of the mixture was similar to the ground snapper. No gas bubble observed in the Durham tube. Fishy and ocean-like aroma were detected.
	40		A reddish brown liquid layer on top of solid sediment observed. Gas bubble was observed in the Durham tube. Pungent and strong fishy smell detected.
1/0 P/S	0		The homogenous mixture of fish heads/frames and salt are in firm texture. The dark grey colour of the mixture was similar to that of the ground snapper heads and frames.
	50		A brown liquid layer on top of solid sediment observed. No gas bubble was observed in the airlock. Pungent aroma and strong fishy smell was detected. Soft textures with visible bones and scales residuals at bottom layer.

The texture of the shell-snapper ferment, observed when handling samples, changed from firm meat with shell particles at the initial fermentation time to a combination of soft meat and shell particles with reddish brown liquid uppermost layer after Week 18. The reddish hue appeared at Week 24 and the colour intensified as the fermentation progressed until it reached the final colour recorded in Table 46. Gas bubbles were observed in the Durham tubes in shell-snapper ferment from Week 2 until the final fermentation time – presumably carbon dioxide was the fermentation gas by-product, indicating that fermentation was taking place. No gas bubble was observed in the Durham tube in any ferment at Week 0 indicating that fermentation had not yet taken place. No further investigation was carried out to determine the type and volume of gas produced.

At the start of the fermentation, the aroma of shell-snapper ferment was described as fishy and marine-like. No marine-like smell was detected in the control ferment. As fermentation progressed, the shell-snapper ferment developed a pungent, fishy and ammoniacal aroma – similar to the control ferment at Week 50. Further investigations were conducted to determine the volatile fatty acids associated with snapper ferment and results obtained will be discussed in the following sections.

10.3 pH in Snapper Fermentations

The pH of snapper ferments was monitored throughout the fermentation process and is presented in Figure 64.

The pH of 1/1 P/S ferment rose gradually from 7.1 to 7.5 over the 40 weeks – similar to the observations made in mussel fermentations with added shell as discussed in Section 9.3. The pH of 1/1 P/S ferment was higher at the final week (Week 40) compared to that at the initial period (Week 0). In comparison, the pH of the control fermentation decreased from 7.0 at Week 0 to 5.3 at Week 50.

The final pH of shell-snapper ferment did not fall within the standard pH range of a traditionally fermented fish sauce outlined by CODEX and described in Section 2.6.

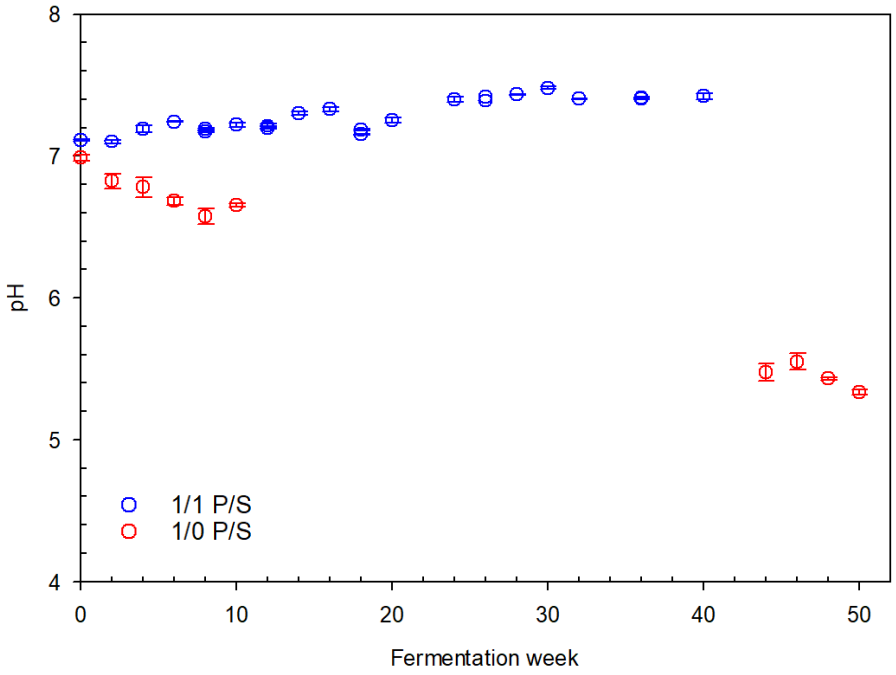


Figure 64: The pH of snapper ferments with and without shell fermented with unstoved salt at 30 °C. Values represent means ± standard error of mean (n=3). n is the number of readings from the same sample bottle. Missing data points for 1/0 P/S ferment was due to the Covid-19 pandemic restrictions.

10.4 Volatile Fatty Acid Formation in Snapper Fermentations

Four VFAs were assayed in snapper ferments: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Figure 65 shows that acetic acid had the highest concentration in 1/1 P/S ferment followed by 3-methylbutanoic acid, propionic acid and finally, butanoic acid. For 1/0 P/S ferment, 3-methylbutanoic acid had the highest concentration, followed by acetic acid and butanoic acid. Propionic acid was not observed above the recording threshold during the 50 weeks fermentation of no-shell snapper ferment (Figure 65).

Figure 65 shows that acetic acid in 1/1 P/S ferment were hovering between 160 – 200 ppm at Weeks 0 – 20. Acetic acid dropped to 110 ± 8 ppm at Week 24, then an increasing trend was observed for the remainder of the fermentation period with the highest value recorded at Week 38 (367 ± 4 ppm). This implied two phases of fermentation. Similar patterns were observed in 3-methylbutanoic where the values were fluctuating at 20 – 80 ppm between Week 0 and 20 before a drop observed at Week 24 (Figure 65). Then, an increasing trend was observed for the remainder of the fermentation time course with the highest value recorded at Week 40 (147 ± 3 ppm). Again, two phases of fermentation were observed. In comparison, for 1/0 P/S ferment, a small increase in 3-methylbutanoic acid was observed at Weeks 0 – 10, then the values remained steady at 60 – 70 ppm for the remainder of the fermentation time course. Acetic acid in the no-shell ferment fluctuated at 10 – 14 ppm during the entire fermentation time course.

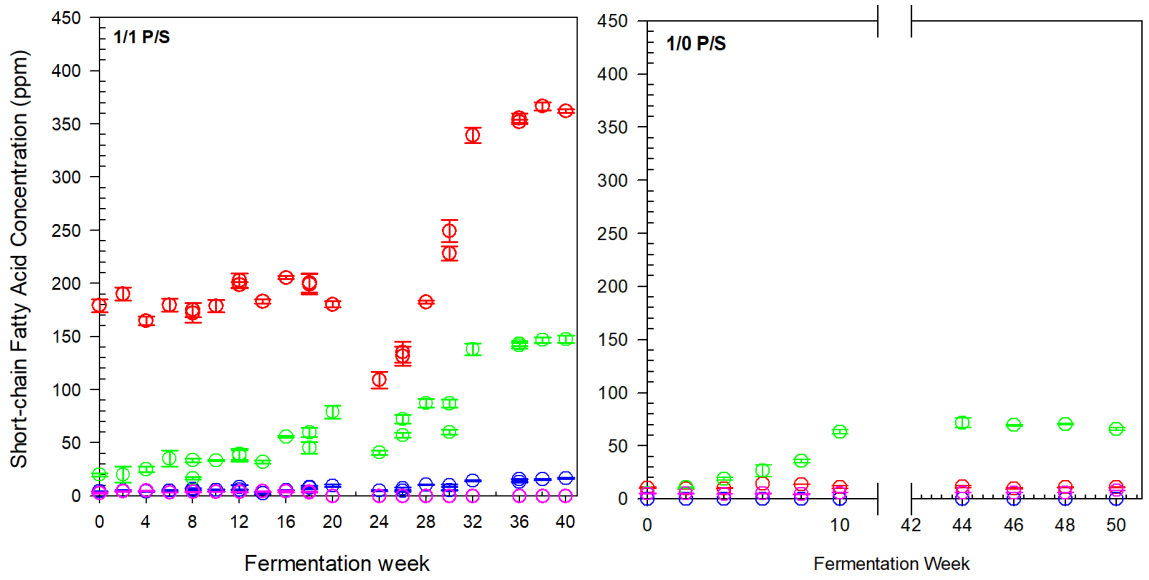


Figure 65: Short chain volatile fatty acids (VFA) in snapper ferments with and without shell fermented with unstoved salt at 30 °C. Values represent means ± standard deviation(n=3). n is the number of injections from the same sample vial. Limit of detection (LOD) for acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid are 9 ppm, 2 ppm, 3 ppm and 2 ppm, respectively. The break in the plot for 1/0 P/S indicates the missing points due to the Covid-19 pandemic restrictions.

Propionic acid in 1/1 P/S ferment was fluctuating at a much lower range (3 – 17 ppm) throughout the entire fermentation period but was not observed at all in 1/0 P/S ferment during its time course.

Butanoic acid in shell-snapper ferment ranged between 3 – 5 ppm from Week 0 to 18, then was not detected above the recording threshold for the remainder of the fermentation period. For no-shell snapper ferment, butanoic acid fluctuated between at 5 – 8 ppm throughout the fermentation.

Figure 65 shows that the overall values of three out of four VFAs in shell-snapper ferment were higher compared to that in no-shell ferment. In mussel fermentation, addition of shell (up to a limit) also increased VFA production (refer to Section 9.4).

Figure 65 also revealed that the values of most VFAs in 1/1 P/S ferment were higher by the end of the fermentation compared to that at the initial period (Week 0), supporting the hypothesis that VFA was accumulated as fermentation product.

10.5 Bacterial Composition in Snapper Fermentations

To understand the influence of shell on bacterial communities during snapper fermentation, metagenomic approaches were adopted as discussed in Section 3.3.9. Bacterial composition in the snapper ferments during the fermentation process is shown in Figure 66.

Figure 66 shows that four family groups predominated in 1/1 P/S ferment throughout the entire fermentation time course: *Carnobacteriaceae* [Relative abundance(RA): 5.0 – 16.3 %], *Enterococcaceae* (RA: 9.0 – 18.2 %), *Moraxellaceae* (RA: 24.5 – 35.1 %) and *Pseudomonadaceae* (RA: 12.5 – 24.1 %). These four predominant groups were also predominant in snapper paste as presented in Section 6.5. On the other hand, only two groups predominated throughout the 1/0 P/S fermentation: *Bacillaceae* (RA: 6.4 – 7.6 %) and *Streptococcaceae* (RA: 7.8 – 8.9 %).

For shell-snapper ferment, several family groups disappeared by the end of fermentation including *Bacillaceae, Brucellaceae, Enterobacteriaceae* and *Spirulinaceae* – while other groups including *Lacipirellulaceae, Lactobacillaceae* and *Staphylococcaceae* appeared above the recording threshold during

mid or late fermentation period. These family groups were present at relative abundances of 2.0 – 8.0 %. *Roseobacteriaceae* and *Halobacteriales* were present only in no-shell snapper ferment with noticeably high values of 11.1 % and 10.6 %, respectively, at Week 0.

