Physicochemical and Microbiological Properties of an Innovative Fermented Mussel Food Product, *Perna*

by

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Abstract

Perna is an edible novel cook-then-lactic ferment product made from mussels, particularly the New Zealand green shell mussel, *Perna canaliculus*. This study characterized *Perna* form a physicochemical and microbiological perspective. *Perna* is made in the sequence: cook mussels by boiling, shuck, comminute, add glucose, salt, a carrageenan thickener and a starter culture; evacuate in barrier bags and incubate at 30°C for 4 days; store as required.

The aim of this thesis was to confirm the potential to develop a safe and stable product. The input variables included annual mussel condition, mussel gender, post fermentation storage conditions and starter culture type. Most work was done with a single lactic culture. Output variables include colour, pH, soluble protein, soluble peptides, amino nitrogen, profiles of free fatty acids and free amino acids, and of volatile compounds. Conventional microbiological plating techniques revealed the microbiological profile at stages of production and storage, and the capability of different starters to inhibit *Listeria monocytogenes* and, indirectly, *Clostridium botulinum.*

In the study of *Perna's* characteristics due to annual variation in mussel quality, *Perna* were prepared over many months and monitored for colour, pH, gender proportion and basic microbiology. The mussels used were as bought at retail with no supply chain history other than date. The pH of *Perna* at day of preparation had no obvious pattern with season. The pH changes at completion of showed no obvious pattern. On subsequent storage at 4°C *Perna* pH continued to decline slowly with storage. The orange/apricot colour for female and creamy white for male gonads was largely swamped by the brown colour of viscera after comminution. Long storage at ambient (19-23 ° C) temperature markedly darkened *Perna*, but colour was stable at chill temperature. Thus, storage at 4°C is best option for *Perna* stability, but the temperature requirements are not particularly rigorous. Limited microbiological showed that during the early phase of fermentation, contaminating microorganisms compete for nutrients with desirable bacteria in starter cultures. Mussels are filter feeders and are known to be highly contaminated with marine microorganisms. Therefore, the cooking step is very important to reduce the native biota.

The changes in protein characteristics from surviving endogenous enzymes of mussels and microflora, and of culture bacteria were investigated in aqueous extracts. Titratable amino nitrogen, a measure of chemically available amino groups, showed little change on fermentation, but storage at higher temperature, 35°C, resulted in big increases in amino nitrogen suggesting enzymatic hydrolysis to the point of spoilage. Long term storage at lower

temperature resulted in slightly reduced amino nitrogen perhaps arising from the Maillard reaction or loss due to microorganisms converting free amino groups to stable products. The similarity of ultraviolet absorbance between 275 to 292 nm and 200 to 400 nm indicated that proteinaceous matter was dominant in the water-soluble portion of *Perna* rather than nucleic acids. A loss in soluble protein by the bicinchoninic assay with storage time was clear but could not be easily reconciled with the amino nitrogen results. One plausible reason was proposed. In free amino acid analysis individual amino acids showed seasonal changes, with some amino acids showing an inverse correlation. Proportions of arginine were particularly striking, revealing three amino acid patterns related to season, and attributable to the role of arginine as an energy reserve when mussel food was not limiting. Alanine, glutamic acid and glycine, which are very flavour active, were present in high concentrations in *Perna* extract. Amino acid analysis work extended to differences due to five Chr Hansen commercial cultures. Two were standouts in respect of acidification rate and changes in amino profile. However, after extended storage amino acid profiles were similar for all five, suggesting a similar ultimate flavour from amino acids (but not necessarily from volatiles).

Cultures 4 and 5 were the fastest metabolizers to create an acidic environment in *Perna*. Lactic acid bacteria and staphylococci contributed to biogenic amine formation in *Perna* when the fermentation process was delayed, but not with advanced storage where biogenic amines were completely absent. Even at early times, biogenic amine concentrations were below regulated food safety limits.

The fatty acid profile of *Perna* was dominated by palmitic acid, followed by docosahexaenoic acid, then eicosapentaenoic acid, and subsequently a host of more minor fatty acids. The fatty acid profile was tracked throughout *Perna* preparation and long-term storage. The profile was extraordinarily stable. Thus, cooking, an anaerobic fermentation step and subsequent storage under vacuum is an excellent way of maintaining fatty acid stability.

The effect of cultures (five), storage time and temperature, and glucose concentration on *Perna* volatiles was explored by solid phase microextraction of the headspace above *Perna*. Different cultures produced different headspace profiles. Storage time and temperature also resulted in differences, conforming that chill storage was best. Below 1.0% glucose, the volatile pattern suggested flavour deterioration, conformed by smell and colour changes. With insufficient glucose it is likely that the surviving microflora successfully competes with the desirable culture microflora. Cooked mussel is not the intended matrix for meat salami cultures; microbial responses are unknown beyond the fact that pH usually fell to preservation levels, provided enough glucose was present.

The microbiological characteristics of *Perna* prepared using one culture stored at 4°C, ambient and 35°C were studied. Also, determination of the basic microbiology of *Perna* prepared using five starter cultures was done. Several pathogens – *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, and *Vibrio parahaemolyticus* – were not detectable in three preparation trials.

The ability of five starter cultures inhibit *Listeria monocytogenes* and *Clostridium botulinum* was examined. In vitro experiment showed certain strain of *Lactobacillus sakei* had a marked inhibitory ability on *L. monocytogenes*. This was in the culture designated T-SC-150. Some lactic acid bacteria have been shown to cause a spontaneous mutation on *L. monocytogenes* but T-SC-150 simply resulted in complete suppression of *L. monocytogenes*.

In situ experiments with supernatant of fermented *Perna* showed to provide favourable condition for *L. monocytogenes* inhibition. In this experiment T-SC-150 was able to reduce pH faster than other cultures but all cultures were able to drop pH below 5.2 within 17 hours of fermentation, greatly faster than the requirement of 48 hours by Ministry of Primary Industry.

T-SC-150 was the standout in the cooked mussel matrix. Its use as starter culture for *Perna* seems obvious, particularly as other quality indicators (pH, free amino acids, fatty acids, and volatiles) did not deviate to any extent from averages.

There is one important merchandising advantage for *Perna*. Provided it is stored cool, with some temperature flexibility, the shelf life would be long with a ‘best by’ date months after preparation. Future work is likely to center on recipe development of several *Perna* formats (very finely comminuted, *Perna* as described here, whole mussel format), but which was beyond the scope of this thesis. That is work for creative chefs.

Statement of originality

I declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no research material previously published or written by another person. Nor has this work been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed Date 20 June 2019

Chapter 1

General introduction and literature review

Introduction

Perna canaliculus is a green shell mussel that is native and unique to New Zealand coastal waters. This large mussel is attractively distinctive (Figure 1) and after cooking in boiling water yields a popular and also nutritious protein-rich food, which coupled with real and perceived health advantages in diet, has led to a major aquaculture industry (M. Miller, Pearce, & Bettjeman, 2014). The export value of green shell mussel in 2011 was NZ$218 million, 73% of the total aquaculture export value. The main export format for green shell mussel is cooked and frozen in a half shell, 84% of mussel by mass (Aquaculture, 2012). A further 10% is simply cooked and frozen mussel meat.

What AUT researchers call *Perna* is a comminuted[1] lactic-fermented green shell mussel product described in a pivotal honors thesis by (Dsa, 2013) at AUT, and which has been subsequently supported by several undergraduate studies at AUT. *Perna* has no known equivalent in any market, nationally or internationally. However, lactic fermentation of foods has been practiced for thousands of years and is recognized as producing storage-stable and usually safe products, and at the same time changing the flavour in often desirable ways. Lactic acid, at a typical final pH of 4.5, has a pleasant flavour, and this flavour is complemented by peptides that are produced by the proteolytic activities of bacteria other than the lactic acid bacteria (LAB). These other bacteria are usually included in lactic fermentation cultures to generate flavour complexity (Ravyts et al., 2010).

[1] Comminuted is a general term for anything cut up to a greater or lesser extent.

Figure 1 New Zealand green shell mussel.

Figure 2 Mature male (creamy white) and mature females (apricot-coloured) mussels. Source: Picture was taken during *Perna* preparation.

Literature review

New Zealand green shell mussel, *Perna canaliculus*

Perna canaliculus, is a bivalve from the family of molluscan Mytilidae. It is characterized by an attractive and unique green-lipped shell (Figure 1) (K. J. Murphy, Mooney, Mann, Nichols, & Sinclair, 2002). It can be up to 260 mm in length and have high meat-to-shell ratio (up to 55%) compared to blue mussels, *Mytilus edulis*, which is widespread throughout South America and Africa, and to *Perna viridis* a species found in the Indo-Pacific region (both up to 25%) (Alfaro, Young, & Ganesan, 2011). Male and female green shell mussel can be differentiated by their gonad colour, apricot for mature females and creamy white for mature male mussels (Figure 2). Green shell mussel was an important food resource for Maoris for hundreds of years and known to them as *kukutai*.

In the modern context, Greenshell^{TM} mussel is a trademarked name of farmed green shell mussel (Aquaculture, 2012). Green shell mussel is the dominant crop of New Zealand's aquaculture industry and is marketed as a pleasurable eating experience coupled with health-promoting properties. Green shell mussel has a high protein content and is low in fat, yet providing 680 mg of the omega-3 fatty acids EPA[2] and DHA in one serving, about 100 g. The mean total fat as g 100 g^{-1} (%) of mussel meat sampled in March 2014 from Marlborough, Coromandel and May 2014 from Bay of Plenty were reported to be 1.3, 1.4 and 0.4, respectively (Romanazzi, 2014). The total lipid content of mussels in different areas may due to variations in the phytoplankton, dinoflagellate and zooplankton populations (Mclean & Bulling, 2005; K. Murphy, Mann, & Sinclair, 2003; K. J. Murphy et al., 2002). Another reason can be variation in temperature. A cooler habitat could result in mussels having higher levels of storage fats (Jeffs, Holland, Hooker, & Hayden, 1999), and can also be an effect of reproductive phase of the mussel, particularly spawning when levels of about 1.2 to 3.7% have been observed (G. MacDonald, 2010).

During frozen storage of fish and shellfish, a loss of omega-3 fatty acids occurs through oxidation, accompanied by an increase in off-flavors. This phenomenon is widely recognized,

[2] Adequate daily intakes and suggested dietary targets for these oils of 500 mg daily of docosahexaenoic acid (20:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) have been proposed by many government and medical authorities throughout the world.

exemplified by the work of Jeong, Ohshima, and Koizumi (1991) on frozen oysters. Importantly, frozen storage is the main export format of green shell mussel.

Green shell mussel is also a rich dietary source of selenium, iron, vitamin B12 and iodine, and a good source of magnesium and calcium (G. MacDonald, 2010). The mussel is also is used to produce high-value nutraceuticals including oil extracts and freeze-dried mussel powders, for example, Lyprinol® and Seatone® (M. Miller et al., 2014). There is considerable clinical evidence of health-promoting effects of these products, including the ability to reduce inflammation and arthritic.

Therefore, green shell mussel has been shown to be not only good to eat, but also to have a high nutritional value and health benefits. These attributes have generated a large green shell mussel farming industry for local and export markets.

Key developments in New Zealand green shell mussel farming

In 1969, green shell mussel aquaculture was established starting with trials conducted with young mussels and grown on ropes suspended from rafts (Figure 3) (Alfaro, Jeffs, & Hooker, 2001). After 12 to 18 months the cultured mussels became ready for harvest, and the first domestic sale of green shell mussel was in 1971 (Aquaculture, 2012). By the mid-1980s, live green shell mussel became readily available in New Zealand supermarkets and were also cheap. Export markets were quick to follow to the extent that these mussels have been exported to over 70 countries (Alfaro, Jeffs, & King, 2014).

Figure 3 Long-line farming of green shell mussel where mussels are attached to the suspended rope system, Deer (2007).

New Zealand's green shell mussel exports

The export value of green shell mussel in 1989 was approximately 40 million N.Z. dollars. By 2000 it increased to sales of $170 million, exporting to over 60 countries to become New Zealand's second most important aquaculture export after salmon (Fisheries, 2012). By 2010, the market was valued in excess of $200 million (both export and domestic supply) and increased to become the highest value aquaculture export product at $218 million in 2011. The export format of green shell mussel is mainly cooked and frozen in a half shell comprising 83.6% of sales by mass (Table 1) (Fisheries, 2012). When other similar cooked-then-frozen formats are included in this frozen category, the fraction rises to about 94%, demonstrating a successful product class but limited in terms of added value.

Table 1 New Zealand green shell mussel exports in 2011.

Product category	Export weight (tonne)	Export proportion by mass (%)
Half shell frozen	31,871	83.60
Meat frozen	3,710	9.70
Whole frozen	1,388	3.64
Preserved marinated	490	1.29
Live	248	0.65
Freeze dried powder	252	0.66
Processed in cans, jars	82	0.22
Other not live/chilled/frozen	30	0.08
Powder in capsule	26	0.07
Whole chilled	15	0.04
Smoked	10	0.03
Crumbed, buttered	8	0.02
Half shell fresh/chilled	4	0.01

Source: Fisheries (2012)

Life cycle effects on green shell mussel meat quality

There is a year-round demand for green shell mussel, nationally and internationally, and in respect of this, the markets always expect a consistent product quality. But green shell mussel quality is affected by spawning. Female and male mussels have a consistent cycle of gamete development, discharge, and redevelopment, where discharge results in a depleted, dark-coloured mussel with inferior appearance and eating quality with obvious implications for mussel products, value added or not.

First consider northern New Zealand, which shows a prolonged spawning season from June to December (Figure 4). Generally, gonad development starts in December, with the early stage becoming increasingly dominant until around February, followed by a later stage of development in March. This continues through to May. Spawning begins in June and July, and spawning continues to December. By April, mussels in the ripe stage became more numerous, and these were most abundant in May. The prolonged spawning season in northern New Zealand differs from that in the northern South Island where spawning occurs in two distinct periods: one in early summer (December) and another in autumn to spring (late spring to early autumn, from May to September). Water temperature differences are the main driver of spawning (Alfaro et al., 2001; Alfaro, Jeffs, & Hooker, 2003).