Figure 66 also shows that bacterial diversities in shell-snapper ferment were higher during mid fermentation compared to that at initial and final period. For no-shell snapper ferment, bacterial diversity at Weeks 10 and 44 were higher compared to that at Week 0 – Several groups including *Clostridiaceae, Lactobacillaceae, Micrococcaceae* and *Pseudomonadaceae* appeared at Week 10.

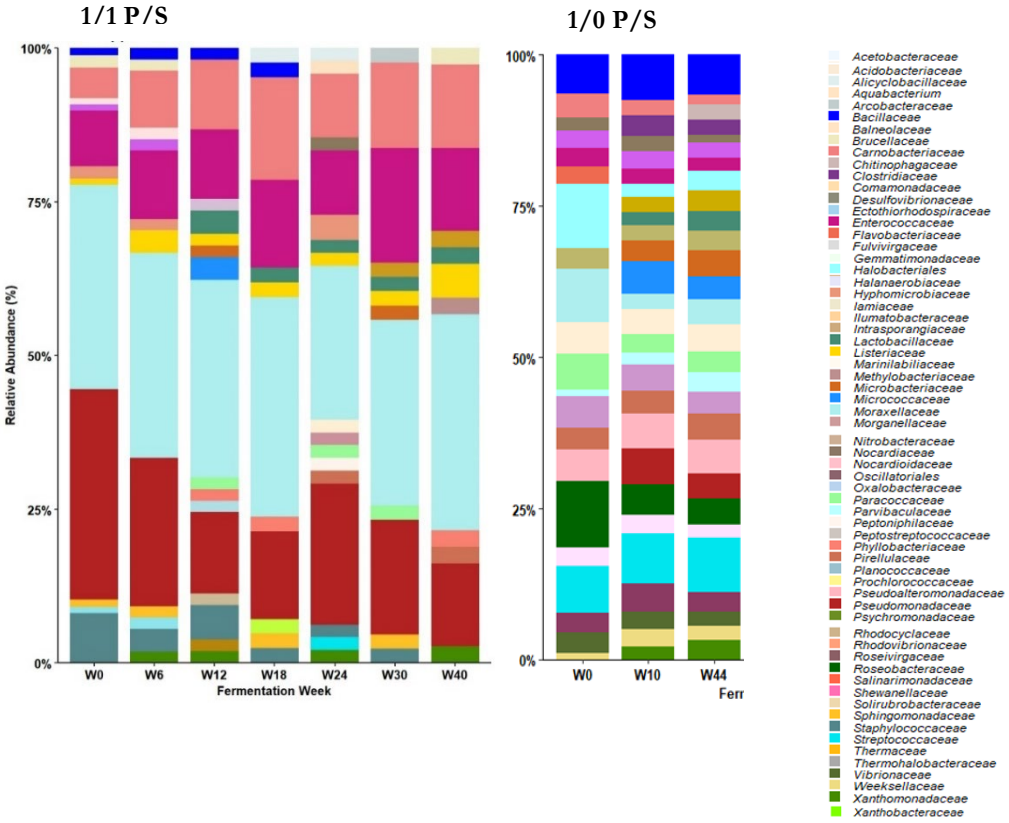


Figure 66: Bacterial taxonomic compositions at the family level showing the bacterial successions during fermentation of snapper ferments with and without shell. Note that the relative abundance for all groups were filtered to a value of at least 1%.

Overall, Figure 66 shows that there were differences in bacterial compositions between snapper ferments with and without shell – particularly the predominant family groups, implying that the presence of shell influenced the bacterial compositions.

In mussel fermentation, different groups predominated throughout the fermentation time course: *Flavobacteriaceae* , *Paracoccaceae, Roseobacteriaceae* and *Vibrionaceae* (refer to Section 9.5). However, similar to 1/1 P/S, *Pseudomonadaceae* had the highest overall relative abundance in 1/1 M/S ferment (refer to Section 9.5). High relative abundance of *Pseudomonadaceae* in both snapper and mussel with added shell may suggest that the group prosper better with higher shell contents. Findings made may also suggest that the presence of shell and the type of raw material influenced bacterial composition, adding to the complexity of the process.

10.5.1 Binary Comparison of the Initial and Final Bacterial Composition in Snapper Fermentations

Binary comparison graphs were employed to spotlight differences in the bacterial communities between Week 0 and Week 40/44 (Figure 67). It is important to reiterate that this approach is only providing a comparison between two time-points and overall bacterial compositions in snapper ferments should be referred to Figure 66.

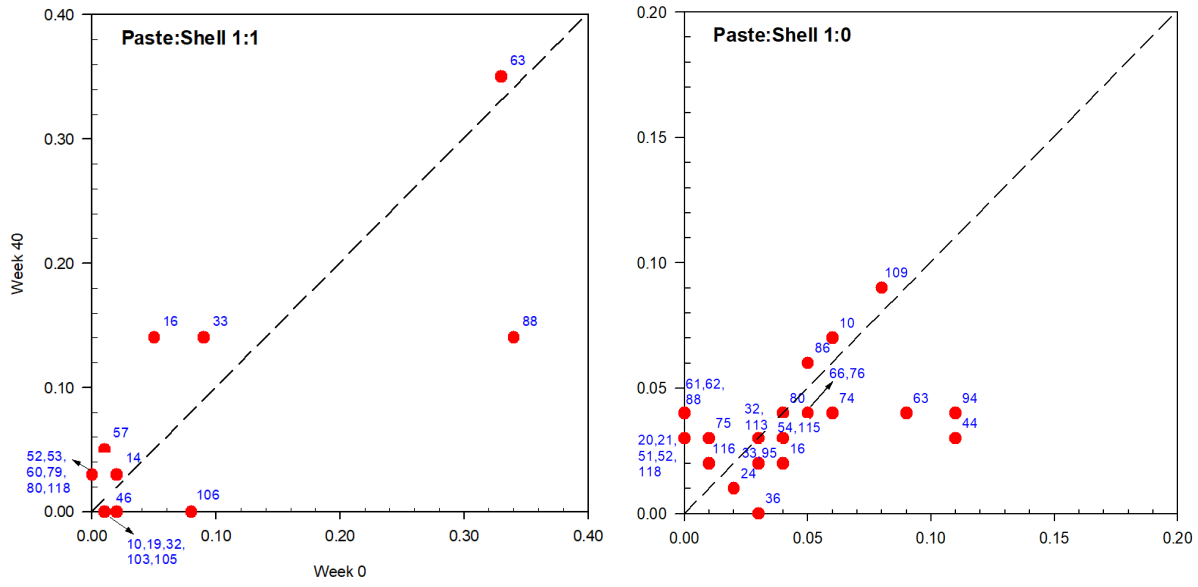


Figure 67: Binary comparison of relative abundance at family level between two fermentation weeks in snapper with and without shell fermented at 30 °C with unstoved salt. A position below the 45° line indicates relative abundance is lower at Week 40/44 compared to Week 0 whereas a family positioned above the 45° line has relative abundance higher at Week 40/44 compared to Week 0. The relative abundance of the bacterial taxon at family level was filtered to a value of at least 0.01.

Family group numbering:

1. *Acetobacteraceae*
2. *Acidobacteriaceae*
3. *Actinomycetaceae*
4. *Alcaligenaceae*
5. *Alicyclobacillaceae*
6. *Arcobacteraceae*
7. *Arthrobacter*
8. *Aquabacterium*
9. *Azospirillaceae*
10. *Bacillaceae*
11. *Bacillales Family XI. Incertae Sedis*
12. *Balneolaceae*
13. *Brevibacteriaceae*
14. *Brucellaceae*
15. *Burkholderiaceae*
16. *Carnobacteriaceae*
17. *Caulobacteraceae*
18. *Cellvibrionaceae*
19. *Chamaesiphonaceae*
20. *Chitinophagaceae*
21. *Clostridiaceae*
22. *Colwelliaceae*
23. *Comamonadaceae*
24. *Corynebacteriaceae*
25. *Deinococcaceae*
26. *Dermabacteraceae*
27. *Dermacoccaceae*
28. *Desulfovibrionaceae*
29. *Devosiaceae*
30. *Dietziaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Enterococcaceae*
34. *Erwiniaceae*
35. *Erythrobacteraceae*
36. *Flavobacteriaceae*
37. *Fulvivirgaceae*
38. *Fusobacteriaceae*
39. *Gemmatimonadaceae*
40. *Geobacteraceae*
41. *Geodermatophilaceae*
42. *Halanaerobiaceae*
43. *Halieaceae*
44. *Halobacteriales*
45. *Haloferacales*
46. *Hyphomicrobiaceae*
47. *Iamiaceae*
48. *Idiomarinaceae*
49. *Ilumatobacteraceae*
50. *Intrasporangiaceae*
51. *Lachnospiraceae*
52. *Lacipirellulaceae*
53. *Lactobacillaceae*
54. *Legionellaceae*
55. *Leptotrichiaceae*
56. *Leucobacter*
57. *Listeriaceae*
58. *Marinilabiliaceae*
59. *Methanobacteriales*
60. *Methylobacteriaceae*
61. *Microbacteriaceae*
62. *Micrococcaceae*
63. *Moraxellaceae*
64. *Morganellaceae*
65. *Mycobacteriaceae*
66. *Neisseriaceae*
67. *Nitrobacteraceae*
68. *Nocardiaceae*
69. *Nocardioidaceae*
70. *Oceanospirillaceae*
71. *Oscillatoriales*
72. *Oscillospiraceae*
73. *Oxalobacteraceae*
74. *Paracoccaceae*
75. *Parvibaculaceae*
76. *Pasteurellaceae*
77. *Peptoniphilaceae*
78. *Peptostreptococcaceae*
79. *Phyllobacteriaceae*
80. *Pirellulaceae*
81. *Planctomycetaceae*
82. *Planococcaceae*
83. *Porphyromonadaceae*
84. *Prevotellaceae*
85. *Prochlorococcaceae*
86. *Pseudoalteromonadaceae*
87. *Pseudoalteromonas*
88. *Pseudomonadaceae*
89. *Psychromonadaceae*
90. *Rhizobiaceae*
91. *Rhodocyclaceae*
92. *Rhodovibrionaceae*
93. *Roseivirgaceae*
94. *Roseobacteraceae*
95. *Rothia*
96. *Rubritaleaceae*
97. *Salinarimonadaceae*
98. *Salinibacteraceae*
99. *Selenomonadaceae*
100. *Shewanellaceae*
101. *Solirubrobacteraceae*
102. *Sphingobacteriaceae*
103. *Sphingomonadaceae*
104. *Spirochaetaceae*
105. *Spirulinaceae*
106. *Staphylococcaceae*
107. *Stappiaceae*
108. *Sterolibacteriaceae*
109. *Streptococcaceae*
110. *Streptomycetaceae*
111. *Thermaceae*
112. *Thermoanaerobaculaceae*
113. *Veillonellaceae*
114. *Verrucomicrobiaceae*
115. *Vibrionaceae*
116. *Weeksellaceae*
117. *Xanthobacteraceae*
118. *Xanthomonadaceae*
119. *Yersiniaceae*
120. *Unclassified*
121. *Spirochaetaceae*

Figure 67 shows that *Moraxellaceae* (No. 63) had by far the highest relative abundance at both Week 0 and Week 40 (positioned close to the 45° line) in 1/1 P/S ferment. It is also shown that *Carnobacteriaceae* (No. 16), *Enterococcaceae* (No. 33) and *Pseudomonadaceae* (No. 88) were predominant at both time-points in shell-snapper ferment as revealed in Figure 66. For 1/0 P/S ferment, *Bacillaceae* (No. 10) and *Streptococcaceae* (No. 109) predominated at both Weeks 0 and 44 – also revealed in Figure 66.