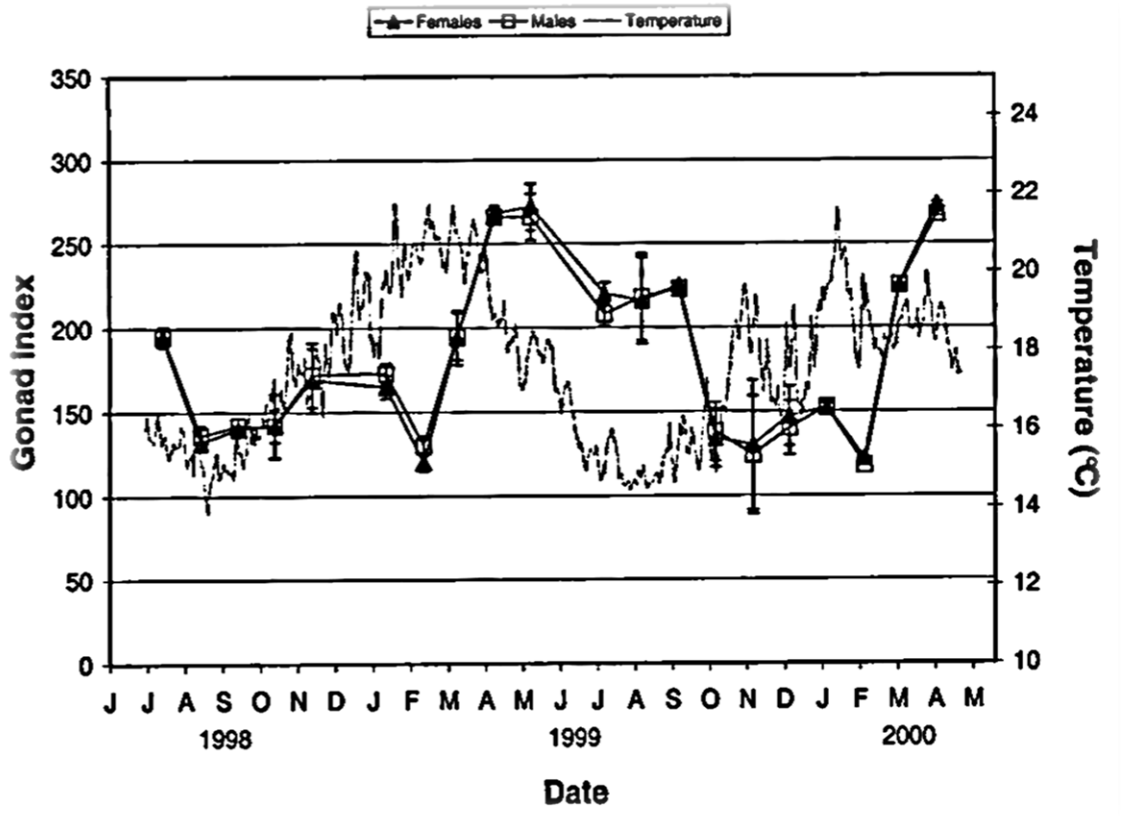


Figure 4 Mean (± SE) gonad indices for female and male mussels (combined data from 6 sites) and water temperature records (jagged line) for Ninety Mile Beach, northern New Zealand between July 1998 and April 2000. The gonad index is a measure of gonad development, where a high value means mussels are in the ripe stage ready to start spawn, lower value means spawning has taken place. Source: Alfaro et al. (2001).

Microbial contamination of green shell mussel and product implications

Spawning is not the only phenomenon affecting mussel meat quality. By filter-feeding from the surrounding water, mussel accumulate contaminants, includes toxins and pathogenic species and arising this contamination to the consumers. Outbreaks associated with a dense algae bloom toxin have been reported worldwide. Outbreaks have been reported in the United States, Europe, Japan, Africa, Australia (Reguera et al., 2014), China (T. Chen et al., 2013), Canada (M. Taylor et al., 2013), Greece (Economou et al., 2007) but Western Europe has probably the highest incidence in the world and may not the most serious problem for

aquaculture sites in the New Zealand (Reguera et al., 2014). Serious algae bloom outbreaks occurred in Western Europe in the1980s. A major algae bloom event occurred in summer 1981, with over 5,000 victims who had eaten Mediterranean mussels (*Mytilus galloprovincialis*) from the Galician Rías Baixas. In June and July 1983, at least, 3,300 people were intoxicated in Brittany and Normandy, France, with mussels (*M. edulis*) from Southern Brittany leading to an enforced sanitary ban. Consumers of mussels from the Skagerrak, Southern Sweden and Norway, 300 in October 1984, 49 in the UK, 1997, 8 in the Netherlands, 1995, became ill after consumption of mussels.

Potentially dangerous conditions of shellfish from microalgal blooms occurs sporadically in New Zealand coastal waters and generate neurotoxins. Monitoring of harmful microalgae in New Zealand began in 1993 following a major neurotoxic shellfish poisoning event in the Hauraki Gulf during the summer of 1992/1993. That event resulted in widespread contamination of shellfish, intoxicating more than 100 consumers (Holland et al., 2012). From March to June in 2011, other major harmful algae bloom affected all shellfish harvesting in the Marlborough Sounds in New Zealand leading to harvesting closure (Rhodes, Smith, & Moisan, 2013).

It is popularly believed that less processed or more ‘natural’ foods are healthier, but less processed/natural mussel consumption represents a greater risk to consumers. Mussels as filter feeders can accumulate high concentrations of microbes in their digestive tract, and where the microflora includes pathogens that pose risks for consumer health. The disease outbreaks due to consumption of shellfish have been attributed to the following: bacterial (*Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Vibrio* spp.) and viruses (norovirus and hepatitis A) that are associated either with human or animal wastes, or to bacterial pathogens indigenous to coastal marine environments (e.g., *Vibrio* spp.). These have been reported in the U.S.A. since the late 1800s; since then, more than 400 outbreaks and 14,000 cases have been reported in that country (Rippey, 1994)

Common pathogens due to contamination of mussels during harvest, transport and processing are *Salmonella* spp., *L. monocytogenes* and *S. aureus*. Faecal coliforms and *E. coli* counts are used to indicate the absence or presence of pathogens but have proved to be inadequate for assessment of enteric viral contamination because they are less resistant to inactivation by environmental factors.

In an episode caused by flooding, ruminant faecal contamination was detected in shellfish located 6 km offshore in Tasman Bay in New Zealand, a large aquaculture management area (Cornelisen et al., 2011). The enterococci and *E. coli* concentrations in

flooded Motueka river water were respectively up to 10,000 and 7,300 Most Probable Number (MPN) 100 mL^{-1} during peak flow. This flood caused microbial counts of enterococci and *E. coli* concentrations in mussel meat as high as 2,200 and 1,300 MPN 100 g $tissue^{-1}$, respectively. Source tracking markers identified the presence of faecal contamination from ruminant animals. These results are an indication of the close connection between land use and the quality of New Zealand's coastal waters.

As a normal inhabitant of the marine environment, *Vibrio* spp. is not a faecal indicator. The *V. parahaemolyticus* shows rapid growth at temperatures between 18 to 40 °C in broth and seafood (Foods, 1996). An earlier outbreak of *V. parahaemolyticus* infection due to indigenous seafood found in shallow water was reported in 1983, involving consumption of recreationally harvested mussels in Southeast Auckland (Thornton, Hazell, & Simmons, 2002). Although the food-borne pathogen *V. parahaemolyticus* has been reported as being present in New Zealand seawaters, and the incidence of *V. parahaemolyticus* has increased over a 30-year period, there have been no current reported outbreaks of food-borne infection from deep water commercially grown mussel due to this bacterium (C. Cruz, Hedderley, & Fletcher, 2015).

Salmonellosis is the most frequently reported bacterial gastrointestinal illness in New Zealand, reported as 'foodborne' of origin, especially through poultry meat. Animals can shed large numbers of *Salmonella* spp. in their faeces and are therefore an important source of contamination in mussels. A case of salmonellosis was reported in 1996, suspected to be caused by the consumption of raw non-commercially harvested mussels growing in the Wellington region (Baker & Roberts, 1996). Between 1997 and 2004, mussels were suspected as being the causative agent for six cases of gastroenteritis due to salmonellosis (Cressey & Lake, 2007).

An outbreak of listeriosis from consumption of contaminated smoked mussels occurred in New Zealand in 1992 and the three affected patients presented with a particular *Listeria monocytogenes* strain (KM92). The same strain was found in isolates from the green shell mussel products spanning the mussel factory environment, at retail and in domestic refrigeration (Brett, Short, & McLauchlin, 1998). The retail brands Pam's and Future Cuisine smoked mussels and frozen green shell mussel meat were found to contain *L. monocytogenes* in 2006 and 2016 (Table 2). An industrial *Listeria* monitoring program from August 2007 to June 2009 confirmed the presence of *L. monocytogenes* strains in raw and processed product, and the importance of cross-contamination during processing (C. D. Cruz & Fletcher, 2011). Specific *Listeria* isolates from mussels and their processing environments were reported to be

identical to those of prior sporadic listeriosis cases in New Zealand (C. D. Cruz, Pitman, Harrow, & Fletcher, 2014) demonstrates the potential health risk associated with mussel processing This, indicating failure of reduction of pathogenic microorganisms through processing .

Table 2 Green shell mussel product recalls due to *Listeria monocytogenes* contamination.

Recall date	Distribution	Product description	Company supplied
August 2006	Nation wide	Smoked	Pam's and Future Cuisine Limited
November 2007	Nationwide and other 11 countries	Meat	Sanford Limited
December 2011	Nation wide	Smoked	Pam's and Future Cuisine Limited
May 2016	Nation wide	Smoked and vacuum-packed	AquaFresh Products Limited

Source: MPI (2019)

Outbreaks due to consumption of New Zealand green shell mussel have also been attributed to enteric viruses. Norovirus is the most frequently reported agent for outbreaks in New Zealand, in terms of both numbers of outbreaks. Uncooked or lightly cooked bivalve shellfish such as oysters and mussels present a risk to health if grown in faecal contaminated waters. The regular identification of outbreaks of infection linked to contaminated oysters over the past 15 years indicates an ongoing risk. Between 1997 and 2004, two outbreaks of norovirus were recorded to be caused by non-commercially harvested mussels (Cressey & Lake, 2007). Mussels grown in aquaculture are probably of lower risk of contamination due to their occurrence in deeper waters (Greening, Lake, Hudson, Cressey, & Nortje, 2003). Another virus is a hepatitis virus, suspected as being the causative agent between 1997 and 2004. Five confirmed cases hepatitis involving green shell mussel have been recorded (Greening et al., 2003). The two sources of danger to human health reported above – pathogenic bacterial and viral contamination and toxins from algal blooms – can be mitigated

by HACCP[3] plans. In the case of bacterial contamination these plans can involve heating mussels, and high-pressure processing, but another entirely different approach is preservation involving non-pathogenic microorganisms that create an environment unsuitable for pathogen growth and without negative impact on the sensory quality of the product. Fermentation is the most common method for achieving this outcome.

Seafood fermentation at high salt concentrations

The earliest historical literature to seafood fermentation was to fish sauce. The Roman cookbook by Apicius in the fourth millennium before present discussed *garum*, a seafood sauce product with that same name in Greek and Latin. Pompeii, near modern-day Naples, was especially famous for *garum* production. Other contemporary names were *liquamen* in Latin, *clazomenai* in Turkey, and *cartagonova* in Spain. The procedure for making *garum* was very similar to that currently used for making any fish sauce in Southeast Asia; salt – typically 20% by weight – was mixed with fresh fish parts including viscera or shellfish and fermented for various times. The simple processing techniques, uniform final fermentation products and uses of the final product are similar throughout the wide geographical area (Yoshida, 1998).

A salted and fermented seafood, called jeotgal in Korean, is widely produced and consumed in Korea. It has equivalents in other Asian countries, shiokara in Japan, yujiang in China, ca mam in Vietnam and pla ra in Thailand (I.-S. Kim et al., 2014). It is often used as an additive to improve the taste or flavour of other foods such as kimchi[4] or it can be served as a stand-alone dish (Jung, Lee, & Jeon, 2014). In Korea, jeotgal is usually made by the fermentation of highly salted, 20 to 30% (w/w), marine animals such as shrimp, anchovy, oyster, clam, fish roe, and squid.

The microflora of high-salt fermented foods varies depending on bacteria originally present in the raw materials, but halophilic and halotolerant bacteria genera are frequently identified. A study by Chuon et al. (2014) has identified to genus level common Gram-positive cocci and rods, such as high salt tolerant bacteria such a *Staphylococcus* spp., *Micrococcus* spp. and *Tetragenococcus* spp., also species of spore-forming bacteria, like *Bacillus* spp. and *Clostridium* spp. The spore-formation ability of these bacteria might be a

[3] HACCP is a management system in which food safety is addressed through the analysis and control of all hazards from raw material production to final consumption.

[4] Kimchi is lactic fermented cabbage, flavoured with chilli and garlic**.**

survival response to high concentration of salt. *Staphylococcus* spp. and *Micrococcus* spp. have proteolytic and lipolytic activities, which are useful in flavour generation.

Tetragenococcus spp. are known to be major lactic acid producers in high-salt-containing fermented foods, such as fish sauce and soy sauce. According to Chuon et al. (2014), *Bacillus* spp., *Clostridium* spp., and *Tetragenococcus* spp., play a major role during the early fermentation, while other *Micrococci* and *Staphylococcus* species thrive during later stages. Itoh (1993), examined the microflora changes to species level during fish sauce fermentation and found that *Bacillus* species such as *B. coagulans, B. megaterium* and *B. subtilis* played a primary role during the early fermentation stage, while other *Micrococcus* spp. and *Staphylococcus* spp. such as *M. colpogenes, M. varians, S. epidermidis and S. saprophyticus* thrived at later stages. However, the basis for the preservation effects is probably primarily due to a high salt concentration and not due to the bactericidal effects of the organic acids described above, as these acids are not sufficiently protonated to diffuse across the plasma membrane of bacterial cells within the mildly acidic or alkaline pH ranges encountered in high salt products.

The biochemical events in producing high-salt fermented seafood products are mainly mediated by enzymatic hydrolysis. Proteolytic enzymes of the viscera of the seafood used to hydrolyse the proteins into amino acids and peptides (I.-S. Kim et al., 2014) Enzymatic hydrolysis with the salt content of higher than 13% (w/v) can also prevent the growth of pathogens and prevent putrefaction from biogenic amine formation. Also, salt concentrations higher than 20% prevent excessive formation of ammonia from organic nitrogen (Owens & Mendoza, 1985). Commercial proteolytic enzymes have been used to speed up the traditional process with reduced salt content, but sensory evaluation has shown the development of undesirable flavour compared to the traditional method (Beddows, 1998). However, a desirable product flavour was achieved when the pH and salt content of the product was close to that of traditionally-made product (Gildberg, Espejo-Hermes, & Magno-Orejana, 1984).

Overall for traditional salt fermented products, whether the final product is a fish sauce or a *jeotgal*-type product, the result is a nutritious although salty food, which is stable at room temperature, and has a delicious flavour attributed to salt and to the umami taste, two of the six recognized tastes experienced by humans (Guan, Cho, & Lee, 2011; I.-S. Kim et al., 2014) glutamate (MSG), guanosine monophosphate, and inosine monophosphate. The umami substances are nominally acids, but which exist in the salt form at neutral pH, where the cation is usually sodium. In a landmark publication, (Noguchi, Arai, Yamashita, Kato, & Fujimaki, 1975) reported that an enzymatic hydrolysate of a fish protein concentrate could be

fractionated to obtain almost 30 oligopeptides with flavour potentiating activity. The complete or partial amino acid sequences, such as Ala-Glu, Asp-Ala, Asp-Gly, Asp-Leu, Glu-Asp, and Glu-Glu etc. frequently contained glutamic acid and were found to have a flavour activity qualitatively resembling that of monosodium glutamate.

While high-salt fermented foods are undoubtedly flavorsome, high salt is not necessary for preservation and flavour, because very many low salt fermented foods – dairy products in particular – are dietary basics throughout the world. These foods are discussed below.

Lactic fermentation of foods at low salt concentrations

Anaerobic lactic fermentation of foods has been practiced for thousands of years and is recognized as producing largely ambient-stable and usually safe products, and at the same time changing the flavour in often desirable ways. The main producers of lactic acid in food the lactic acid bacteria (LAB). Growth to at least $\log_{10}$ 7 colony forming units per gram of food is required to generate enough lactic acid to lower pH to a typical final value of around 4.5 (Paludan-Müller, Huss, & Gram, 1999). At this pH, the growth of most pathogenic bacteria is inhibited. Lactic acid has a pleasant flavour, and this flavour is complemented by peptides that are produced by the proteolytic activities of bacteria other than the LAB. Lactobacilli as one of microorganism included in LAB are common in the human environment, most notably the vagina where the microflora maintains an acid pH. When the material to be fermented is comminuted to some degree with salt and a fermentable carbohydrate, and then blended by hand – as was always traditionally done – LAB on hands and equipment are incorporated throughout the mixture. The added salt concentration must be less than about 9%, above which the most of the lactic acid bacteria will not grow (Guan et al., 2011). Upon the exclusion of air, usually achieved by tight packing, the LAB quickly dominates the microflora and lactic acid accumulates. However, as with any wild-type fermentation the results are somewhat unpredictable[5]. The solution to variable outcomes is the use of microbiologically defined starter cultures.

Starter cultures have been developed from the study of the microflora involved in traditional fermentation (Alagić et al., 2011; Babić et al., 2011). A starter culture can be defined as ‘a microbial preparation of many cells of at least one microorganism that is added

[5] Lactic fermentation of milk left undrunk in plastic bottles variably yields some pleasant tasting outcomes and some very unpleasant outcomes.

to a raw material, to produce a fermented food by accelerating and guiding its fermentation process' (Saithong, Panthavee, Boonyaratanakornkit, & Sikkhamondhol, 2010). In general, terms, the preservation effect is due to a combination of low pH, typically around 4.5, the antibacterial activity of lactic acid in its protonated form (Mahdi Ghanbari, Mansooreh Jami, Konrad J Domig, & Wolfgang Kneifel, 2013). Another mechanism of suppressing the growth of undesirable microorganisms is through the production of bacteriocins (Daeschel, 1989). Bacteriocins are hydrophilic antimicrobial peptides produced by lactic acid bacteria during fermentation (Jang, Lee, Jung, Choi, & Suh, 2014). Bacteriocins have been isolated and characterized from many different bacterial genera; however, few bacteriocins have been analysed in application studies in food systems for their ability to inhibit undesirable bacterial flora. Two important bacteriocins, nisin and pediocin, have been reported (Vandenbergh, 1993). Nisin, for example, is a low molecular mass antimicrobial protein synthesized by *Lactococcus lactis* subsp. *lactis*. Nisin-producing starter cultures prevented clostridia gas formation in cheese and have been observed to possess a wide spectrum of activity against Gram-negative and Gram-positive bacteria. Pediocin, produced by *Pediococcus acidilactici*, can inhibit a variety of pathogenic bacteria (Vandenbergh, 1993). Many studies have reported bacteriocin production by different *L. sakei* strains (Avaiyarasi, Ravindran, Venkatesh, & Arul, 2016; Castellano, Belfiore, Fadda, & Vignolo, 2008; Møretrø et al., 2005).

Commercial dry-fermented sausage meat starter cultures can be grouped into two classes: heterofermentative and homofermentative (Figure 5). Homofermentative LAB that produce almost only lactic acid from fermentable carbohydrates present in meats or added to meats, but heterofermentative species produce quantities of additional fermentation products, such as ethanol, acetic acid, butanoic acid, acetoin and CO_2 gas (Zdolec, Vidaček, Marušić, & Medić, 2011). The homofermentative and heterofermentative classification originates from the metabolic pathways that microorganisms use to generate the resulting end products (Figure 5). While homofermentative follow the glycolytic pathway, heterofermentative use the 6-phosphogluconate/phosphoketolase pathway (Garvie, 1984). The primary role of homofermentative LAB starter cultures is to ferment available carbohydrates to produce lactic acids, and in most foods heterofermentative are not wanted, one important reason being their ability to produce CO_2. A fermented sausage is not expected to contain gas bubbles, but a Swiss-style cheese is.

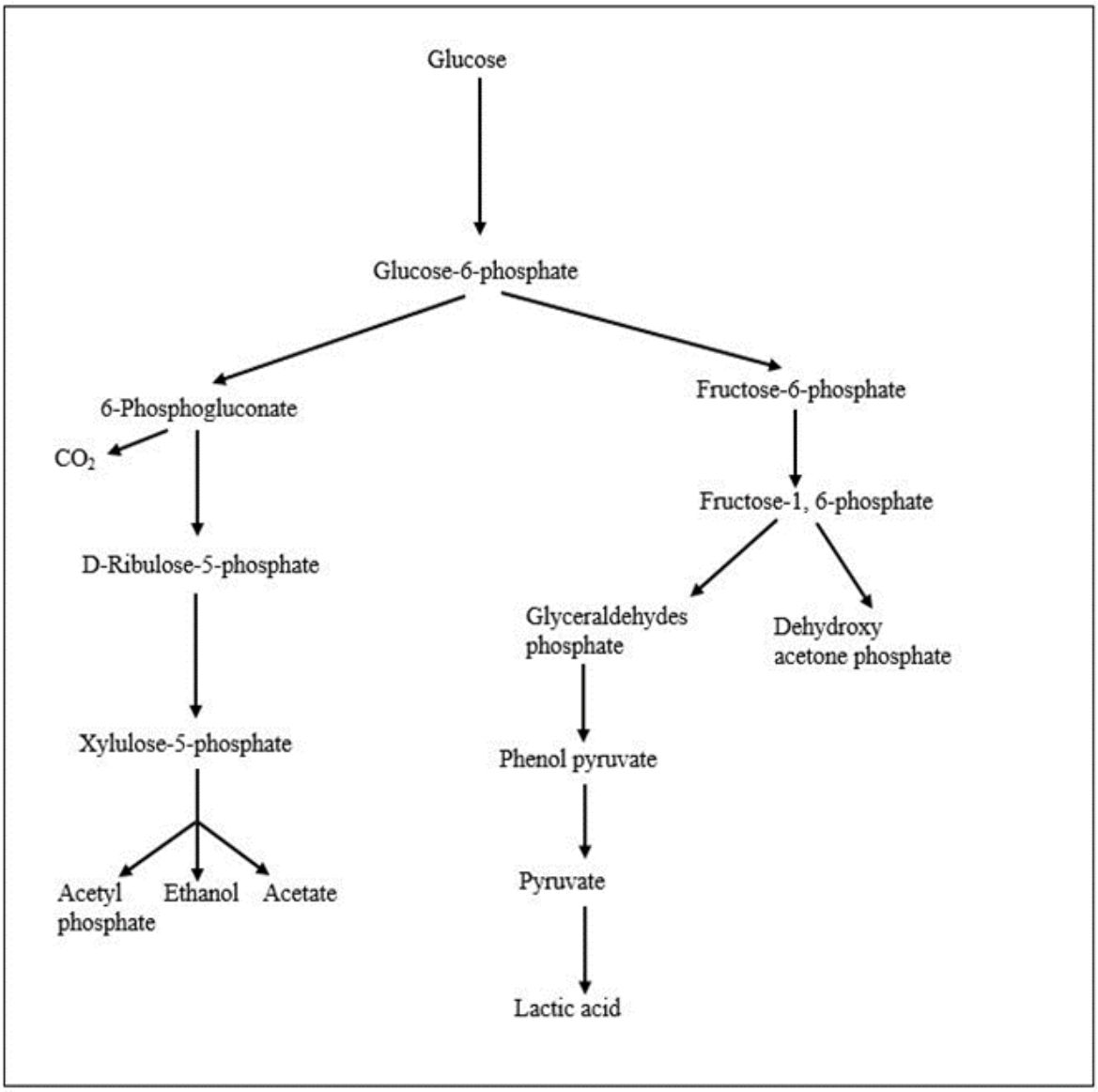


Figure 5 Glucose metabolic pathways of LAB. Source, Garvie (1984). Homofermentative metabolism follows the glycolytic pathway (right side) while heterofermentative follow the 6-phosphogluconate/phosphoketolase pathway (left side).

LAB used for fermentation of sausages in the USA are *Lactobacillus* spp and *Pediococcus* spp., while in Europe, *Lactobacillus* spp., *Pediococcus* spp., *Micrococcus* spp. and *Staphylococcus* are usually used (Zdolec et al., 2011). The different bacteria have various roles in lactic acid formation, flavour generation and food safety. For example, *Pediococcus* spp. has been shown to inhibit undesirable microorganisms including *Staphylococcus aureus,*

Clostridium botulinum, some species of the genus *Salmonella*, *Escherichia* and *Bacillus*, and certain molds (El Adab, Essid, & Hassouna, 2015; Wang et al., 2015; Yoo, Na, Park, Seo, & Son, 2015). The inhibitive action is affected by the rate of production and the final concentration of lactic acid (Vatanyoopaisarn, Prapatsornwattana, Kuhakongkeat, & Phalakornkule, 2011). For example, Wang et al. (2015) showed that fermented sausages inoculated with higher numbers of LAB showed a significant decrease in pH compared to control sample without added LAB and a rapid increase in numbers to log 8.7 cfu g^{-1} within 2 hours, attributed to a short lag phase. The rapid decrease in pH seriously inhibited the Enterobacteriaceae and *Pseudomonas* spp., and it was concluded that early lowering of pH is an essential requirement to ensure the hygienic stability of sausages. In short, the early domination of LAB is a key factor in suppressing the growth of potential pathogens and spoilage microorganisms.

The role of bacteria other than LAB in flavour generation from protein, fat and carbohydrate

In the previous section, it was noted that bacteria other than LAB have a role in flavour generation beyond that due to lactic acid alone. Very many proteins are insoluble and as such do not contribute to flavour, the combination of taste and smell. However, as proteins are hydrolysed to polypeptides, peptides and ultimately amino acids, solubility increases with increased flavour potential (Noguchi et al., 1975). Thus, protein hydrolysates are used for flavoring, and at the same time contribute to functionalities and antioxidative properties in food systems (Thiansilakul, Benjakul, & Shahidi, 2007).

During fermentation endopeptidases endogenous to the (uncooked) food, or from culture microorganisms such as staphylococci, hydrolyse large protein molecules mostly to poly- or oligopeptides, and at the same generating very few free amino acids (Figure 6). As hydrolysis progresses, hydrolysis of protein continues, and polypeptides continue to hydrolyse to oligopeptides and eventually individual amino acids (Figure 6). This progression can be readily monitored in detail by liquid chromatography (Villas-Bôas, Smart, Sivakumaran, & Lane, 2011), but a classical biochemical method also yields useful information. The α-amino nitrogen method quantifies the free amino groups that increasingly become titratable as peptides and amino acids accumulate (Wang, Zhang, Cao, Zhang, & Chen, 2012). (The importance of α-amino nitrogen in flavour is clear from the fact that this assay is often used as the quality indicator for (high salt) fish sauce products (Yokotsuka, 1986). The higher the α-amino nitrogen concentration, the better the quality.)

Turning now to fats and amino acid catabolism, it is useful to consider *Staphylococcus* spp. as an example of how flavour is generated, using fermented raw red meat sausage as the food example. Before fatty acids can be flavour active, either as free fatty acids or as derivatives of free fatty acids, they must be hydrolysed from the triacylglycerols, commonly called fats or oils. Hydrolysis of fats occurs spontaneously under moist acid conditions (M.-C. Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996), and hydrolysis is greatly accelerated by fat lipases and muscle phospholipases. For example, (M.-C. Montel et al., 1996) showed that inoculation of sausage with *Staphylococcus* spp. alone (no LAB) appeared to increase the free fatty acid concentration 5-fold. However, when they included antibiotics to inhibit the *Staphylococcus* spp., the 5-fold free fatty acid concentration was not reduced. This indicated that the source of lipase enzyme was not bacterial. Other researchers have reported hydrolysis to be controlled by lipases and phospholipases of added bacteria, which has led to the release of free fatty acids (Gandemer, 2002; Molly et al., 1997). But the relative contribution of endogenous enzymes and microorganism to lipolysis and flavor development is still controversial (Xu et al., 2018). Although both endogenous enzymes and microbial have been reported to be responsible for the release of free fatty acid during fermentation of fish, endogenous enzymes play a major role (Xu et al., 2018). But the inclusion of adjunct microbial species in starter culture is no accident. Microbial species with higher hydrolytic activity such as *Staphylococcus* spp. is used as starter cultures to promote development of good flavour. In addition, Q. Chen, Kong, Han, Xia, and Xu (2017) has observed that *Pediococcus pentosaceus*, *Lactobacillus curvatus* and *Staphylococcus xylosus* are also able to enhance lipid hydrolysis and improve flavour development.