For 1/1 P/S ferment, at Week 0, *Staphylococcaceae* (No. 106) had relative abundance of 8.0 % and interestingly, the group disappeared at Week 40. There was small difference in the number of bacterial family groups present exclusively at either Week 0 or Week 40 indicating that there is small difference in the bacterial diversities between the two time-points.

Meanwhile, in 1/0 P/S ferment, eight groups were present exclusively at Week 0 indicating bacterial diversity initially was higher compared to that at Week 44. In mussel fermentation as discussed in Section 9.5.2, *Roseobacteriaceae* (No. 94) and *Paracoccaceae* (No. 74) predominated at both Weeks 0 and 40 in all ferments regardless of shell content – suggesting that the presence of shell and type of raw material influenced the bacterial composition.

10.5.2 Alpha Diversity Analysis of the Snapper Fermentations

Alpha diversity analysis was employed as described in Section 3.5.8 and Section 5.5.3 to further understand the bacterial diversities in snapper ferments. Table 47 summarizes the sequencing data reads from snapper ferments with and without shell, post-software trimming procedures, and alpha diversity indices calculated from the obtained sequence reads.

Table 47 shows that Chao1 index in 1/1 P/S ferment peaked at Week 24 (Chao1 = 64.50) and dropping to its lowest point at Week 40 (Chao1 = 22.50) – having been 24.53 at Week 0.

Consequently, the highest Simpson's index in shell-snapper ferment was at Week 24 (D = 1.34) and the lowest at Week 40 (D = 0.97) with little difference in Simpson's indices between initial and final weeks.

Chao1 index in 1/0 P/S ferment was far higher throughout its fermentation time course remaining between 173.25 to 177.36 from Week 0 to Week 44. This is reflected in Simpson's index at Week 44 of $D = 1.78$ and at Week 0 of $D = 1.76$.For 1/1 P/S ferment, the Shannon-Wiener indices at Weeks 0 and 30 have values close to 1 (H values ≥ 0.97), indicating that the abundances of bacterial groups at the respective weeks were evenly distributed. Lower Shannon-Wiener indices at other weeks imply that the abundances of bacterial groups at the respective weeks were not evenly distributed and a few groups dominated during the time points. Table 47 revealed that bacterial diversities in shell-snapper ferment were higher during mid-fermentation compared to that during initial and final fermentation period, supporting the observations made in Figures 66 and 67. For 1/0 P/S ferment, bacterial diversities remained high throughout.

Overall, Table 47 also revealed that bacterial diversities in 1/0 P/S ferment were higher compared to that in 1/1 P/S ferment implying that the presence of shell influenced bacterial diversities during snapper fermentations. Section 9.5.3 shows that all mussel ferments regardless of shell content had Chao1 indices between 265 and 461 throughout the fermentation time course indicating that bacterial diversity in mussel fermentations were much higher than both snapper ferments with and without shell.

Table 47: Summary of the sequencing data sets derived from snapper with and without shell fermented with unstoved salt at 30 °C for 40/44 weeks and statistical analysis of bacterial diversity (alpha-diversity).

Paste/shell ratio	Fermentation week	High quality reads*	Average read length, bp	OTU*	Chao1	Shannon Index, *H*	Simpson's Index, *D*
1/1	0	20156	468	25	24.53	0.98	0.99
	6	25973	467	14	23.33	0.82	0.99
	12	22468	468	20	46.00	0.88	1.06
	18	19468	467	13	49.00	0.83	1.09
	24	21064	466	19	64.50	0.88	1.34
	30	16615	466	13	49.00	0.98	1.08
	40	13999	466	12	22.50	0.84	0.97
1/0	0	40127	467	121	173.25	0.98	1.76
	10	41236	466	127	173.74	0.98	1.76
	44	38975	465	133	177.36	0.99	1.78

*High quality reads is the number of sequence reads after trimming of the PCR primers and removal of low quality reads using Geneious Prime Software.
*OTU = operational taxonomic unit.

10.6 Discussion

Summary of the overall results from snapper fermentation with shell is presented in Table 48. Section 10.3 revealed that 1/1 P/S ferment (with shell present) had high pH (7.1 – 7.5) throughout the entire fermentation time course. On the other hand, 1/0 P/S (with shell absent) had lower pH (5.1) by the end of fermentation, as expected for fish sauce. The same behaviour was reported in Chapter 9 with mussel fermentations in the presence of shell.

Figure 66 shows that *Moraxellaceae* had the highest overall relative abundance throughout the fermentation time course appointing it to be the predominant group in 1/1 P/S ferment. Many of genera belong to *Moraxellaceae* have optimum growth temperature of 25 – 30 °C (Yang, 2014). *Psychrobacter cibarius* species isolated from Korean fermented seafood (*jeotgal*) had optimum growth of 25 – 30 °C at pH 6.0 – 7.5 in the presence of 0 – 9 % NaCl (w/v) (Yoon et al., 2003). Another species belongs to *Moraxellaceae* family, *Psychrobacter jeotgali* had optimum growth of 25 – 30 °C at pH 7.0 – 8.0 in the presence of 1 – 10 % NaCl (w/v) (Jung et al., 2005). It was also found that the *Psychrobacter* genus can utilize acetic acid for growth (Jung et al., 2005).

The microbial communities in a fish sauce can influence the formation and concentration of volatile fatty acids (VFA) which consequently influence the overall sensory profile and quality of the final product (Ma et al., 2022). The absence of butanoic acid in the shell-snapper ferment from Week 20 to Week 40 may be explained by the disappearance of *Bacillaceae* from Week 24 until the remainder of the fermentation time course (Figures 65 and 66). For no-shell snapper ferment, butanoic acid was observed at 5 – 8 ppm during the 50 weeks fermentation which may be associated with the presence of *Bacillaceae* during the time course (Figures 65 and 66) – also discussed in Section 6.7.

When comparing bacterial composition between shell-snapper and mussel ferments with shell present there were differences in predominant bacterial family groups (refer to Sections 9.5 and 10.5).

However, there were several groups that present in both shell-snapper and shell-mussel ferments particularly for *Pseudomonadaceae,* where it was found to prosper better in high shell content – as already discussed in Section 10.5. In addition, alpha diversity indices indicate that bacterial diversity in all mussel fermentations (regardless of shell content) were far higher than snapper fermentations with and without shell. It was reported that raw mussel paste had higher bacterial diversity than snapper paste with Chao1 indices of 146.15 and 22.50, respectively (refer to Sections 6.6 and 7.6). These findings imply that the presence of shell and type of raw material used influence the bacterial diversity in a fermentation, as a result of complex interactions.

Figure 65 indicates that there were hints of two phases fermentation based on acetic acid and 3-methylbutanoic acid behaviours. The production of 3-methylbutanoic acid can be associated with the presence of lactic acid bacteria (LAB) including *Carnobacteriaceae, Streptococcaceae, Lactobacillaceae* and *Enterococcaceae* via leucine degradation (Liu et al., 2014). There was a slow increase in 3-methylbutanoic acid during Week 0 to Week 20 which may be explained by the gradual increase in aggregated relative abundances of *Carnobacteriaceae* and *Enterococcaceae* during the same period. Concurrently, a drop in 3-methylbutanoic acid at Week 24 may be associated with the drop in aggregated abundances of *Carnobacteriaceae* and *Enterococcaceae* at the same week.

Figure 65 shows that the concentrations of acetic acid and 3-methylbutanoic acid in snapper ferment showed an increasing trendline during the fermentation time supporting the hypothesis that the concentration of VFA increases as the fermentation time increases. For propionic acid, the values fluctuated during the fermentation time, but it is important to point out that the value of the respective VFA was higher by the end of the fermentation compared to its concentration at Week 0. It was noted that butanoic acid was not observed above the recording threshold from Week 24 and as previously discussed, it may be associated with the complex bacterial interactions and dynamics during the fermentation. In mussel fermentation, addition of shell (up to a limit) also increased VFA production (refer to Section 9.4).

Table 48: Summary of the overall results from snapper fermentation with shell.

Paste/Shell Ratio	Parameter	Snapper with Unstoved Salt	
1P/1S	pH		
	Acetic acid		
	Propionic acid		
	Butanoic acid		
	3-methylbutanoic acid		
	Chao1		
	Predominant family groups	**Week 0**	**Week 40**
		Enterococcaceae *Moraxellaceae* *Pseudomonadaceae* *Staphylococcaceae*	*Carnobacteriaceae* *Enterococcaceae* *Moraxellaceae* *Pseudomonadaceae*

*Predominant bacterial family groups were filtered to at least 6.0 %
*Chao1 index is a measure of diversity

10.7 Conclusion

In conclusion, presence of shell increased the pH of snapper ferment which consequently may influenced the bacterial diversity and compositions as well as the VFA accumulated. Also, the complexity of natural fermentations influenced by the combination of type of raw material used and the presence of shell has influenced the bacterial diversity and compositions.

Chapter 11: Overall Discussions

11.0 Introduction

The current study is aimed at understanding the fish sauce fermentation from New Zealand's raw materials. The overall findings discussed in this chapter are based on the results obtained from different fermentation set-ups investigated in this current study as summarized in Table 49.

11.1 The Effect of Different Fermentation Conditions on Volatile Fatty Acid

Flavour and aroma in fish sauce are formed as a result of proteins and lipids degradation via various biochemical metabolic pathways by microorganisms and by enzymatic hydrolysis (Ding et al., 2020). As mentioned in Section 2.6.2.2, aroma is often used to assess the quality of a fish sauce because the salty taste can overpower other flavours that may be present in the product (Beddows, et al., 1980). Dougan and Howard (1975) described that one of the major factors contributing to the aroma of a fish sauce is short-chain volatile fatty acids (VFA).