The degradation of amino acids into volatile molecules plays an important role in the characteristic flavour of fermented sausage. M.-C. Montel et al. (1996) and Ravyts et al. (2010) showed that staphylococci increased the concentration of the flavour-active methyl ketones – arising from beta oxidation of fatty acids – and helped to generate fermented sausage flavour. Staphylococci were able to degrade the branched chain amino acids leucine, isoleucine, and valine into flavour active methyl branched aldehydes, alcohols, and acids (Larrouture, Ardaillon, Pépin, & Montel, 2000; Ravyts et al., 2010).

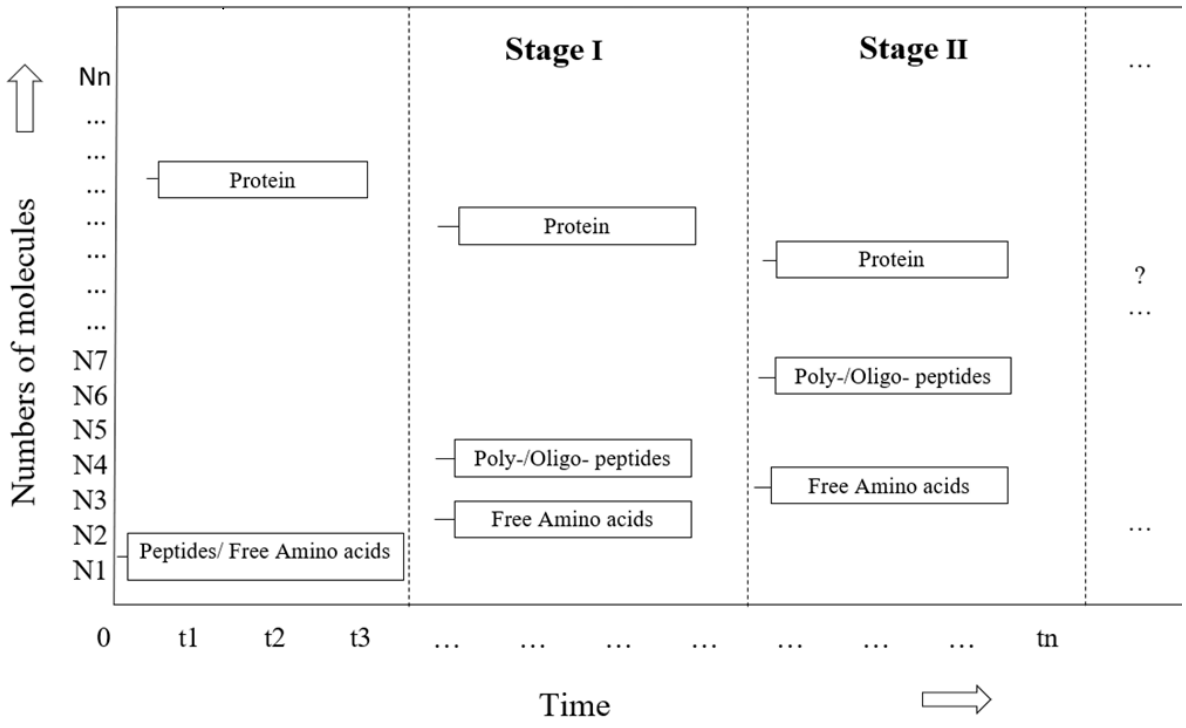


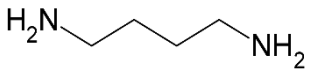
Figure 6 Schematic representation of the changes of different components (protein, poly-/oligo- peptides, and free amino acids) during the process of hydrolysis. The X-axis is hydrolysis time; Y-axis is the number of the molecules. Source: Wang et al. (2012).

The coagulase-negative staphylococci are common adjuncts to LAB cultures, and their roles have been outlined above. One deserves special attention. *Staphylococcus carnosus* is very tolerant to acidity (Janssens, Myter, De Vuyst, & Leroy, 2012; Ravyts et al., 2010). During meat sausage fermentation, Janssens et al. (2012) reported that in the presence of acidifying LAB, *S. carnosus* colonies count remained high and constant despite low pH. Low pH did not prevent catabolism because *S. carnosus* produced 3-methylbutanol at pH 4.5, improving flavour by producing volatiles from amino acids catabolism and pyruvate metabolism as well as methylketones from beta oxidation of fatty acids (M.-C. Montel et al., 1996; Ravyts et al., 2010). This result was supported by El Adab et al. (2015). They reported the *S. carnosus* to be dominant microflora after LAB, and their number remained higher throughout at all stages of maturation, also called ripening, after fermentation.

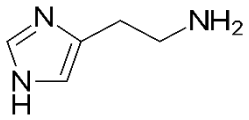
Fermentation and biogenic amine production

As discussed earlier, LAB, *Staphylococcus* spp. and *Pediococcus* spp. are variable responsible for proteolysis and release of free amino acids and free fatty acids, which are a further substrate for a range of flavour generating compounds (Freiding, Gutsche, Ehrmann, & Vogel, 2011; Wang et al., 2015). If microorganisms with decarboxylation ability are present in a lactic fermentation system, undesirable biogenic amines can be produced from free amino acids (Spano et al., 2010). These biogenic amines – histamine, putrescine, cadaverine, and tyramine – are organic bases (Figure 7) and are commonly used as chemical indicators of fish decomposition.

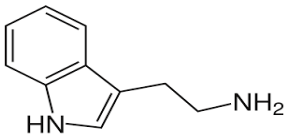
In higher concentrations they are toxic to humans, so in New Zealand, the concentration of histamine in fish and their products must not be more than 10 mg 100 g^{-1}. While lactic acid fermentation is not a precondition of biogenic amine formation, they can accumulate in fermented meat sausage (Spano et al., 2010) and in fermented fish sausage (Khem, Young, Robertson, & Brooks, 2013). Food fermenting LAB are generally considered to be non-toxic and non-pathogenic, but some species of LAB can produce biogenic amines.



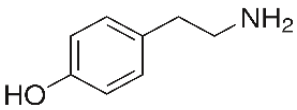
Cadaverine



Histamine



Tryptamine



Tyramine

Figure 7 Structures of four representative biogenic amines. Source (Gubartallah, 2018)

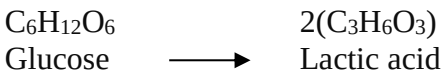
Statement of the commercially inspired opportunity

There is a high and increasing international demand for high-quality aquatic food products (G. MacDonald, 2010). New Zealand has a good reputation for eco-friendly production practices and strict quality assurance programmed with an emphasis on shellfish, and because of that, the shellfish are acceptable in many international markets. The New Zealand Government, through the Aquaculture Strategy and Ministry for Primary Industries' 2012 plan, is aiming to grow annual aquaculture export sales to NZ$1 billion value by 2025 (Alfaro et al., 2014). As noted earlier, the current highest value aquaculture export category is green shell mussel (Aquaculture, 2012). Some of the focus in marketing mussels has been on the health properties of mussel fat (usually called oil), which is high in eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and other omega-3 fatty acids (Gupta, 2012; Mclean & Bulling, 2005; McPhee et al., 2007; M. Miller et al., 2014; K. Murphy et al., 2003; K. J. Murphy et al., 2002; A. G. Taylor & Savage, 2006) with little emphasis on other more mundane components such as protein.

Seafood is a risky group of foodstuffs in terms of consumer safety. The nutrient-rich composition of seafood creates a favorable environment for the growth of spoilage microorganisms and common food-borne pathogens (Mahdi Ghanbari, Mansooreh Jami, Konrad J. Domig, & Wolfgang Kneifel, 2013). This is particularly true of the filter-feeding molluscs, where the entire shellfish is commonly eaten thus including the animals' entire microflora. The tradition solution to this is cooking the shellfish, which not only destroys endogenous bacteria but also conveniently opens the shell and from a nutritional perspective makes proteins more available for digestion (Evenepoel et al., 1998). Cooking is thus the green shell mussel industry's first step in the preservation and is normally followed by freezing (Fisheries, 2012), which greatly minimizes recolonization of the mussel meat by micro-organisms. Problems remain, however, the first being that the category frozen mussels (usually on a half shell) have minimal value added, and thus are a commodity category, and is greatly subject to variable financial returns (Brett et al., 1998; C. D. Cruz & Fletcher, 2011). A second problem relates to fats. EPA and DHA are both in the category of long-chain polyunsaturated fatty acids (PUFAs). PUFAs in green shell mussel oil comprise more than half the oil extract (M. Miller et al., 2014), and are highly susceptible to oxidation. Oxidation can be minimized in several ways, usually involving oxygen exclusion through modified gas atmospheres (CO_2, N_2), or more commonly through vacuum packaging. Both methods can be and often are combined with cooling that further slows – but never fully prevents – microbial

growth that develops from recolonization during product preparation.

In response to the first problem of lack of value addition, Professor Owen Young of AUT Applied Sciences proposed that lactic fermentation of mussels could be a means of adding value in a way different from all other methods applied in New Zealand. Lactic fermentation occurs where a class of bacteria, the lactic acid bacteria (LAB) anaerobically metabolize a monosaccharide, usually glucose, to lactic acid. The pH falls to a point, typically around 4.5, where other microorganisms cannot grow, including pathogens. The overall equation is:



Moreover, he proposed that vacuum packing that is helpful in promoting fermentation would also be useful in minimizing oxidation because most air is removed by the vacuum, and because the LAB scavenge remaining oxygen. His early work was supported by two AUT students, Dsa (2013) and Qiang (2012) extending that research is the topic of my proposed research.

In the traditional lactic fermentation, the raw material is not normally subject to heat treatment prior to fermentation. An obvious exception to this is the modern pasteurization of milk before yoghurt/cheese fermentation because pasteurization destroys pathogens like *Mycobacterium tuberculosis*. However, pasteurization also destroys endogenous LAB before fermentation, so LAB must be added to the pasteurized heated milk to start the fermentation. In the case of yoghurt, whether made industrially or domestically, the heated milk is inoculated with specific strains of LAB to optimize fermentation.

By contrast, yoghurts and cheeses were and still can be traditionally made only with endogenous LAB, along with other opportunist microorganisms of unknown ‘parentage’. For this to occur the milk is not pasteurized. In other foods, prior cooking can be followed by fermentation with unknown LABs and other microorganisms. Consider a traditional lactic-fermented maize, sorghum or millet non-alcoholic meal, for example, *amahewu* and *incwancwa* in South Africa, *ogi* in West Africa, *togwa* in Tanzania, and *munkoyo* in Zambia. The processing is similar. It involves the addition of water to flour (9:1 ratio), boiling for 10 to 15 minutes, cooling to around 40°C before addition of small amount of dry flour as a

source of inoculum[6], followed by fermentation for three days in a warm place (Chelule, Mokoena, & Gqaleni, 2010). (As the author of this thesis, I never anticipated that my *togwa* skills in Tanzania would lead to mussel fermentation at AUT.)

Other traditional foods that depend on an endogenous microflora are German fermented cabbage (sauerkraut), its Korean equivalent that also contains garlic and chili (kimchi), and fermented raw fish.

Kimchi is a traditional Korean food, which is made by the fermentation of vegetables such as cabbage, radish, and cucumber with various seasonings including salts, red pepper powder, garlic, leek, and ginger (Jang et al., 2014). Based on the major raw materials and preparation methods, kimchi is classified into hundreds of varieties, which have their own unique features in biochemical, nutritional, and organoleptic properties (Cheigh, Park, & Lee, 1994). Preparation is simple, vegetables are soaked into water containing salt for two hours, then drained, and mixed by hands with various spices. The spiced vegetable is then covered and left in room temperature for one to two days to ferment. The vegetables once it starts to ferment, stored in the refrigerator to use as needed. Refrigeration slows down the fermentation process, which will make the kimchi more and sourer as time goes on. Because kimchi ferments without specific inoculants or starters, this leads to the growth of various LAB species or strains, originated from raw materials, hands and utensils.

Fermented fish models were developed at AUT University using marine fishes, hoki, kahawai and trevally to emulate multiple Asian examples (Khem et al., 2013), where fish fermentation is a widespread cottage industry. The formulations comprise raw ground fish, carbohydrate, garlic and salt, but no added culture, and are well described from Riebroy, Benjakul, Visessanguan, and Tanaka (2005). The carbohydrate is cooked rice. Processing by Khem et al. (2013) was done using equipment which had been thoroughly cleaned but not sterilized. Although the number of the endogenous LAB from raw material, clean hands and clean equipment were low, about $\log_{10}$ 3 cfu g^{-1}, these LAB were able to increase to $\log_{10}$ 5 cfu g^{-1} after incubation at 30°C for 96 days. It resulted in successful fermentation using glucose as source of carbohydrate, but not cooked rice, possibly bacteria associated with the New Zealand fish supply chain to retail and beyond are unable to hydrolyse gelatinized starch to fermentable glucose. After glucose, physiochemical changes in pH, colour, fat oxidation,

[6] LAB will be on hands, domestic equipment and the flour itself.

and texture depended on the fish species.

Now consider a traditional European salami, which is fermented chopped meat and fat. The process is simple. Chopped meat and fat are mixed with about 2% salt and 1% glucose or similar sugar. The mixture is extruded into a casing that excludes most air, and the mixture ferments at ambient or moderately warmer temperatures. Just as for the African examples above, the microorganisms responsible are the LAB and others that are present on hands and processing equipment, producing lactic acid from the sugar.