During fermentation, raw materials are degraded into primary metabolites such as glucose, amino acids and fatty acids (Wang et al., 2020). These primary metabolites then act as precursors for the metabolism of microorganisms providing an energy source for microbial growth and also resulting in the production of secondary metabolites (Lee et al., 2015). Primary metabolite such as amino acids can further degrade producing volatile fatty acids (Lee et al., 2015). Various studies have been reported seeking to understand and identify the metabolic pathways of amino acids during fermentation which were considered crucial in understanding the production of volatile compounds associated with the key aroma of fermented products (Sanceda et. al., 2002).

Table 49: Summary of different fermentation set-ups to investigate different aims in this study.

	Laboratory Scale Fermentation			**Pilot Scale Fermentation**			**Shell-on Mussel Fermentation**			**Shell on Snapper Fermentation**		
Refer to	**Chapters 5,6 and 7**			**Chapter 8**			**Chapter 9**			**Chapter 10**		
Aim	To investigate the influence of different variables (seafood material, type of salt and temperature) on fermentation			To investigate the influence of sampling depth on fermentation			To investigate the influence of shell on mussel fermentation			To investigate the influence of mussel shell on snapper fermentation		
Fermentation set-up	Seafood material	Salt	Temperature	Seafood material	Salt	Temperature	Mussel meat/shell ratio	Salt	Temperature	Snapper/shell ratio	Salt	Temperature
	Snapper, Hoki and Mussel	Stoved	18 °C	snapper	Stoved	30 °C	1M/0S	Unstoved	30 °C	1P/1S	Unstoved	30 °C
			25 °C		Unstoved		1M/.5S					
			30 °C				1M/.75S					
		Unstoved	18 °C				1M/1S					
			25 °C									
			30 °C									

In this current study, four VFAs were quantified in all fermentations: acetic acid, propionic acid, butanoic acid and 3-methylbutanoic acid. Butanoic acid was detected only in unstoved fermentations regardless of seafood material and fermentation temperature. Acetic acid had the highest concentration in all fermentations – except in laboratory scale snapper fermentation where 3-methylbutanoic acid had the highest concentration (refer to Section 6.4). Acetic acid is associated with pungent and sour odour – mainly produced as microbial metabolites (Ding et al., 2020). Sanceda et al. (2002) investigated the source of VFAs during fish sauce fermentation had found that acetic acid production increased with the addition of valine and leucine in fermented fish sauces (Sanceda et al., 2002). The possible metabolic pathways leading to the production of acetic acid are presented in Figures 68, 69 and 70 as sourced from MetaCyc Database, an online database compiling metabolic pathways (source: https://metacyc.org/).

In addition, the current study detected that all four VFAs at Week 0 in all fermentations despite no fermentation yet having taken place. A study by Beddows et al., (1980) found that acetic, *n*-butanoic, propionic and 3-methylbutanoic acids were detected in *ikan bilis* (*Stolephorus sp.)* used as the raw material for *budu* (a Malaysian fish sauce). The same study also found that acetic acid concentration increased significantly (p-value < 0.05) during the initial 48 hours prior to salting with *n*-butanoic, propionic and 3-methylbutanoic acids increasing at lower rates (Beddows et al., 1980). The study suggested that the formation of VFAs was influenced by bacterial activity present before the addition of salt (Beddows et al., 1980). Based on these literature findings, it may be suggested that the presence of all four VFAs at Week 0 and particularly high acetic acid concentration in majority of fermentations may be due to bacterial activity prior to salting – presumably via one/more of the metabolic pathways shown in Figures 68 – 70.

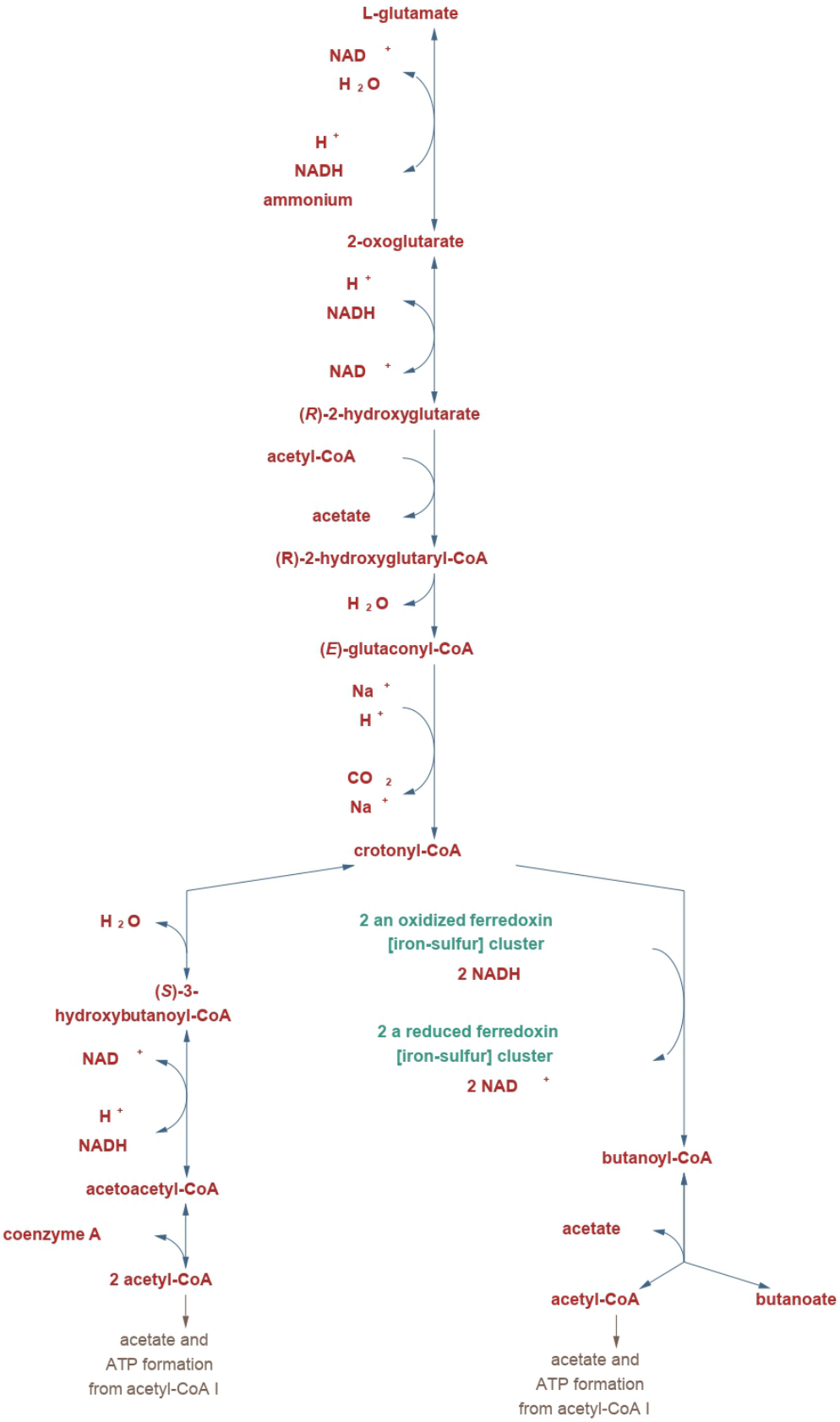


Figure 68: The degradation of L-glutamate that leads to the production of acetate (also known and acetic acid as the neutral compound) (sourced from Metacyc database, 2024).

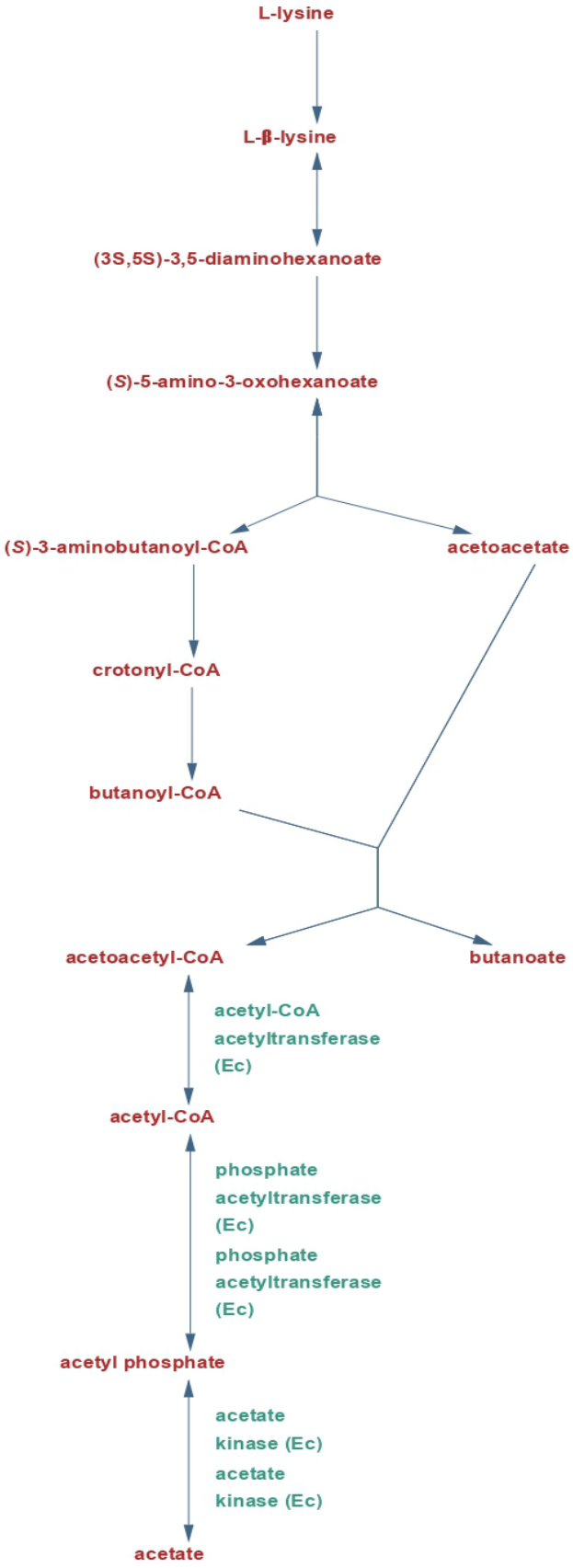


Figure 69: The degradation of L-lysine that leads to the production of acetate (also known and acetic acid as the neutral compound) (sourced from Metacyc database, 2024).