A fundamental problem with traditional fermentation methods is that, during the early phase of fermentation which is associated with the lag phase of microbial growth, contaminating microorganisms on raw materials, utensils and from the environment slowly increase in number and compete for nutrients to produce metabolites. The lower initial number and slow growth of naturally occurring LAB, allows the other wild microorganisms to grow and dominate, resulting in long periods of fermentation. Human activity has been fostering this problem by intentionally adding a portion of previously fermented food to the new batch of raw materials (back slopping) to promote successful fermentation. The competing microorganisms are thus continually present.

These problems are eliminated in modern commercial lactic fermentations using a starter culture. In their modern expression, starter cultures usually contain more than one microorganism (Hansen, 2014) and details have been reported above. In summary, the LAB's role is production of lactic acid and bacteriocins, while the additional microorganisms – mostly coagulase-negative staphylococci – contribute to the sensory quality of fermented foods through the generation of flavour active compounds and their precursors, such as amino acids, free fatty acids, aldehydes, amines, ketones, and alcohols.

The research to date on *Perna* has been aimed at developing a microbiologically stable value-added product from comminuted mussel meat for national and international markets. The concept was market-led in the knowledge that lactic-fermented foods are well accepted in Asia (Yoshida, 1998). However, when raw mussels were lactic fermented with a commercial culture in the presence of 2% salt and 2% glucose, the outcome was a stinking sulphurous slurry, although the pH did fall indicating that the LAB were active among other the metabolic activities (Figure 8).

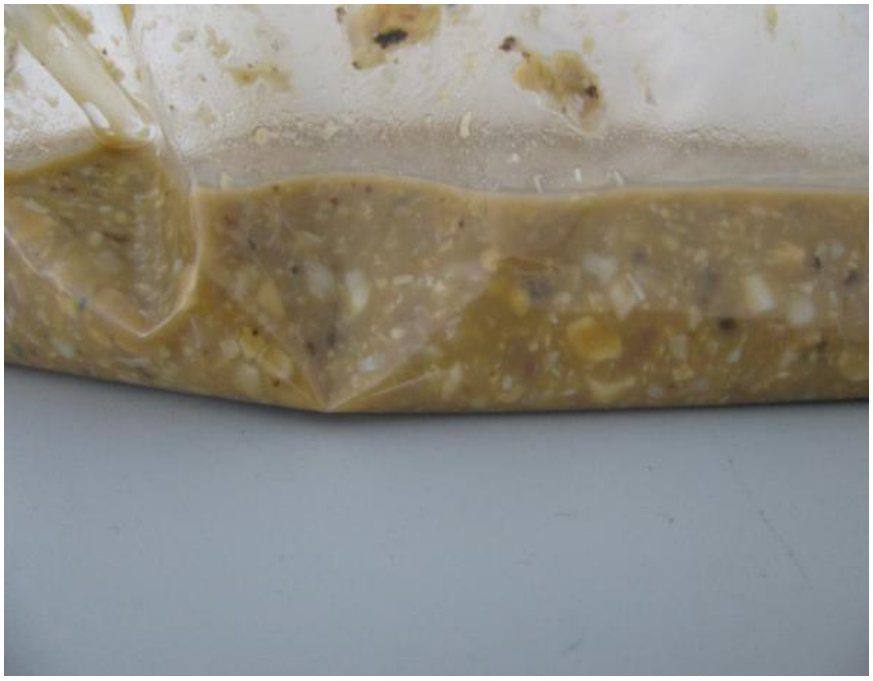


Figure 8 Fermented mussel mince with uncooked mussel packed in a vacuum barrier bag. Source: Qiang (2012).

It was quickly proposed that the cause of the liquefaction was due to the prolific microflora of the mussel gut, and/or the digestive enzymes of the same gut. The solution was obvious (Dsa, 2013). Cooking before fermentation destroyed the microflora (but not spores) and denatured the enzymes. Moreover, the meat was greatly easier to access from a cooked mussel shell. Although good results were not immediately obtained (Figure 9), success was finally achieved by vacuum packing the container and its surrounding vacuum bag prior to fermentative incubation at 30°C for an arbitrarily-chosen four days (Figure 10).

Figure 9 A dense growth of moulds on the surface of the fermented mussel mince. Source: Dsa (2013).

Figure 10 A packaged container with fermented mussel mince in a format intended for consumer appeal. In the present study, the degree of comminution was similar to that of a coarse pâté to facilitate good replication, Source: Dsa (2013).

Scope of the study

Consider now some variables of interest in the characterization of *Perna*. It might be assumed that the physicochemical properties of *Perna* will not change throughout a year because one green shell mussel looks very much like another. However, the mussel has a life cycle that affects the morphology of the animal within the shell. The endemic New Zealand sea urchin *Evechinus chloroticus* similarly shows seasonal changes in biochemical composition(Verachia et al., 2012) and sensory quality (Phillips et al., 2010), changes that are likely to occur in many temperate climate species. Variations in physicochemical and consequently nutritional components of *Perna* and understanding the magnitude of these variations are important in defining a commercial product.

Another outcome of the trials with different starter cultures also demands attention. Food-fermenting LAB are generally considered to be non-toxic and non-pathogenic, but some species of LAB can produce biogenic amines. The use of starter cultures with amine oxidase activity such as *Staphylococcus carnosus* and *Bacillus amyloliquefaciens* in fish sauce fermentation (Zaman, Bakar, Jinap, & Bakar, 2011) and in Chr.-Hansen starter cultures SM-194 and SM-181 (with five and three active bacterial species respectively) have been reported to be effective in reducing biogenic amines accumulation (Wang et al., 2015). Thus, as part of the screening process biogenic amines needed to be monitored.

The three most important activities of these starter cultures are conversion of lactose into lactic acid by glycolysis, hydrolysis of protein chains into peptides and amino acids (proteolysis), hydrolysis of fatty acids (lipolysis) and subsequent metabolism into keto-acids, ketones and various esters many of which are responsible for odor and flavour.

First consider is proteolysis. Starting with Chr Hansen's BFL-F02, peptides and amino acids needed to be monitored in water extracts. After high gravity centrifugation, and very finely filtering the following assays were done: colorimetric ultraviolet light absorbance indicating concentration of aromatic amino acids (and nucleic acids); soluble protein by a colourimetric method involving reduction of copper ions in alkaline solution; and finally gas chromatographic analysis of volatiles are important. This last assay allows inspection of amino acid and peptide profiles that are likely to be important in flavor.

Another assay involving protein is the simple formaldehyde titration method for amino nitrogen, where 'amino nitrogen' refers to α-amino acids, and the overwhelming dominant amino groups in food. The concentration of α-amino nitrogen is often used as the quality indicator for fish sauce products (Yokotsuka, 1986) and is an indicator of protein hydrolysis.

The higher the value, the better the quality. There was no idea of what the results was going to be in *Perna*, but significant concentrations were expected because of proteolysis due to adjunct bacterial activity.

Fat, or, more importantly, its component fatty acids, was of interest for several reasons. Fats are prone to two types of spoilage. Both are termed rancidity, but the causes and the results are different. The first is fatty acid oxidation. Of the approximately 2% fat in mussels, about one-third is comprised of unsaturated fatty acids dominated by the omega-3 polyunsaturated class, of which eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are of special interest from a dietary perspective (K. J. Murphy et al., 2002). Like all polyunsaturated fatty acids, they are very prone to oxidation, which at the very least generates off-flavours from oxidation products such as aldehydes and ketones.

After extraction of total fat by the chloroform-based Bligh and Dyer (1959) method, the fatty acid profile usually determined after conventional methyl derivatisation using a gas chromatograph. Experimentally, the profile was tracked through *Perna* preparation and subsequent long-term storage. Any significant and thus deleterious oxidation would be a fall in the proportion of EPA and DHA.

Another way of determining oxidation has been by solid phase micro extraction (SPME) of the headspace above extracted fat. In outline, volatiles in the headspace are bound to an absorbent fiber, from which the volatiles are later heat-desorbed in the injection port of a gas chromatograph. Volatiles are subsequently determined by elution times and mass spectrometry. As for the fatty acid profile, the changes with time is the important issue.

Another parameter of great interest is the effects of storage temperature post fermentation. *Perna* is routinely stored at refrigeration temperatures, but limited trials have previously shown that the *Perna* can be equally well stored at ambient temperatures. Ambient storage possibilities are particularly important from a commercial perspective because maintaining an unvarying cold chain from post fermentation to consumption is costly and prone to failure. Therefore, the effects of storage temperature were important to study.

Although green shell mussel are grown in clean coastal waters as required by food safety standards (Authority, 2006), the potential for health risk remains, along with the associated loss of reputation and costs of a product recall due to pathogenic bacterial contamination. Contamination of green shell mussel has been linked to land use and flooding, sewage contamination of ocean water, and microbial cross-contamination during processing (Aquaculture, 2012). These threats are mitigated to a great degree by blanching/cooking before freezing and then cooking a second time before eating (Gupta, Farid, Fletcher, &

Melton, 2015).

The alternative to cooking and subsequent freezing as means of preservation of green shell mussel is biological preservation by lactic acid fermentation, as discussed in some detail above. In this study, the microbiological status of *Perna* products throughout the entire production and storage events has been done according to New Zealand microbiological criteria set by the New Zealand Food Safety Authority to confirm or otherwise that *Perna* met safety standards.

A lactic culture suited to raw meat fermentation (BFL-F02) was chosen for *Perna* work simply because its use was suggested by Chr.-Hansen Ltd., the world's leading culture supplier, and although the results are largely acceptable, the effects of other cultures were completely unknown, and may be superior, or inferior, to BFL-F02 in flavour acceptability. For example, a BFL-F02 starter culture can ferment to a pH just below 4.0, which some consumers have described as 'too sour' in *Perna*. Chr.-Hansen's catalogue of starter cultures for fermented meat (Hansen, 2014) lists about five different cultures comprising a range of lactic acid bacteria, micrococci species and coagulase-negative staphylococci, and representative examples of these were tested in *Perna* production. Some LAB can produce bacteriocins, which can inhibit even the stubborn pathogens like *Listeria monocytogenes*. Some LAB may be undesirable due to their ability to generate biogenic amines; others can prevent accumulation of biogenic amines generated by other microorganisms (Alvarez & Moreno-Arribas, 2014) by degrading it.

The extent of pH fall, which is governed by the availability of carbohydrate, by the resilience of the microbes to lower pH, and lactic acid itself, needed investigation. The ability of starter cultures to drop pH quickly was also important. Rapid pH fall can prevent growth of the anaerobic, spore forming pathogenic bacteria, *Clostridium* spp., which is likely to grow in environment of evacuated seafood products.

The research questions

All the above can be summarized in two high level research questions:

1. How are the physiochemical and microbiological properties of *Perna* affected by storage time, storage temperature, and how do they vary with the annual cycle of mussel harvest?
2. How do different lactic acid bacteria cultures affect the basic physicochemical, microbiological and sensory properties of *Perna*?

Significance of the study

The study provided detailed physicochemical and microbiological insights into what is a completely new food product. There is no prior knowledge regarding lactic-fermented molluscs, and a new body of knowledge has been generated. At a commercial level, the study has identified the best cultures for use in production and has been shown, why they are the best in physicochemical and microbiological terms.

Specific objectives of the study

Objective 1 (Chapter 3)

Determination of *Perna*'s physicochemical and microbiological characteristics due to annual variation in mussel quality, and to long-term refrigerated storage. For each trial, three batches of *Perna* were prepared, monitoring for colour, pH, male mussel proportion and basic microbiology.

Objective 2 (Chapter 4)

Determination of amino nitrogen, UV absorbance of peptides and soluble protein and basic microbiological profiles of *Perna* stored at chill (4°C), ambient (between 19 and 23°C) and higher ambient (35°C) temperatures. The mussels were fermented with a single lactic acid culture.

Objective 3 (Chapter 5)

Determination of fatty acid profile of *Perna* stored at 4°C, ambient and 35°C temperatures, fermented with a single lactic acid culture.

Objective 4 (Chapter 6)

Determination of effect of culture, storage day and temperature on volatiles compound profile by using five starter cultures. Also, an investigation of the effect of glucose concentration on *Perna* pH and volatiles using one culture only.

Objective 5 (Chapter 7)

Determination of microbiological characteristic of *Perna* prepared using one culture stored at 4°C, ambient and 35°C. Also, determination of the basic microbiology of *Perna* prepared using five starter cultures.

Objective 6 (Chapter 8)

Investigation of the ability of five starter cultures used to prepare *Perna* to inhibit *Listeria monocytogenes* and *Clostridium* species.

Chapter 2

Materials and methods

Experimental details

Routine preparation of *Perna*

Live mussels in shells were routinely bought from one supermarket, PAK'nSAVE, Wairau, Auckland, New Zealand. One kilogram lots were cooked from cold in 1500 mL of boiling water for 7 minutes, by which time all shells were open. The resulting mussel meat, free of beard (byssus) and representing between 20 and 25% of the original mussel mass, was minced twice through a hygienically prepared 4-mm plate mincer assembly on a Kenwood mincer (KM 200, Kenwood, U.K.). Subsequently, for each 100 g of mince, 2 g of glucose monohydrate (Thermo Fisher), 2 g of NaCl, 1 g of iota carrageenan (Gelcarin GP 379, FMC BioPolymer, USA), and 2 mL water added to the carrageenan was blended into the mince for 5 minutes using the mixer tool. To this mixture was added 10 mg of starter culture (BFL-F02, Chr. Hansen) dispersed in 1 mL of water and mixed for a further minute. This culture comprises *Pediococcus pentosaceus* and *Staphylococcus carnosus*. Then 70 grams of prepared mince was then filled into 140 x 210 mm with barrier bags, 70 µm-thick (Dunningham, New Zealand), which were evacuated in vacuum packer (DZ400/2D, Hongzhan, Zhejiang, China), and hermetically sealed. The bags were incubated for 96 hours at 30°C followed by refrigerated storage as default but at other temperatures too. Attempts to find the gas transmission properties of the bags were fruitless.

Long term storage trials and seasonal changes in mussels

Determination of *Perna*'s physicochemical and microbiological characteristics on long-term refrigerated storage was explored, as well as the effect of annual variation in mussel condition. The mussels were bought every three weeks from the single supermarket between 1 August 2014 and 6 July 2015 and fermented with a single lactic acid culture (BFL-F02) obtained from Chr. Hansen in three deliveries.