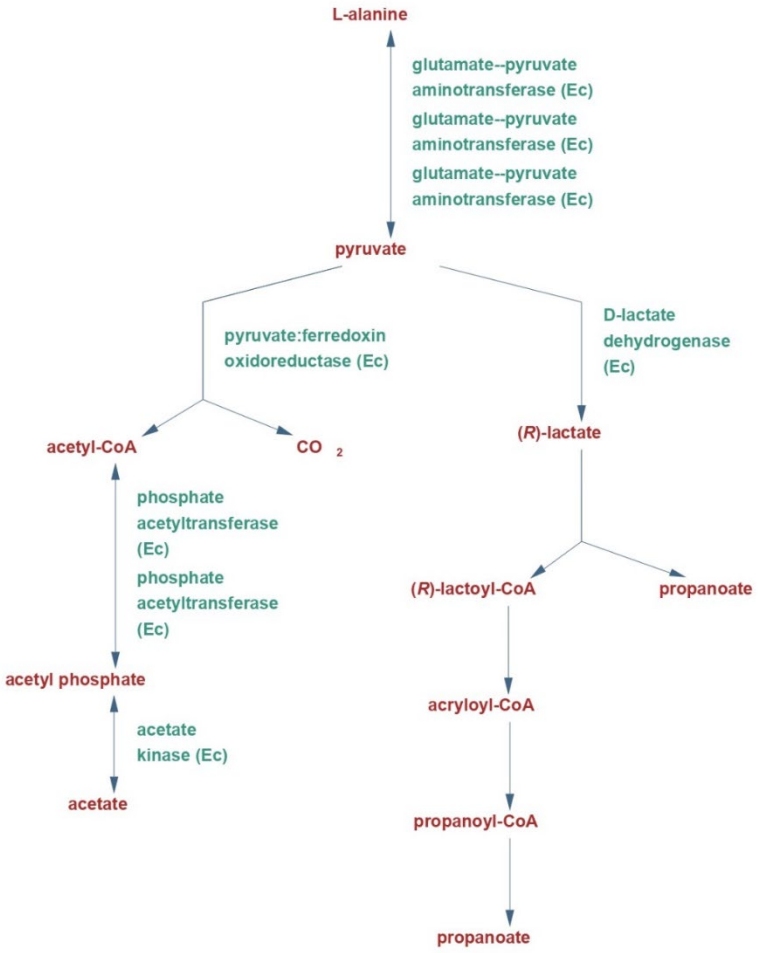


Figure 70: The degradation of L-alanine via Stickland reaction that leads to the production of acetate (also known and acetic acid as the neutral compound) (sourced from Metacyc database, 2024).

On the other hand, metabolic pathways producing propionic acid can be categorized into three classes: 1) sugar fermentation pathways (includes acrylate, Wood-Werkman cycle and succinate pathways), 2) amino acid catabolic pathways and 3) biosynthetic pathways (Gonzalez-Garcia et al., 2017). The sugar fermentation pathways result from the catabolism of glucose, lactate and/or succinate (Gonzalez-Garcia et al., 2017). Figure 71 shows the production of propionic acid via amino acid pathways involving the degradation of valine, theonine, isoleucine and methionine in the presence of propionyl-CoA (Gonzalez-Garcia., 2017).

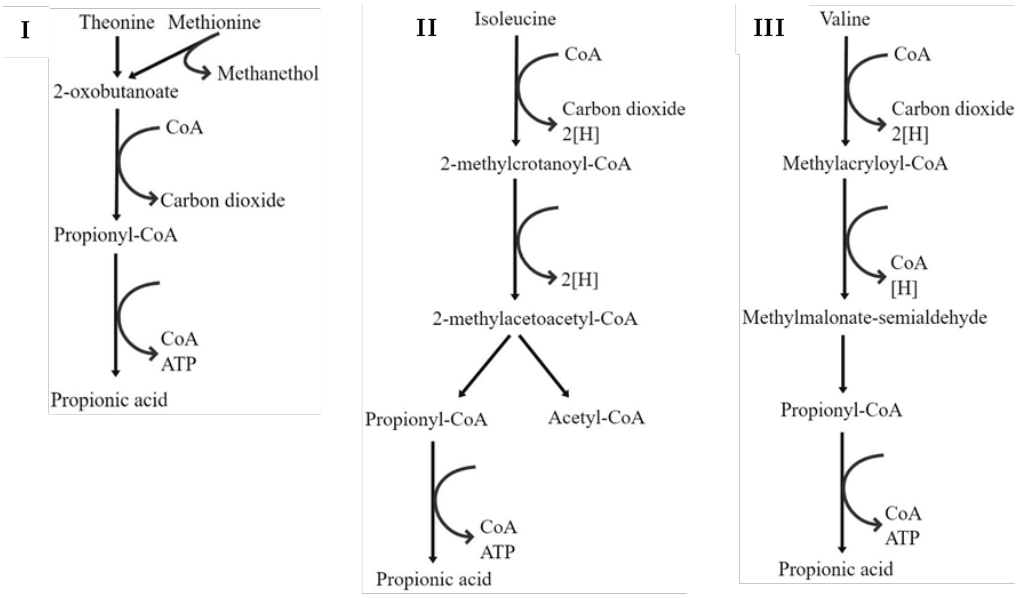


Figure 71: Amino acid metabolic pathways producing propionic acid (Gonzalez-Garcia at el., 2017)

In a study to investigate the source of VFAs during fish sauce fermentation, production of 3-methylbutanoic acid increased with the addition of leucine to fish sauces (Sanceda et al., 2002). The reactions between α-dicarbonyl compounds in amino acids are classed under Strecker reactions which involve deamination of amino acids into amino ketones, aldehydes and carbon dioxide (Sanceda et al, 1984). This reaction commonly occurs chemically in food products containing higher concentrations of amino acids and under extreme conditions such as high temperature and pressure (Sanceda et al., 2002). The possible pathway of 3-methylbutanoic acid via leucine deamination is presented in Figure 72.

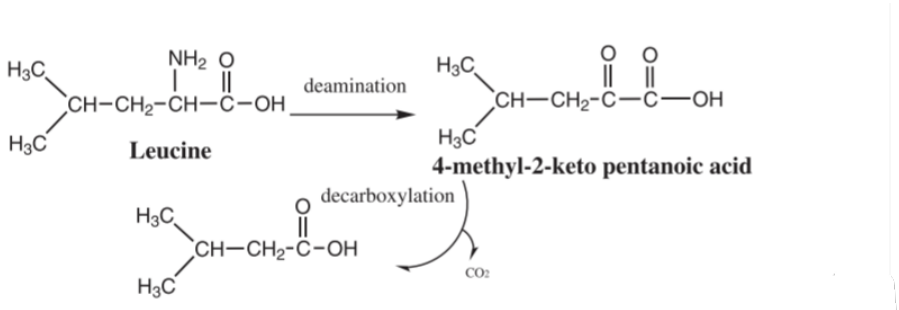


Figure 72: Possible metabolic pathways of leucine deamination via Strecker degradation producing 3-methylbutanoic acid (Sanceda et al., 2002).

Section 6.4 showed that 3-methylbutanoic acid had the highest overall concentration in all laboratory lab scale snapper fermentations which may be explained by the highest leucine content in snapper paste than other seafood materials. Based on literature values, leucine content in snapper scrap meat, Hoki and mussel meat were 2.60, 0.78 and 1.19 % (w/w), respectively (Prayudi et al., 2020; Saritha et al., 2015; Watanabe and Kawai, 2018). However, 3-methylbutanoic acid was not the highest VFA in pilot scale snapper fermentations suggesting that leucine is not the only source for 3-methylbutanoic acid production.

The current study showed that butanoic acid was consistently detected only in unstoved fermentations regardless of the seafood material and fermentation temperature used. A study on the development and origin of VFA in Malaysian fish sauce (*budu)* by Beddows et al., (1980) reported that butanoic acid could be produced by the oxidation of glutamate via the action of *Clostridium kluyveri*. On the other hand, butanoic acid is also produced as the end-product of sugar fermentation by obligate anaerobic bacteria such as *Clostridiaceae, Peptococcaceae* and *Eubacteriaceae* (Golberg and Rokem, 2009). The metabolic pathway for butanoic acid production via sugar fermentation is presented in Figure 73.

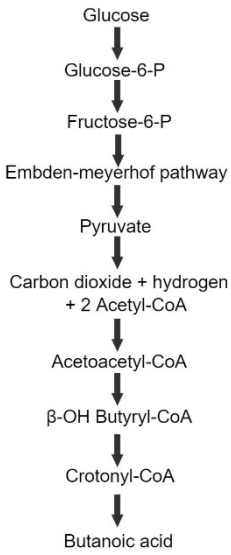


Figure 73: Metabolic pathway leading to the production of butanoic acid via sugar fermentation (Goldberg and Rokem, 2009).

The presence of butanoic acid exclusively in unstoved fermentations may be associated with the presence/high abundance of bacterial groups associated with butanoic acid production in these fermentations which were already discussed in Sections 5.7, 6.7, 7.7, and 8.7. The presence of butanoic acid only in unstoved fermentations may also be associated with the presence/high abundance of bacteria groups unique to unstoved salt. However, no further conclusion can be made on this postulate since insufficient intact nucleic acid could be extracted from unstoved salt to identify bacterial present as discussed in Section 5.6.

The colour and aroma of most of the experimental fish sauce fermentations after about 60 weeks, were not atypical of Southeast Asian style fish sauce even though temperate fish stocks and temperate fermentation conditions were employed. VFA levels analysed and pH measure were also not atypical. However, fermentation was slow.

Non-metric multidimensional scaling (NMDS) plots were used to visualize the effect of different fermentation conditions on VFA production. NMDS is a multivariate statistical method used to visualize the similarity/dissimilarity between data points (Saeed et al., 2018). The quality of NMDS solution is measured by a "stress" value to indicate how well the configuration of points in the plot reflects the original data. According to Clarke (1993), the interpretation of "stress" value is as follow:

- Stress < 0.05 gives an excellent representation with no prospect of misinterpretation.
- Stress < 0.10 indicates good ordination with no real risk of drawing false inferences.
- Stress < 0.20 shows useful representation but with a potential mislead, so it is suggested to not put too much reliance on the details of the plot.
- Stress > 0.20 means the plot is less likely to be reliable.

It was noted that the "stress" interpretation is over-simplistic as the value tends to increase with increasing number of samples – however, it is still a useful guideline in interpreting an NMDS plot (Clarke, 1993). In this study, the dissimilarity matrix was calculated using the Bray-Curtis method. NMDS plot factors in all four VFAs identified during fermentation process and reduced the information into 2 dimensional spaces (NMSD1 and NMDS 2) as presented in Figure 74. The axes scales are arbitrary, as is the orientation of the plot. Ellipses that are closer to one another are more similar that those further apart. Each ellipse represents a different fermentation set-ups.

Figure 7(A) shows that the "stress" value < 0.20 which is common for higher numbers of samples. Figures 7(B) and 7(C) had "stress' values < 0.10 which indicate good ordination with no real risk or misinterpretation. Figure 7(A) shows overlapping ellipses for fermentations with similar salt type regardless of seafood material used. Similar observations were made in Figure 7(B) where fermentations with similar salt type overlap with each other regardless of the sampling depth. Overall, Figure 7(A) and 7(B) indicate that salt type had more influence on VFA productions than other variables (seafood material and sampling depth).