Methods of analysis used were standards methods; some methods were modified depending on laboratory conditions in order to utilize tools commonly available in the laboratories. Some published work of similar food product, their suitable modified standard methods for were also opted to be used. Fifteen trials were performed and for each trial *Perna*

was monitored for pH, colour, proteinaceous matter, and microbiological profile. The logic for monitoring these four major attributes is now described. During fermentation microorganisms metabolize available carbohydrate and lower the pH (Pérez-Alvarez, Sayas-Barberá, Fernández-López, & Aranda-Catalá, 1999). The extent of pH fall is governed by the availability of carbohydrate, which was not limiting in these experiments, and by the resistance of the microbes to lower pH values, lactic acid itself, temperature, and other factors (Gaenzle, 2015).

The colour of food is one of the major attributes affecting consumer acceptability. *Perna* colour was measured in L*, a*, b* space (CIE 1976). L*, a* and b* indicate lightness, redness/greenness, and yellowness/blueness, respectively. Lightness L* is the measurement of reflectance on a scale of 0 (no light reflectance) to 100 (complete light reflectance). Positive a* values indicate red while negative values indicate green. Positive b* values indicate yellow colour and negative indicate blue. As absolute a* and b* values increase, the colour becomes more intense.

Microbiology was also monitored for the last three of the 15 seasonal trials, but details of the techniques are not described until later in this chapter under the heading: Determination of microbiological characteristics and quality of *Perna*.

pH and colour of *Perna*

Determination of pH of Perna

The pH of the mussel was determined according to the destructive method of ISO 2917:1999 with modification as per Benjakul, Seymour, Morrissey, and AN (1997). A 2-g sample was blended using a T-25 Ultra-Turrax (Jankee & Kundel, Germany) with 18 mL of deionised water, and the pH was measured by glass electrode meter (Meterlab, PHM-201, UK.)

Determination of Perna *colour*

Colour was determined in L*, a*, b* space with a Hunter meter (Model 45/0 Hunterlab ColourFlex, Virginia, USA.) Plastic bag containing the *Perna* was placed over the Hunter meter light source and the entire assembly covered with a black cloth. Three readings of L*, a*, b* were recorded at different areas of the bag. An empty barrier bag was also measured in triplicate for blank values. The mean blank value was subtracted from each experimental datum before statistical analysis.

Amino nitrogen, UV absorbance of peptides and soluble protein of *Perna*

Proteins are usually tasteless (Solms, 1969) but peptides and amino acids are well known for their often sweet, bitter or savoury flavour (Belitz & Grosch, 1999) and important for the flavour profile. Protein can be hydrolysed by endogenous mussel proteases that may survive the cooking step, by culture and opportunist microbes (L. Wang, Tian, Gyawali, & Lin, 2013) and by the acidity generated in fermentation and subsequent storage.

Four methods were used to quantify or semi quantify *Perna* proteins, peptides, and particularly amino acids.

Amino acids tryptophan, tyrosine, and phenylalanine absorb strongly at wavelengths in the ultraviolet (UV), and because their contribution to the amino acid profile is a constant for one species of mussel, absorbances at representative wavelengths in the UV range are a guide to the extent of soluble proteins, peptides and amino acids.

A measure of amino nitrogen was used to determine amino groups that become chemically available due to protein hydrolysis. Amino nitrogen is an old method described by Wadsworth and Pangborn (1936) as more recently defined by Li, Kim, and Kang (2014) . In that assay, the pH of a water extract of *Perna* adjusted to pH 8.4 falls in response to the reaction of free amino groups with formaldehyde in a Schiff's reaction. The alkaline amino groups are blocked, and the pH falls in response to the free carboxyl groups. The formaldehyde-treated mixtures are back titrated with NaOH to pH 8.4. Because every amino group was matched by one carboxyl group on each α-amino acid, the titration is an indirect measure of amino groups and, therefore, amino nitrogen.

Another analysis method – the bicinchoninic acid method – extends the classic Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). This type of assay measures the concentration of peptide bonds, a measure soluble proteins and peptides, but not free amino acids.

The fourth method aimed to identify exactly what amino acids were in the aqueous extracts using liquid chromatography-mass spectrometry (LCMS). This method is also capable of determining biogenic amines. Food-fermenting lactic acid bacteria (LAB) are generally considered to be non-toxic and non-pathogenic, but some species of LAB can produce biogenic amines (Spano et al., 2010), which are toxic in high concentrations(Smith, Kenney, Kastner, & Moore, 1993; Stratton, Hutkins, & Taylor, 1991; S. L. Taylor & Organization, 1985).

Details of the above four assays are described in sections below.

Determination of amino nitrogen in *Perna* by the formaldehyde titration method

Amino nitrogen was measured according to Li et al. (2014) with minor modifications. Two grams of each sample was mixed with 50 mL of distilled water and dispersed using a T-25 Ultra-Turrax. The filtrate (25 mL) was added to 25 mL of 35% (w/w) formaldehyde (Thermo Fisher Scientific) previously adjusted to pH 8.4 with 0.1 M NaOH, plus 30 ml of distilled water. The back titration with NaOH was done until the pH of the solution returned to 8.4. In a blank titration, 25 mL of water was used instead of formaldehyde. The concentration of amino nitrogen was calculated using the following equation:

Amino nitrogen (mg/g) = (A-B) (mL) x 280/S (g)

where A is the volume in mL of 0.1 M NaOH solution used in titration in the experiment; B is the volume of the blank titration; the value of 280 includes the factor for only 25 mL being used of 50 mL, the molar mass of nitrogen (14 g $mole^{-1}$), and the concentration NaOH (0.1 M). S is the weight of the sample. The final dimensions of amino nitrogen are mg g^{-1}.

Extraction and determination of peptides by ultraviolet absorbance

Extraction of *Perna* samples was done according to Faithong and Benjakul (2014) with minor modifications. Exactly 2 g of comminuted *Perna* was mixed with distilled water (18 mL) and the mixture was dispersed at 10,000 rpm for 10 seconds with an Ultra-Turrax dispersing element. (This useful dispersal time was established in a pilot experiment.) The slurry was centrifuged at 10,000 gravities for 60 minutes at 4 °C using a Sorvall RC-5B refrigerated centrifuge to remove undissolved debris. A sample of the supernatant was recovered avoiding the fat layer and filtered through a 0.1 µm syringe filter (Millex-VV from Thermo Fisher). The filtrate was frozen in suitable aliquots, and later used for three of the four proteinaceous assays: UV absorbance, the bicinchoninic assay for peptide bonds, and liquid chromatography mass spectrometry (LCMS).

Ultraviolent absorbance method, however, is only applicable to proteins, peptides amino acids that contain tryptophan or tyrosine residues. Proteins usually show absorption maxima between 275 and 280 nm. The aromatic amino residues tyrosine, phenylalanine and tryptophan absorb at various wavelengths in the UV, so monitoring of *Perna* extract was done in wider range, between 200 and 400 nm in spectrophotometer (Ultraspec 3300, Amersham Bioscience, UK) using quartz cuvettes after a 1:10 dilution.

Determination of extracted protein by the bicinchoninic assay

Determination of soluble protein was performed using the Thermo Fisher Pierce Microplate BCA Protein Assay Kit. There were two steps in the BCA protein assay. Peptides containing three or more amino acid residues form a blue coloured chelate complex with Cu^{2+} in an alkaline environment containing sodium potassium tartrate. This is the Biuret reaction (Lowry et al., 1951). In the second step two molecules of bicinchoninic chelate with one Cu^{+} molecule resulting in an intense and stable purple colour measurable at 562 nm. Detection of protein concentration in microplate reader with 96 samples or standards in Nunc™ Edge 2.0 96 well plates (Thermo Fisher), following the defined procedure. A calibration curve was prepared with bovine serum albumin (BSA) in 0.9% saline and 0.05% sodium azide, with working range of 20 to 2000µg mL^{-1}. Data are expressed as BSA equivalents.

Determination of extracted amino acids by liquid chromatography-mass spectrometry

Amino acid was determined as described by Salazar, Armenta, Cortés, and Shulaev (2012). The basis of this method is the reaction of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate with amino groups of amino acids (Figure 11). The AQC-amino acid complex is detected by mass spectrometry after liquid chromatography (LCMS).

Borate buffer comprising (200 mM) sodium tetraborate and 10% (v/v) acetonitrile was adjusted to pH 8.8 with HCl. Accutag reagent was made from 2.8 mg 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Waters, USA) in 1 mL of dry acetonitrile. Accutag solution was kept in 2-mL amber glass vials at room temperature. The neutralising solution was 1% (v/v) formic acid in water.

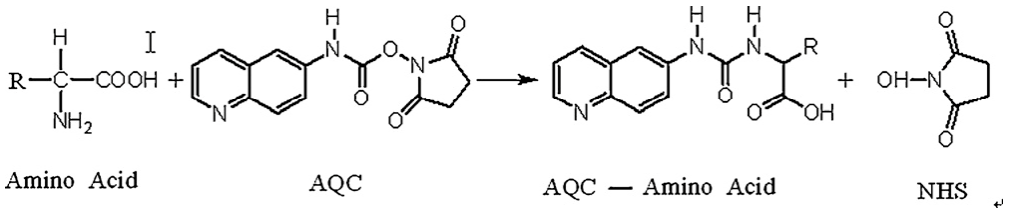


Figure 11 Derivatisation of an amino acid with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Water will also react with AQC in a competing reaction, but the amino reaction is much faster and can occur before AQC hydrolyses to useless products. Any primary or secondary amine will react in this way. NHS is N-hydroxysuccinimide. Source: Hou, He, Zhang, Xie, and Zhang (2009).

D4-Alanine was the internal standard. It met all requirements of good internal standard; well-resolved, with a stable isotopic label, and with a chemical relationship to analyte of interest.

The external standards were based on Sigma A9906, a mixture of 37 stable amino acids and other amines like urea, each at 500 µM. The protocol required initial dilution of a 20 µL aliquot with water to 100 µL. This initial dilution gave the opportunity to add aliquots of the relatively unstable asparagine and glutamine to the mix, as well as the biogenic amines tyramine, histamine and tryptamine (ThermoFisher), plus the internal standard d4-alanine. These were added as small volumes such that the now 42 external standards and d4-alanine were each at 100 µM. Serial dilutions were made to generate 100, 50, 25, 12.5 etc. using neutralizing solution.

For derivatisation, standards, samples and solvent blank (neutralizing solution) were deproteinised by cold (-20°C) acetonitrile and centrifugation at 4°C. The supernatant was used for derivatisation. The 15 µL of d4-alanine solution was spiked into 1485µL borate buffer. For samples and blanks, 10 µL of sample or blank (neutralizing solution) was mixed with 70 µL of spiked borate buffer and 10 µL Accutag reagent. The mixtures were vortexed and incubated at 55°C for 15 minutes, and finally centrifuged at 10,000 gravities for 5 minutes at 4°C. Then, 50 µL of supernatant from each reaction mixture was neutralized by 450 µL of neutralizing solution, in 1.8 mL autosampler vials (Supelco Co., Bellefonte, USA). The final concentration of internal standard was 0.0389 mgL^{-1} in the vials. The vials were caped stored at -20°C until analysed.

The LC-MS/MS system included an Agilent 1260 Infinity Quaternary LC System (Agilent, Santa Clara, USA) connected to and Agilent 6420 triple quadrupole mass spectrometer with multimode ionization source (model G1978B) operating in negative electrospray ionization mode. Mobile phase A was 1% formic acid in ultrapure water and B was 0.1% formic in acetonitrile. The column was a Waters Cortecs C18 2.7 µm particle size, 2.1 × 100 mm (Waters, Ireland). The total run times were 31.0 min. The autosampler temperature was maintained at 4°C and injection volume was 10 µL. The multimode ionization (MMI) source parameter for temperature was 300°C. Other parameters were: atmospheric pressure chemical ionization heater at 200°C, gas flow of 6 L min^{-1}, nebulizer pressure of 60 psi, and capillary voltage of 2,500 volts. The 10 µL of samples were injected in triplicate. Amino acids were detected using the most common method for quantitation of analyte by LC/MS/MS; the multiple reaction monitoring transitions were optimized using Agilent Mass Hunter Optimizer software. Data collection was done with Agilent Mass Hunter

Acquisition software. After correcting for injection volume variations with the internal standard responses, the calibration curves (Appendix I and Appendix II) were used in a spreadsheet exercise to calculate concentrations of each analyte in each injection volume. The relationship of the injection volume to the initial extraction volume (18 mL water + 2 mL of *Perna*) allowed the data to be finally expressed µmole of analyte per gram of *Perna*.

Fatty acid profile of *Perna* fermented with a single lactic acid culture

As the dominant triacylglycerols and other classes like phospholipids, the long chain polyunsaturated fatty acids (PUFAs) in New Zealand green mussel oils make more than half the fat in extracts (M. Zhou, Balaban, Gupta, & Fletcher, 2014). PUFAs are highly susceptible to oxidation ultimately generating mainly malodorous aldehydes, ketones and alcohols that are typically the cause of unpleasant fishy flavors. At the same time fatty acid ester hydrolysis can yield free fatty acids, another index of fat deterioration. For *Perna* some question was these: does fermentation under anaerobic conditions stabilize the PUFAs?; what is the effect of temperature for longer term storage?

Three batches of *Perna* were made using the same standard procedure. The fatty acid profile and acid value of *Perna* product was monitored for the three independent trials at Day 0, then after 4 days fermentation at 30°C and then at Days 11, 18, 46 and 74 when stored at 4°C, ambient (19-23°C) and 35°C.

The total fat in *Perna* sample was extracted by the chloroform-based Bligh and Dyer (1959) method. This method isolates nonpolar fats such as glycerides (primarily triacylglycerol) and cholesterol, as well as free fatty acids and other more polar fats. The key to this assay is the ratio of wet tissue (in this case *Perna*) to a 1: 2 (v/v) $CHCl_3$: MeOH extraction solvent. The fats ultimately reside in the lower (denser) chloroform phase that was recovered. The chloroform is evaporated leaving a dry fat residue. The amount of fat recovered per gram of *Perna* was of no interest. The objective was to determine fatty acid profile after methyl esterification and hydrolytic fat rancidity.