Meanwhile, Figure 7(C) shows overlap between fermentations with different meat/shell ratios and a clear separation of snapper-shell fermentation from mussel fermentations, indicating that seafood material had more influence on VFA production for shell fermentations.

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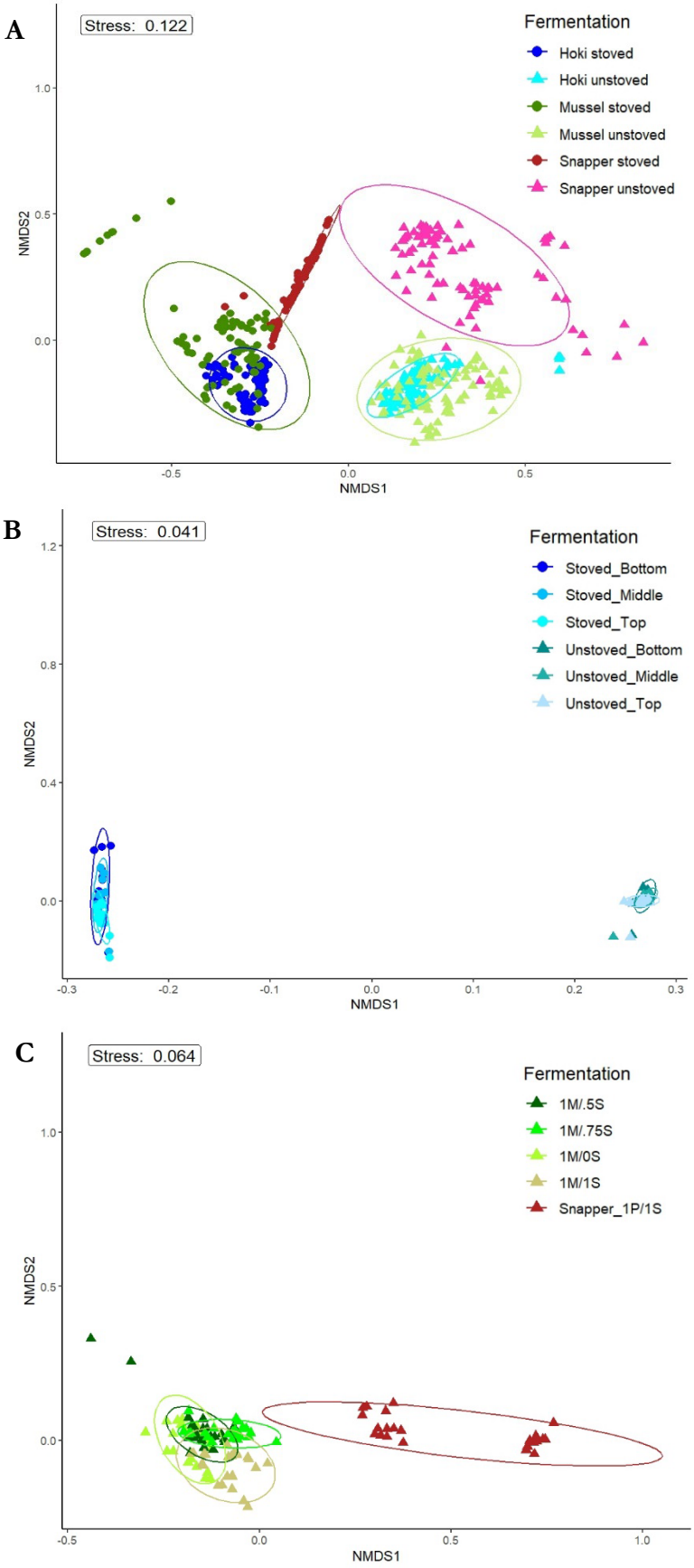

Figure 74: NMDS plots visualizing the effect of different fermentation conditions on volatile fatty acid production. A: Laboratory scale fermentations with different variables (seafood material – Hoki, snapper and mussel; salt type – stoved and unstoved solar salts; temperatures – 18, 25 and 30 °C); B: Pilot scale fermentation using snapper frames and heads with stoved/unstoved salt at 30 °C sampled from 3 different depths of fermentation barrel; C: Mussel fermentation with different meat/shell ratios fermented at 30 °C with unstoved salt.

11.2 The Effect of Different Fermentation Conditions on Bacterial Composition and Diversity

As mentioned in Section 2.6.2.3, microorganisms play an important role in protein degradation and the development of flavour and aroma in fish sauce (Lee et al., 2015). The current study focuses on bacterial composition and diversity during fermentation. Findings from this study revealed that bacterial composition differed based on fermentation conditions (refer to Sections 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5). A common observation made across all fermentations was that the seafood material used appeared to be the main determinant for the predominant bacteria family groups for the fermentations. The predominant groups in the seafood materials were: Hoki paste – *Micrococcaceae* and *Moraxellaceae* (refer to Section 5.6); snapper paste – *Carnobacteriaceae, Moraxellaceae* and *Pseudomonadaceae* (refer to Section 6.6); mussel paste – *Flavobacteriaceae, Moraxellaceae, Paracoccaceae, Pseudomonadaceae* and *Roseobacteriaceae* (refer to Section 7.6). Similar findings were reported in a study on a fermented Chinese soybean product (*gray sufu)*, where the raw soymilk used in the fermentation was found to be the main determinant of *Lactococcus, Leuconostoc, Cronobacter, Apiotrichum,* and *Trichosporon* – the predominant genera in the final fermented product (Ding et al., 2023). The same study also found that at least 108 bacteria genera present in the *gray sufu* were detected in the raw materials used (Ding et al., 2023). The findings made in this current study supported by the literature findings indicate the importance of raw material in shaping the bacterial communities in the fermented products. However, it is important to emphasize that natural fermentation process is complex, and it involves intricate interactions between bacteria, enzymes, metabolites and environmental conditions. In general, bacteria can co-exist, dominate, or synergize with other species occupying the same environment. Multiple microorganisms within a food system can result in competition for the same nutrients.

The dominant growth of certain species can alter the chemical characteristics of the food system, resulting in the inhibition of one or more competing types of microorganisms while supporting the growth of others – which was observed in laboratory scale snapper fermentation as discussed in Section 6.6. Extrinsic factors such as temperature can also influence microbiota structure and function, altering interactions among community members and metabolic characteristics during fermentation (Zhao et al., 2022). Together, those factors explain the different composition and succession of microbial communities in a food system (Lestari et al., 2023). In current study, bacterial composition of fermentations with similar seafood material and salt type were similar/almost similar at Week 0 – which was expected as fermentation was not yet taken place. The ferments at Week 0 represent triplicate samples prepared with either stoved or unstoved salts. The agreement among these triplicates across all fermentation conditions indicates the consistency of sample preparation and validates the experimental design. This demonstrates low variability and provides a reliable starting point, ensuring that any subsequent changes observed are due to fermentation effects rather than initial inconsistencies. It highlights the robustness of the methodology and supports the reproducibility of the results. As fermentation progressed, bacterial composition changed – as observed in pilot scale snapper fermentation where certain bacteria predominated by the end of the fermentation process which resulted in lower bacterial diversity during late fermentation period (refer Section 8.6).

Another similar observation made across all fermentations was that *Halobacteriales* was present only in unstoved salt fermentations regardless of seafood materials used, fermentation temperature, sampling depths and shell content. *Halobacteriales* was not present in any raw seafood material used thus suggesting that it may be originated from unstoved salt. However, no conclusion can be made on this assumption since no information was able to be generated on bacterial composition in either stoved/unstoved salt.

To help visualize the effect of different fermentation conditions on bacterial composition, NMDS plots were used as shown in Figure 75. Bray-Curtis dissimilarity method (Calle, 2019) was used to construct a matrix that quantifies the differences in relative abundances of bacterial communities between all fermentations . All NMDS plots had "stress" values < 20 which indicates good ordination and are common for higher numbers of samples (Figure 75).

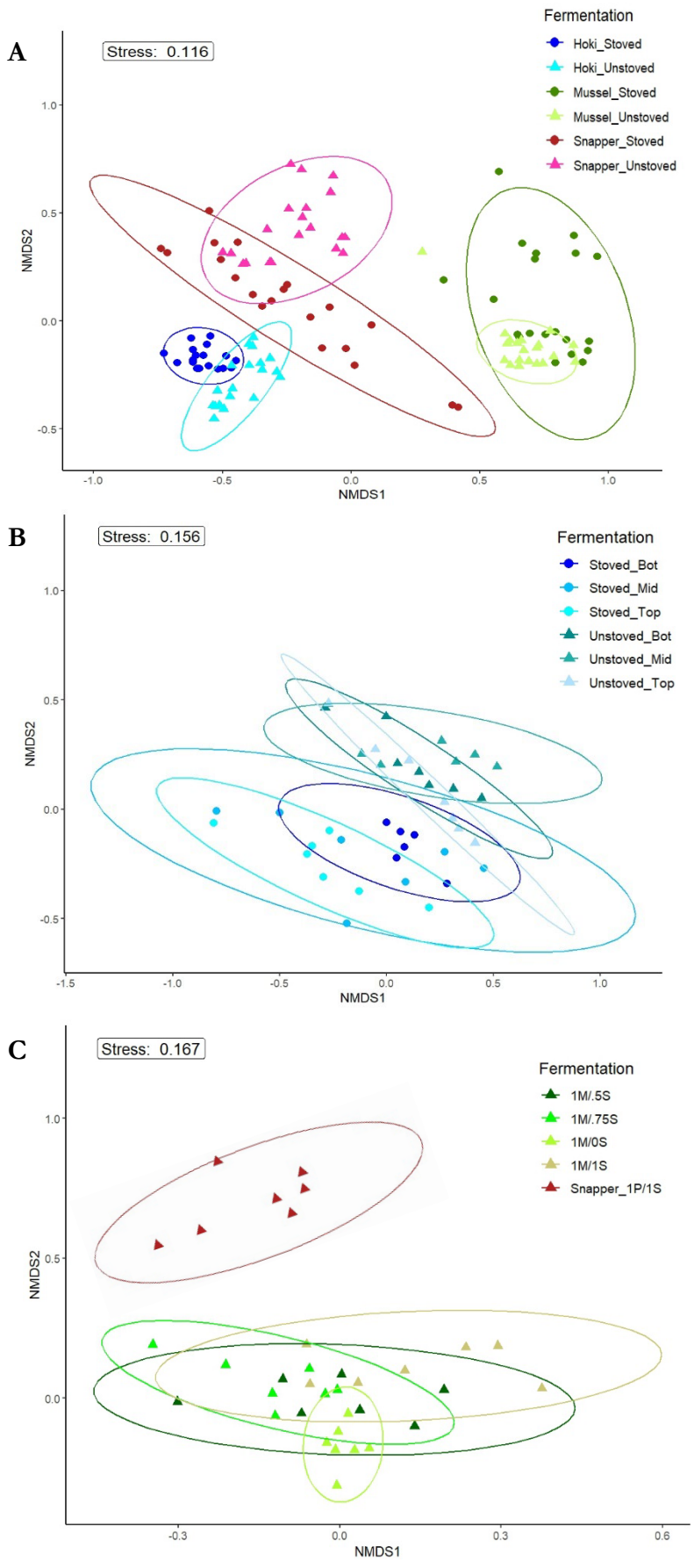

Figure 75: NMDS plots visualizing the effect of different fermentation conditions on bacterial composition. A: Laboratory scale fermentations with different variables (seafood material – Hoki, snapper and mussel; salt type – stoved and unstoved solar salts; temperatures – 18, 25 and 30 °C); B: Pilot scale fermentation using snapper frames and heads with stoved/unstoved salt at 30 °C sampled from 3 different depths of fermentation barrel; C: Mussel fermentation with

Figure 75(A) shows overlaps in fermentations with similar seafood material regardless of the type of salt used. Similarly, Figure 75(B) shows that all pilot scale snapper fermentations are overlapping with each other. However, despite the overlaps, it is noted that ellipses of similar salt type fermentations were positioned slightly closer together. Figure 75(C) shows all mussel fermentations with different meat/shell ratios are overlapping while snapper-shell is not. Overall, Figure 8 shows that seafood materials had more influence on bacterial compositions than other variables including salt type, sampling depths and shell content.

The current study found that high bacterial diversity is often seen in fermentations with close to/neutral pH – an exception being no-shell mussel fermentation where bacterial diversity was high despite a low pH range (5.4 – 5.9) which may be partially explained by a high bacterial diversity in raw mussel paste (refer to Sections 7.6, 9.3 and 9.5.3). Table 50 summarizes the pH and Chao1 indices (a measure of species richness where a high value indicates high diversity) in all fermentations.

Table 50: Summary of pH and Chao1 indices (a measure of species richness where high value indicates high diversity) in all different fermentations.

Set-up	Fermentation	Chao1	pH	Reference
Laboratory scale	Hoki Stoved/Unstoved	140 – 300	4.8 – 6.5	Sections 5.3 and 5.5.3
	snapper Stoved/Unstoved	152 – 201	5.1 – 7.0	Sections 6.3 and 6.5.3
	Mussel Stoved/Unstoved	188 – 463	7.0 – 7.3	Sections 7.3 and 7.5.3
Pilot scale	snapper Stoved/Unstoved	90 – 220	5.0 – 7.0	Sections 8.3 and 8.5.3
Shell on mussel	Mussel Unstoved with different meat/shell ratios	265 – 461	7.0 – 7.6	Sections 9.3 and 9.5.3

In some cases, neutral/close to neutral pHs are considered favourable for bacterial diversity as these conditions are often associated with a broader range of available nutrients and a more stable environment (Wu et al., 2017).

Many bacterial groups can adapt to this relatively mild pH, leading to increased diversity as various organisms find suitable niches for growth (Wu et al., 2017) – thus may explain the observations made in this current study. However, the pH values of fish sauce can vary between 4.5 to 7.5 (Park et al., 2001). Fish sauce produced from basic raw ingredients consisiting of only fish and salt with no additional ingredients introduced to enhance or manipulate the aroma and flavour of the final product normally have neutral or alkaline pH while fish sauce with added carbohyrate such as rice, wheat and sugar have more acidic pH (Lopetcharat et al., 2001).

11.3 The Effect of Different Fermentation Conditions on Volatile Fatty Acid Production, Bacterial Diversity and pH

A Principal Components Analysis (PCA) biplot was used to visualize the relationships between different fermentation conditions and their influence on VFA, bacterial diversity (represented as Chao1 index) and pH collectively. Vectors represent the parameters used in assessing fermentations with long vector indicates a high variation in the overall data (more contribution to the principal components). Two vectors that form a 90° angle indicate no correlations.

Figure 76 shows that the first two principal components (PC1 and PC2) explain a total of 92 % of the variance in the dataset, with PC1 capturing 55 % and PC2 capturing 37 %. All dependent variables for mussel fermentations regardless of salt type, fermentation temperature and shell content are clustered on the left side of the biplot while all Hoki and snapper fermentations regardless of salt type, fermentation temperature and sampling depth are spread across the right, suggesting that seafood material is the major factor influencing the joint impact of bacterial diversity, VFA production and pH.

Figure 76 also shows that the vectors for bacterial diversity and pH are closely aligned suggesting positive correlation between the parameters. The result supports the findings discussed in Section 11.2 that high bacterial diversity is often seen in fermentations with high pH.

Meanwhile, VFA is almost perpendicular to Chao1 indicating that these parameters are uncorrelated. This is because VFA may be correlated with bacterial compositions (relative abundances of bacterial family groups present in the fish sauce), not diversity (varieties of bacterial family groups present in fish sauce). Note that VFA also has a positive correlation with pH (vectors did not form 90° angle).

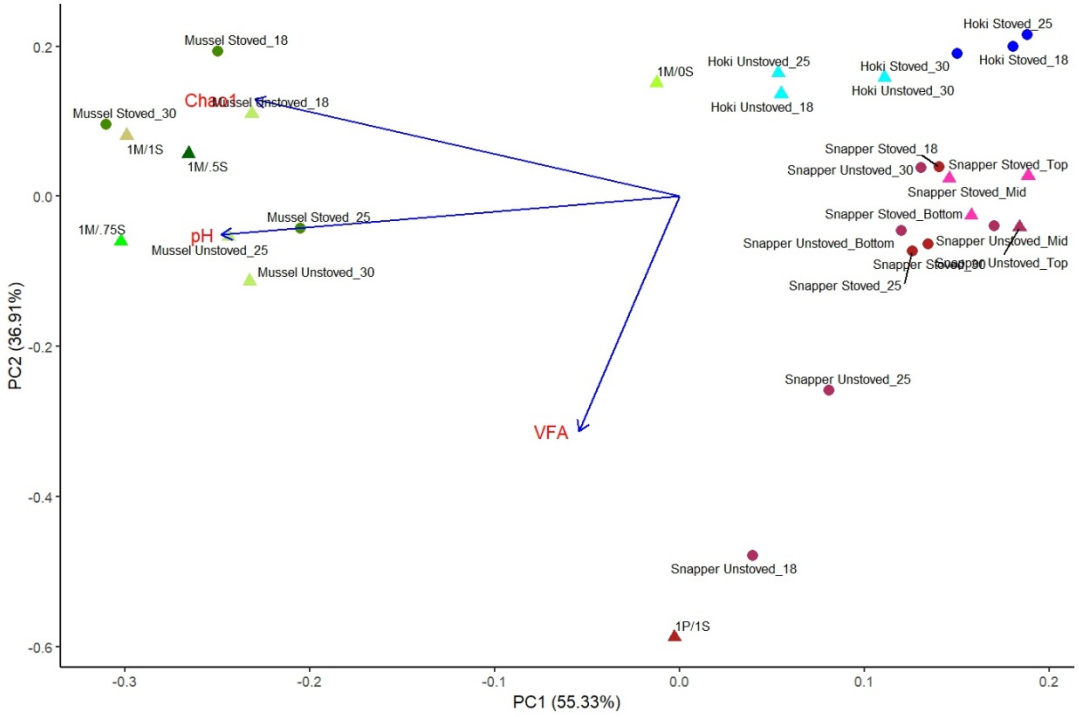

Figure 76: The Principal Component Analysis (PCA) showing the effect of different fermentation conditions on volatile fatty acid production (VFA), bacterial diversity (presented as Chao1 index) and pH. Each datapoint represents different fermentation where, green variation = green-shell mussel; blue variation = Hoki paste; red variation = snapper paste; and assigned shapes "●" = stoved salt and "▲" = unstoved salt.

Overall, Figure 76 shows that fermentations are clustered based on seafood material used indicating that it plays a significant role in influencing bacterial diversity and VFA productions. Note that the influence of different fermentation conditions on bacterial composition should be referred to Figure 75. The biplot also shows the relationship between parameters during fermentation process.

Chapter 12: Overall Conclusions and Recommendations

12.0 Overall Conclusions

This study generated experimental results regarding the effect of different fermentation conditions using New Zealand fish raw materials under different temperature condition. Volatile fatty acid (VFA) productions as well as bacterial composition and diversity were measured. Findings from this study conclude that:

1. VFA concentrations increased as the fermentation time increased
2. Salt type (solar salt that had been heat-treated (stoved) or not) had more influence on VFA productions than other single variable including seafood material used, fermentation temperature and sampling depths.
3. Seafood materials had more influence on the bacterial compositions than other variables including salt type used, fermentation temperature, sampling depths and shell content. Seafood materials also appeared to be the primary determinant of the predominant bacteria family groups for the fermentations.
4. Based on Principal Component Analysis (PCA), seafood material is the major factor influencing the complex of VFA production, pH and bacterial diversity (but not bacterial composition – relative abundances of bacterial family groups present in the fish sauce).
5. The presence of butanoic acid exclusively in unstoved fermentations may be associated with the presence or abundance of bacteria groups associated with butanoic acid production in these fermentations, though it was not determined if they originated from the unstoved solar salt.

6. *Halobacteriales* were found only in unstoved salt fermentations suggesting that salt type may be responsible for these results. No validation can be made of this postulate due to no bioinformatics information being generated on the salts used.
7. The presence of mussel shell is associated with high pH in fish sauce fermentation. However, no significant difference in pH was found in fermentations with different shell contents.
8. In pilot scale snapper fermentation, sampling depth did not have major influence on VFA production. However, sampling depth may influence the growth of some bacterial families to become the predominant groups by the end of the fermentation.

Overall, it can be concluded that the findings from this study can provide better understanding on the fermentation technology using New Zealand raw materials. Findings from this study can also provide initial background towards further investigation on the bacterial dynamics and compositions during the fermentation process, VFA production associated with key aroma and flavours of a fish sauce and eventually, the optimum fish sauce fermentation condition. General observation during this work indicate that Thai-style fish sauce could be made with New Zealand stocks and temperature. However, no conclusion can be made yet on their quality perceptions in market. In addition, overall findings from this study may provide a stepping stone towards the commercialization of fish sauce unique to New Zealand by utilising New Zealand's fish waste by-product and iconic seafood material as well as New Zealand's sea salt.