To methyl esterify, duplicate aliquots of fat around 23 mg were added to 3 mL of 5% Sulphuric acid solution in 1: 1 methanol: toluene in glass tubes with Teflon-lined caps. The tubes were heated at 100°C for 1 h. After cooling, 5 mL of saturated NaCl solution was added and the mixture vortexed to encourage phase separation. The upper toluene layer was recovered, and the residue was reextracted with 1 mL of toluene to recover fatty acid methyl esters (FAMEs). FAMEs were separated and analysed on a Phenomenex FAME-WAX capillary (0.25 mm x 0.32 µm x 30 m) fitted to a Shimadzu GC-2010 plus GLC (Japan) with

flame ionization detection after auto injection of 1 µL in split less mode. Initial oven temperature was 40°C, increasing to 140°C at 50°C per minute, held there for 5 min, then increasing to 245°C at a rate of 3.5°C per minute and held there for 3 min. A Supelco (USA) 37 component FAME Mix was used to identify fatty acid methyl esters.

Determination of flavor compounds in *Perna*

Flavour compounds were monitored by the solid phase microextraction (SPME) method according to Tuckey *et al.* (2013) with minor modifications made after optimization trials. Optimization of method was done using divinylbenzene (DVB), carboxen (CAR), polydimethylsiloxane (PDMS) fibre coatings, ultimately settling on a combination DVB/CAR/PDMS fibre coating. A DVB/CAR/PDMS fibre coating absorbed the greatest number of volatile, with high responses. The fibre used was thus Supelco (57329-U). This is a common choice where the volatiles are a mixture of polar and non-polar materials (Boyce & Spickett, 2002). Optimization trials to *Perna* volatiles showed the following. Cylindrical vial volumes of 10 mL and 20 mL made no different to peak size. The 10 mL vial was chosen with a 2-g sample placed on the base. This combination allowed full fibre exposure without contacting the sample and generated large reproducible peaks after exposure at 40°C for 10 minutes. No internal standard was included because of the heterogeneity inherent in *Perna*. An alkane series was used to calibrate retention times.

Perna samples were weighed to 2.0 ± 0.1 g, evenly spread on the base of 10 mL flat bottom vials fitted with PFTE septum beneath a magnetic crimp cap (Gerstel, Linthicum, USA), and placed in a 32-place tray (VT32, Gerstel). Using the multi-purpose sampler, each 10 mL vial was sequentially moved to an agitated heating block set to rotation speed of 250 rpm, for exposure at 40°C. The fibre was exposed to the sample headspace for 10 minutes. The fibre was subsequently inserted into the chromatograph's injection port and held for 25 minutes that released all volatile compounds and avoided carryover of volatile compounds from the previous exposure. An Agilent 7890 mass spectrometer (model 5977B MSD) was linked to the Trace GC Ultra (Thermo Scientific), fitted with a Zebron - Guardian capillary column, ZB-1701 (Phenomenex); 30 m + 5 m guard column, 0.25 mm internal diameter, 0.15 µm film thickness. To resolve the peaks, the initial temperature of the oven was set at 40°C, then increased at 5°C min^{-1} until reaching 140°C, which was maintained for 5 min then increased again at 3.55°C min^{-1} to 245°C and held for another 3 min. The detector was set at 250°C. The mass spectrometer was operated at 70eV in the electron ionization mode with a mass range of 48 to 400 m z^{-1} at 3.4 scans s^{-1}.

Identification of compounds was done with Agilent Mass Hunter Workstation software instrument was used for data acquisition and qualitative data analysis and identified was done according to NIST/EPA/NIH mass spectral library (Kramida, Ralchenko, & Reader, 2014).

Effect of glucose concentration on *Perna* pH

The ability of starter cultures and varying glucose concentrations to lower pH was investigated in this study. After comminution and subdivision, batches of *Perna* were conventionally prepared with different five glucose concentrations, 0.25, 0.5, 0.75, 1, and 2%, fermented for four days at 30°C, and subsequently stored at 4° and ambient temperature for 46 and 74 days after preparation and recorded the pH change. Volatiles for samples in each glucose concentration were monitored by SPME as explained above.

Determination of microbiological characteristics of *Perna*

Common pathogenic bacteria in seafood can be placed into three general groups (Calo-Mata et al., 2008; Mejlholm et al., 2008) (1) Bacteria that belong to the natural microflora of fish, such as *Clostridium* botulinum, pathogenic *Vibrio* spp., *Aeromonas*hydrophila; (2) Enteric bacteria from faecal and/or environmental contamination, such as *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*; and (3) bacterial contaminants during processing, storage, or preparation for consumption, (such as *Bacillus cereus*, *Listeria monocytogenes*, *S. aureus*, *Clostridium perfringens, C. botulinum, Salmonella* spp.). The microbial investigations of *Perna* was based on the general requirements in New Zealand Microbiological Reference Criteria, section 5.26 SEAFOODS, part (c) Shellfish - processed, requiring no further cooking (Food Administration Manual, (1995) and Food Standards Australia New Zealand (FSANZ) (2001), using American Public Health Association (APHA) (2001) methods (Table 3).

Methods for microbiological analysis of *Perna*

Determination of microbiological safety was done as indicated in Table 3. Ten gram of comminuted sample of *Perna* was added to 90 mL of 0.1% peptone (Thermo Fisher) in a sterile plastic stomaching bag (Thermo Fisher Scientific, New Zealand). The sample was then stomached for 2 minutes. The sample was then serially diluted in 0.1% peptone and used for the following microbiological analysis.

Determination of total aerobic plate count

To determine the total aerobic plate count of *Perna*, the dilutions were plated on

Aerobic plate count (APC) agar (Oxoid) using the pour plate method (APHA, (2001). Plates were incubated at 37ºC for 48 h, after which colonies was counted.

Table 3 Representative sampling and interpretation of data according to Microbiological Reference Criteria (1995).

Microorganism	n	c	m	M
Aerobic plate count at 35°C (/g)	5	2	10^4	10^5
Coagulase producing *Staphylococcus* (/g)	5	2	10^2	10^3
Faecal coliform(/g)	5	2	10	10^2
Listeria monocytogenes (/25 g)	5	0	0	
Salmonella (/25 g)	5	0	0	
Vibrio parahaemolyticus (/ g)	5	2	10^2	10^3

Where:

n = the number of sample units which must be examined from a lot of food to satisfy the requirements of a sampling plan

c = the maximum allowable number of defective sample units. When more than this number are found, the lot is rejected by the sampling plan

m = represents an acceptable level and values (CFU/g) above it are marginally acceptable or unacceptable in the terms of the sampling plan

M = a microbiological criterion which separates marginally acceptable quality (CFU/g) from defective quality. Values above M (CFU/g) are unacceptable in terms of the sampling plan and detection of one or more sampling exceeding this level would be cause for rejection of the lot.

Determination of faecal coliforms

The faecal coliforms and *Escherichia coli* were determined by the most probable number (MPN) method, divided into three steps: presumptive MPN test for faecal coliforms and *E. coli*; confirmed MPN test for faecal coliforms and *E. coli* and; completed MPN test for *E. coli*.

The presumptive MPN test for faecal coliforms and *E. coli* was performed as follows. The dilutions were used for a 5-replicate, 3-dilution MPN procedure for analysis of coliforms. In this procedure, 1 mL of three consecutive dilutions from the prepared dilutions were inoculated into five replicate 9 mL volumes of selective enrichment broth, lauryl tryptose broth (LST) (Thermo Scientific, Oxoid), and incubated at 35°C for 24 hours then examined for growth and gas production. Growth was shown by turbidity of tubes and gas production was measured by gas displacement in the inverted Durham tubes. Negative tubes were re-incubated after recording the results, for an additional 24 hours and re-examined.

Presumptive-positive tubes were tested for confirmation: presumptive tubes were sub-cultured to EC-MUG Medium (*E. coli* selective broth with 4-methylumbelliferyl-β-D-glucuronide) (Oxoid). Nine millilitres of EC-MUG medium were inoculated with 1 mL from presumptive tubes and incubated at 45°C for 24 h. All tubes that showed turbidity and gas production were confirmed for faecal coliforms.

Positive EC-MUG tubes were sub-cultured by streaking on L-EMB agar plates (Oxoid) and incubated aerobically for 20 h at 35°C. Plates were examined for typical nucleated, dark-centered colonies with or without a metallic sheen, which was indicative of *E. coli.*

MPN method for Staphylococcus aureus *and* Staphylococcus carnosus

An MPN procedure was used for determination of *Staphylococcus aureus* and *Staphylococcus carnosus* (APHA (2001). From each of three useful serial log dilutions, 1 mL was inoculated into five replicate tubes containing 9 mL of sterile trypticase soy broth (TSB) (Oxoid) supplemented with 10% NaCl (Thermo Fisher) and 1% of sodium pyruvate (Thermo Fisher) and incubated for 48 h at 37ºC. Tubes showing dense growth were streaked onto Baird Parker medium supplemented with egg yolk and incubated for 48 hours at 37°C. Two types of colony were found. Five replicates of each were isolated and streaked onto Baird Parker medium (Oxoid) supplemented with rabbit plasma fibrinogen (X086) (Oxoid) and incubated for 48 hours at 37°C. Black colonies surrounded by a zone of coagulase activity were considered positive for *S. aureus* (coagulase positive) and differentiated from coagulase-negative *S. carnosus.*

Determination of Vibrio parahaemolyticus

Dilutions were spread onto plates of thiosulphate citrate bile salts sucrose (TCBS) agar (Oxoid) and incubated for 24 h at 35°C. *V. parahaemolyticus* appears as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on this medium.

Determination of Pediococcus pentosaceus

Ten grams of *Perna* were stomached and serially diluted as for the two determinations above. Dilutions was plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid), incubated aerobically at 30ºC for 48 hours and the colonies counted.

Test for Listeria monocytogenes

Listeria monocytogenes in *Perna* was detected a presence/absence test was used. Twenty five grams of comminuted sample of *Perna* was added to 225 mL of primary

enrichment broth (Neogen Corporation, USA) in a sterile plastic stomaching bag and stomached for 2 min. The bag was then closed with some air trapped in the bag and incubated at 30°C for 24 h. One millilitre of the mixture was added to 10 mL of Fraser secondary enrichment broth (Neogen) and examined for blackening after 48 h at 35°C. A loopful of blackened Fraser culture was sub cultured by streaking onto triplicated plates of Modified Oxford medium (MOX) agar (Neogen) in a four-phase streak to obtain single colonies. Plates were incubated at 35°C for 24 h. MOX colonies were examined for morphology typical of *Listeria* spp. Suspect colonies were typically small (about 1 mm) and were surrounded by a zone of darkening due to esculin hydrolysis. If no suspect colonies were evident, the MOX plate was reincubated for an additional day. Purified isolates of suspected colonies were subjected to a *Listeria* API test system and the β-haemolysis test.

Test for Salmonella

Salmonella in *Perna* was also determined by a presence/absence test. Twenty-five grams of comminuted sample of *Perna* was added to 225 mL of lactose broth (Oxoid) in 250 mL bottle, sealed, mixed by shaking, held for one hour at ambient temperature, remixed then incubated at 37°C for 16 h. An aliquot (100 µL) of broth was transferred to 9 mL of Rapport-Vassiliadis (RVS) broth (Oxoid) at 42°C which was further incubated for 24 h. Tubes that showed turbidity were subculture to duplicate xylose, lysine, and desoxycholate (XLD) (Oxoid) agar plates and incubated at 37°C for 24 h. Pink colonies with or without black centers, or completely black colonies were presumptive *Salmonella.*

Basic microbiology of Perna *prepared using five starter cultures*

Perna prepared using five starter culture were analysed for APC, pH and *Listeria monocytogenes* using methods explained above. After fermentation bags were distributed to two groups, one stored at 4°C temperature and the other at ambient for 74 days.

During fermentation, homofermentative LAB metabolize glucose through the Embden-Meyerhof-Parnas glycolytic pathway, producing lactic acid as main product, leading to a lowering of pH. pH is one of the major factors affecting the viability of microorganisms (Mathipa & Thantsha, 2015). Many pathogenic bacteria do not grow in low pH, but LAB and some *Staphylococcus* species can grow in acid conditions as well as some relatively stable bacteria like *Listeria*. The study included monitoring of pH and the microbiological profiles with a focus on *L. monocytogenes* and *Clostridium* pathogens.

During and after fermentation, lowering and low pH due to lactic acid accumulation and bacteriocins produced by LAB, both contribute to the inhibition of undesirable

microorganisms (Rhee, Lee, & Lee, 2011). *L. monocytogenes* however, can grow at low temperatures (a psychrotrophic bacterium), under both aerobic and anaerobic conditions and it can grow in a pH range between 4.5 and 9.0. *L. monocytogenes* is widespread in food processing facilities and can persist for long periods under adverse conditions (C. D. Cruz & Fletcher, 2011; Farber & Peterkin, 1991; Vogel, Huss, Ojeniyi, Ahrens, & Gram, 2001). The spores of *Clostridium* survive the processing and are activated by cooking, anaerobic conditions allow vegetative cells to grow to high levels and produce toxin that cause illness when ingested. Faster pH drops preventing pathogenic bacteria growth, at 28°C to 32°C (range commonly used in New Zealand), fermentation usually takes 16 to 24 hours. If it takes longer than 48 hours for the pH to drop below 5.2, or if the pH does not drop far enough, then there is a likelihood that pathogenic bacteria or toxins will still be present (MPI, 2017)

The input variables for this study were: the five cultures in Table 4, six glucose concentrations and two storage variables up to 74 days after preparation. Output variables were: pH, soluble amino acids and biogenic amine by LCMS; microbiological profile for APC and LAB as described above, volatiles by solid phase micro extraction (SPME); inhibition of *L. monocytogenes* by in situ and in vitro assays and comparing rate for LAB growth through reduction of pH. Details of assays for some of these variables have been described earlier, and others are described below.

Comparison of starter culture inhibitory capabilities on *Listeria monocytogenes*

In situ experiments

The five starter cultures in Table 4 were compared for their inhibitory capability with in situ assays.

The in situ experiments are summarized in Figure 12, with detailed descriptions following.