12.1 Recommendations on Future Work

Areas on which more focus can be directed and further development can be carried out are listed below:

1. Further investigation on microorganisms present in stoved and unstoved salt should be conducted to validate the postulates arising from this study. Further work on appropriate development and validation of molecular methods focusing on fungus/microalgae should be conducted to investigate other taxa that may be present in salts and other raw materials used in the fermentation process. Filtration may be employed as pre-treatment step prior to DNA extraction. The filter residue can then be subjected to DNA extraction and subsequent sequencing to determine the microorganisms present in the salts.
2. Further investigation on the effect of unstoved solar salt season-to-season variability should be conducted to provide better understanding of fish sauce production for commercialization.
3. Further work on the relationship and dynamics between predominant bacteria and metabolites present during fermentation process could be employed to further understand the role of predominant bacteria groups and metabolites in the formation of key flavours and aromas in fish sauce for the commercialization of a high quality final product.
4. Amino acid monitoring during fermentation can be employed to further investigate their roles in VFA productions associated with key flavours and aromas of a fish sauce.
5. As mentioned in Section 2.6.1.2, biogenic amines including histamine, cadaverine, putrescine and tyramine are commonly found in fish sauce and their consumption at particular concentrations may cause health problems (Tanasupawat and Visessanguan, 2014; Zaman et al., 2011). Further investigation on biogenic amines and monitoring of their contents during fermentation process can be conducted to ensure a safe for

consumption fish sauce. Biogenic amines investigation would also help to determine the appropriate fermentation duration.

6. As described in Section 2.6.2.2, other volatile compounds that are associated with the aroma of a fish sauce include trimethylamine (TMA) – contribute to ammoniacal aroma; 2-methylpropanal, 2-methylbutanal and 2-ethylpyridine – contribute to meaty note; pyrazines, pyridines, pyrimidines, amines and nitrile – contribute to a burnt aroma (Beddows, 1998; Dougan and Howard, 1975; Lopetcharat et al., 2001). Further investigations on other volatile compounds present in current fish sauces can be employed to understand their flavour profiles and their roles for high quality products and help decide which traditional Asian fish sauce New Zealand can best emulate.

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Appendices

Appendix 1 – Protein Content in Seafood Raw Materials

Nitrogen and protein content of seafood raw materials used in this study were determined using the Kjeldahl method based on the AOAC Official Method 2001.11 (AOAC, 2005) described in Section 3.4.1.

Table 51: Nitrogen and protein contents of seafood materials used in the fermentation.

Seafood Material	Nitrogen content (g of nitrogen/100g wet sample)	Protein content (g of protein/ 100g wet sample)
Hoki paste	2.25 ± 0.19	12.82 ± 0.46
Snapper paste	3.42 ± 0.03	19.54 ± 0.09
Mussel (whole)	1.91 ± 0.08	10.93 ± 0.20
Mussel meat	2.03 ± 0.10	12.7 ± 0.11
Mussel shell	0.86 ± 0.12	5.37 ± 0.12

Seafood materials were prepared as described in Section 3.1. prior to Kjeldahl method.
All experiment was conducted in triplicate.
Values represent means ± standard error of mean (n=3)

Appendix 2 – Proximate Composition of Seafood Raw Materials

Proximate analyses were conducted by external laboratory - Nutrition Laboratory, Massey University, Palmerston North, New Zealand.

Table 52: Proximate composition of seafood materials.

Seafood Material	Moisture (%)	Ash (%)	Crude protein (%)	Fat (%)	Carbohydrate (%)
Hoki paste	60.37 ± 0.08	22.63 ± 0.07	13.17 ± 0.20	0.47 ± 0.05	3.37 ± 0.11
Snapper paste	71.11 ± 0.04	6.91 ± 0.11	15.87 ± 0.11	5.3 ± 0.11	1.45 ± 0.22
Mussel meat	78.13 ± 0.11	2.23 ± 0.09	13.01 ± 0.07	0.57 ± 0.08	6.51 ± 0.08
Mussel shell	6.73 ± 0.07	87.03 ± 0.15	6.07 ± 0.14	0.20 ± 0.04	0.77 ± 0.15

Methodology:
Ash: Furnace 550 °C AOAC 920.153, 923.03 (meat)
Moisture: AOAC 950.46B (Meat)
Crude protein: AOAC 968.06 (Dumas method). N-P = 6.25
Fat: Soxtec (Meat), AOAC 991.36
Carbohydrate (Carb): By difference
All results are by wet basis.
All experiment was conducted in triplicate.
Values represent means ± standard error of mean (n=3)

Appendix 3 – Determination of Seafood to Salt Ratio for Laboratory and Pilot Scale Fermentation

Seafood to salt ratio were determined based on nitrogen content.

Based on literature values for fish sauce fermentation using 3:1 fish: salt, in average (Alfonzo et al., 2016; Bagthasingh et al., 2015; Detkamhaeng, 2016; Dissaraphong et al., 2016; Lee et al., 2015; Lopetcharat, 1999):

For every 100 g of fish = 3.2 g nitrogen

For every 100 g of fish = 33.3 g sodium used

Hence,

$$\frac{3\ g\ nitrogen}{33.3\ g\ sodium} = \frac{A\ (g\ nitrogen\ of\ seafood\ material)}{x\ (g\ sodium\ required)}$$

Where A = nitrogen content of seafood material as determined in Appendix 1.

For every 100 g Hoki paste, 23 g sodium is required.

Hence, salt to fish ratio = 1/4 salt/Hoki paste

For every 100 g snapper paste, 35.6 g sodium is required.

Hence, salt to fish ratio = 1/3 salt/snapper paste

For every 100 g whole mussel paste, 20.0 g sodium is required.

Hence, salt to mussel ratio = 1/5 salt/mussel paste

Appendix 4 – Determination of Seafood to Salt Ratio for Shell Fermentation

Mussel fermentation with different meat/shell ratio were described in Section 3.3.3. The unstoved salt added to each fermentation was based on salt/water ratio.

Table 53: Mussel and snapper fermentation with different meat/shell ratios as described in Section 3.3.3.

Fermentation set-up	Meat or paste content	Shell content	Ratio
Shell on mussel	1	0	1M/0S
	1	0.5	1M/.5S
	1	0.75	1M/.75S
	1	1	1M/1S
Shell on snapper	1	1	1P/1S

Total moisture content (TM %) of seafood materials:

Sample	%TM
Mussel meat	73.54 ± 0.15
Mussel shell	8.99 ± 0.22
Snapper frames	69.10 ± 0.32

The TM % of salt is assumed to be 0.

Based on literature values, TM % to salt ratio is 3/1 (Faisal et al., 2014; Je et al., 2005; Lee, Jung and Jeon, 2015; Lopetcharat et al., 2001; Lopetcharat et al., 2002; Lopetcharat et al., 2003; Park et al., 2001; Lee et al., 2015).

For 1M/1S fermentation:

In 1000 g total raw material = 500 g meat + 500 g shell

Hence, TM% for 1000 g raw material:

$= (500\ g\ of\ meat\ \times 75.57\ \%TM) + (500\ g\ of\ shell\ \times 8.99\ \%TM)$

= 390.43 %TM

Using 3/1 %TM/salt ratio,

$$\frac{1\ salt}{3\ \%TM} = \frac{x\ (g\ of\ salt\ to\ be\ added)}{390.43\ \%TM\ for\ 1M/1S}$$

Thus, salt to be added (g) = 130 g. (#repeated for other shell fermentations)

Appendix 5 – Estimation of Liquid Level Drop for Pilot Scale Fermentation Design

Estimation of the volume sampling port valve:

Sampling port valve dimensions = 12 mm length x 12 radius

$V = \pi r^2 h$

$V = \pi(0.12^2 \times 0.12)$

$V = 5.43 \times 10^{-3}$ litre ~5.43 ml

Hence, 10 ml will be discarded prior to sample collection.

*****

Total sample that will be taken from each sampling port:

V_{port} = 20 ml (ferment liquor) + 10 ml (trapped liquid in sampling port valve)

= 30 ml

For a total of 12 time sampling time, total sample for each sampling port is:

$V_{Total\ from\ each\ port}$ = 30 ml (V_{port}) x 12 (sampling time)

= 360 ml ~0.36 litre

*****

To estimate liquid drop in fermentation barrel:

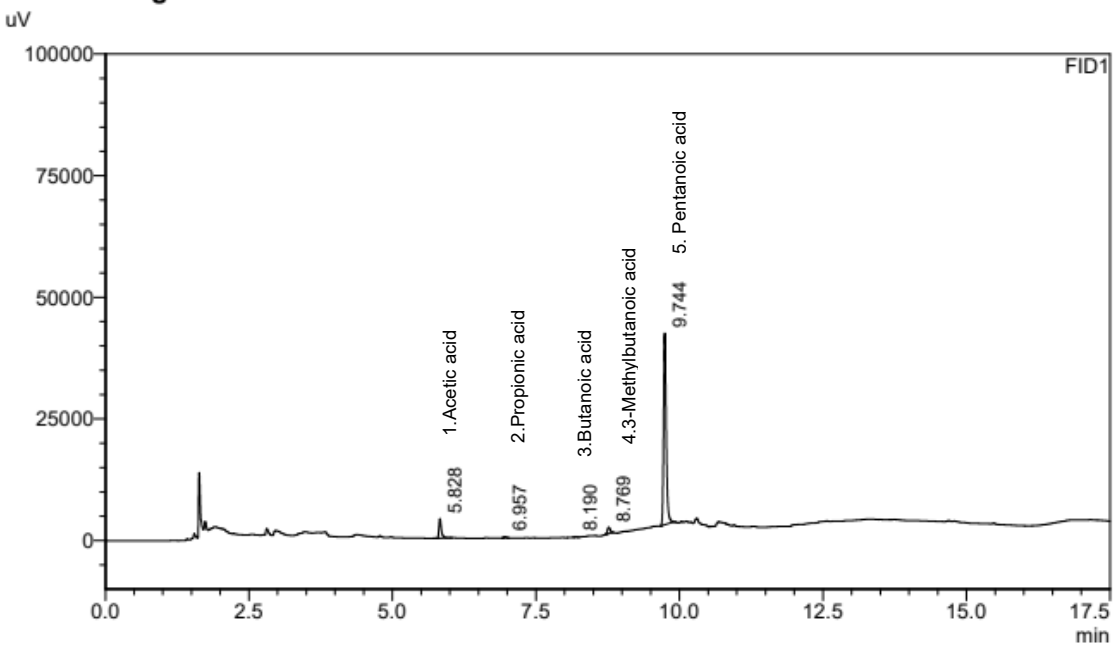
$h_{liquid\ drop} = \frac{V_{Total\ from\ each\ port}}{\pi r^2}$, where r = radius of fermentation barrel (1.5 m)

$h_{liquid\ drop} = \frac{0.36\ L}{\pi \times 1.5^2}$

$h_{liquid\ drop} = 0.051\ m \sim 5.1\ cm$ for each sampling depth

Appendix 6 – Example of Chromatogram from Fish Sauce Sample

<Chromatogram>



<Peak Table>

FID1

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.828	11556	3985	8.127			
2	6.957	678	244	0.477		M	
3	8.190	319	123	0.224		M	
4	8.769	4227	1305	2.973		M	
5	9.744	125405	39140	88.199		M	
Total		142184	44797				

Figure 77: Example of chromatogram from 25 °C unstoved Hoki ferment at Week 67.