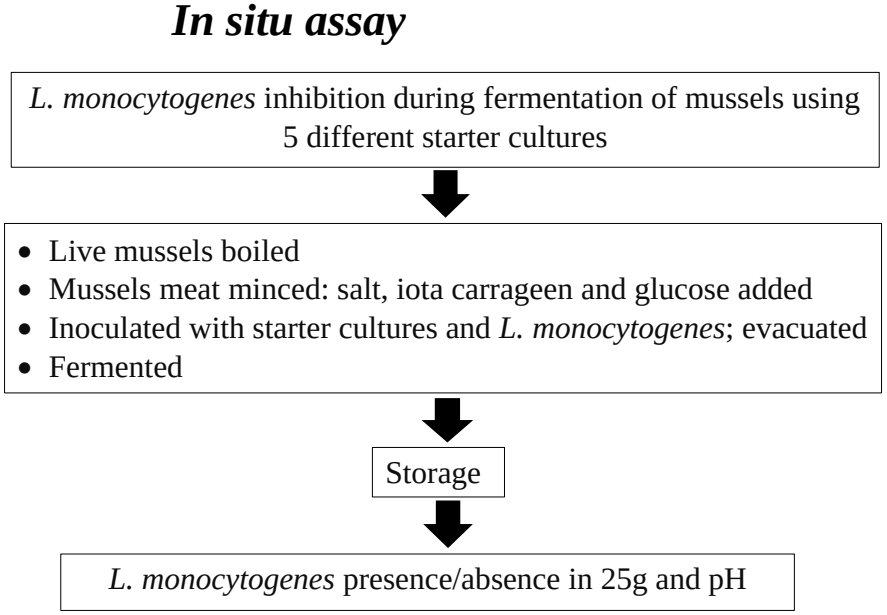


Figure 12 In situ assay for the inhibition capability of five starter cultures from Chr Hansen on *L. monocytogenes* in *Perna.*

For the in situ assay, *Perna* was conventionally prepared to the point of culture addition, then divided into five portions for culture inoculation (Table 4). Using standard safety protocols, the mixtures were then distributed in six 70 g aliquots into barrier bags, evacuated and sealed. Next, 1 mL of *Listeria monocytogenes* culture (around log 7 cfu mL^{-1}) was injected into each bag using 1 mL disposable syringes fitted with a narrow-gauge needle. The injected *Listeria* was distributed by massage. Each bag was then placed in a new barrier bag, the surface of which was wiped with 75% alcohol before evacuation and sealing. Then the samples were incubated at 30°C for 4 days.

After fermentation bags were distributed to two groups, one stored at ambient temperature and the other at 4°C. *Listeria monocytogenes* colonies were counted on Days 0,

4, 46 and 74 using Listeria Oxford agar supplemented with listeria selective supplement which is specific for *Listeria*. Suspect colonies were typically small (about 1 mm) and surrounded by a zone of darkening due to esculin hydrolysis. Plates with no suspect colonies were reincubated for an additional 26 h.

Table 4 Details of five starter cultures supplied by Hansen (2014).

Experimental code	Industrial code	Name of microorganisms
1	BFL-FO2	*Pediococcus pentosaceus*, *Staphylococcus carnosus* ssp.,
2	SM-194	*Pediococcus pentosaceus*, *Lactobacillus sakei*, *Staphylococcus xylosus*, *Staphylococcus carnosus* and *Debaryomyces hansenii*
3	F-LC	*Pediococcus acidilactici*, *Lactobacillus curvatus* and *Staphylococcus xylosus*
4	BFL-FO4	*Lactobacillus sakei*, *Staphylococcus carnosus* ssp.
5	T-SC-150	*Lactobacillus sakei*, *Staphylococcus carnosus*

The identity of *L. monocytogenes* was confirmed by sequencing 16s ribosomal DNA. Full length primers, 27F and 1492R primers and Sanger were used to sequence the product. DNA extraction: Colony was picked from culture plate and suspended in 100µl ultrapure water in Eppendorf tube. Eppendorf tube was microwaved for 45 seconds to crack the cells releasing the DNA and the centrifugation at 10,000rpm for 1 minute. Supernatants containing DNA were transferred to new Eppendorf tubes. Extracted DNA was suspended in Ultrapure water and then concentration in each sample adjusted to 5 ng uL^{-1} before amplification by PCR process. PCR was conducted with the primer set targeting the full length regions of bacterial 16S rRNA gene. The primer was set as follows: 1492r primer 5′-TACCTTGTTACGACTT and 27f-CM 5′-AGAGTTTGATCCTGGCTCAG. Master mixes contained 5 ng μL^{-1} of total DNA and 2X KAPA HiFi Hotstart Ready mix (Kapa Biosystems, Wilmington, MA, USA) and Amplicon PCR Forward primes 1 µM and Amplicon Polymerase chain reaction (PCR) reverse primes 1 µM primers with volumes of 2.5, 12.5 5 and 5 uL respectively. The thermocycles parameters were:

1. 95°C for 3 min,
2. 25 cycles of 95°C for 30 s, 55◦C for 30 s, ◦C for 30 s, 72◦C for 5 min, and
3. Holding the samples at 4°C.

The amplicons were then indexed using Nextera XT index kit (Illumina). PCR was then cleaned up with AmpureXP beads (Beckman-Coulter, Brea, CA, United States) at a 1:1 ratio. Amplicon was quantified with Qubit High Sensitivity. Sequencing was conducted with an Illumina MiSeq system (Illumina) with the 500 cycle V2chemistry at University of Auckland. A 5% PhiX spike-in was used, as per manufacturer's recommendation.

Sequenced data bioinformatics software tools were used to read the sequence. Sequencing reads were assembled using the Geneious (commercial software). The reverse complement of reverse sequence was obtained. Pairwise alignment of forward sequence and reverse sequence was done by aligning/assembling the forward sequence and reverse complement of reverse sequence. The highest quality portion of the full-length consensus sequence was taken. This best match was searched using either BLAST.

In vitro experiments

There were four in vitro assay experiments, summarised in Figure 13 with detailed descriptions following.

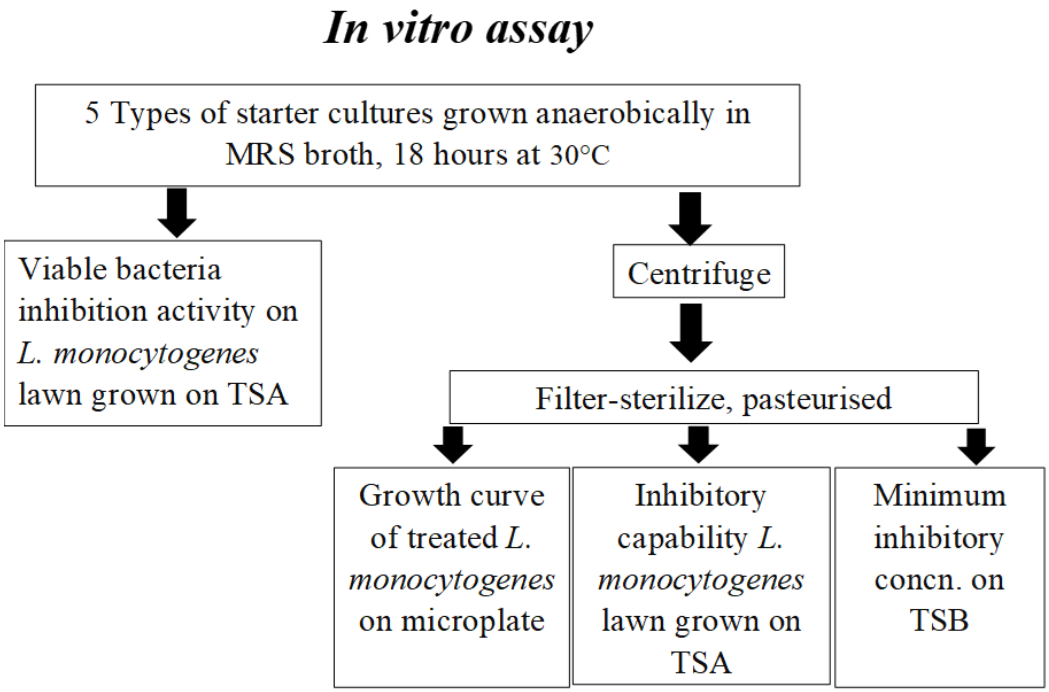


Figure 13 In vitro assays for the inhibition capability of five starter cultures from Chr Hansen on *L. monocytogenes* in *Perna*.

To start these, the five starter cultures were grown anaerobically in MRS broth (Thermo Fisher) for 18 hours at 30 °C and used for the following assays. Assay numbers are bolded to

link details with Chapter 8's method summaries (page 147).

1. To determine the inhibition activity of starter culture spots on a *Listeria* lawn, the bacterium at range of 10^4 to 10^7 cfu mL^{-1} (based on McFarland standards) was seeded at 1:100 dilution into soft tryptose soy agar (TSA) agar (0.75% agar); 5 mL of this seeded agar was overlaid normal TSA agar plates (1.5% agar). A 10 µL aliquot of each starter culture grown on MRS broth was spotted on triplicated seeded plates and incubated for 18 hours at 30°C. The inhibition zone around each starter culture spot was measured. Inhibition of starter culture supernatant grown in broth was determined as follows. After 18 hours of starter culture growth (see above), sample volumes were centrifuged at 1790 gravities for 15 min. The supernatant was filtered through Millex-VV syringe filter unit, a gamma sterilized 33 mm diameter sterile syringe filter with a 0.1 µm pore size hydrophilic polyvinylidene fluoride membrane. The supernatant was sterilized in a water bath at 80°C for 10 minutes. This sterilized supernatant was used in a microplate inhibition experiment in a lawn experiment, and in an experiment to determine minimum inhibition concentration of each starter culture.

2. To determine the inhibition activity of starter culture supernatants by turbidity, 100 µL of filter-sterilized supernatant of each culture was distributed in a sterile clear 96-well flat bottom micro plate (Thermo Fisher). *L. monocytogenes* was diluted by double strength TSB broth (10^8 to 10^4 cfu mL^{-1}), from which 100 µL were added on wells with filter-sterilized supernatant. The 96-well plate was sealed with clear sealing tape to prevent evaporation and well-to-well contamination. Settings for the growth curve/turbidity analysis using a using a microplate reader (BioTek Instruments, USA) were as follows: measurement mode absorbance; measurement wavelength 595 nm; temperature range 33 to 35°C; orbital shake 10 s; total measurement time 48 h. The absorbance values, which represented turbidly, were plotted against time.

3. To determine the inhibition activity of starter culture supernatants on a *Listeria monocytogenes* lawn, lawns as prepared above were spotted with 10 µL of supernatant incubated for 18 h at 35°C and monitored for inhibition zones.

4. To determine the minimum inhibition concentration of starter culture supernatants on *Listeria*, sterile filtered preparations were serially diluted with sterilized double strength TSB broth. A *Listeria* culture at 10^4 cfu mL^{-1} was inoculated (100 µL) into each dilution with sterile TSB as control. Tubes were incubated for 18 hours at 35 °C.

The growth was determined by turbid absorbance at 595 nm. The tubes with low absorbance were regarded to have high inhibitory concentration and the lowest concentration with no growth was regarded as the minimum inhibition concentration.

Acidification activity during fermentation to observe ability of starter cultures to inhibit *Clostridium botulinum*

The ability to form spores enables *C. botulinum* to survive a wide range of unfavorable conditions, such as heat and chemicals. The spores survive many heat processes that kill other pathogens in foods. In fact, certain types of *C. botulinum* spores are able to survive 5 to 10 hours in boiling water. In canned products, oxygen is excluded from sealed containers, thus providing the anaerobic environment for spores to germinate, become vegetative cells, multiply, and produce toxin when the foods are stored at temperatures that allow growth. It is important to recognize that it is not the spore that produces the toxin, but the vegetative cell. If spores are present, but are prevented from forming vegetative cells, as in acidified foods, the toxin will not be produced. It has been traditionally accepted that spores of *C. botulinum* will not germinate and grow at pH 4.8 or below (Wong, Young-Perkins, & Merson, 1988). In summary, *C. botulinum* is of great concern to home, commercial canners (USDA, (2007) and fermented foods (Chiou, Hennessy, Horn, Carter, & Butler, 2002; O'Mahony et al., 1990). The five starter cultures in Table 2 were compared for their rate for LAB growth through reduction of pH.

Perna was conventionally prepared to the point of culture addition, then divided into five portions for culture inoculation (Table 4). Samples for each culture type were subdivided into 10 gram in barrier bags (20 bags for ach starter culture type). Bags were evacuation and sealing. Incubated was at 30°C. From time 0 of incubation the pH was measured for interval of one hour for 17 hours and then the rate of pH decrease for each culture was observed and compared to other starter cultures.

Batches of *Perna* were prepared by different five starter cultures (Table 4) using the same method for *Perna* preparation explained previously. To test acidification activity of starter cultures, the pH was analysed by using pH meter, hourly during fermentation for 17 hours. Ability of starter cultures to drop pH quickly can prevent growth of dangerous anaerobic and spore former pathogenic bacteria, *C. botulinum* which are likely to grow in environment of vacuumed packaged seafood products. The faster acidification capability of starter culture was observed in this experiment.

Chapter 3

Seasonal changes in mussels and long-term storage trials

Introduction

There is a year-round demand for green shell mussel, nationally and internationally. Mussel quality changes with season due to spawning, and this phenomenon explained more clearly in Chapter 1. However, it was important to investigate quality of *Perna* in respect of consistency throughout a year.

Material and methods

The detailed methods are presented in Chapter 2. However, relevant to this work, live mussels in shells were routinely bought for every three weeks from the single supermarket between 1 August 2014 and 6 July 2015 and fermented with a single lactic acid culture (BFL-F02) obtained from Chr. Hansen in three deliveries. The mussels' source was unknown, the sea conditions were unknown, and the time between harvest and sale was also unknown. After fermentation for samples were stored in 4°C, ambient temperature and 35°C. Samples were analysed for change of pH, colour and microbial counts with season and storage. The effect of proportion of male mussel were observed.

Data visualization and statistical analysis

Various graphical and statistical methods were used to study the relationships between date of trial (15 of these), proportion of males by weight, storage time, and pH and colour. Routines with XLSTAT were used to explore these relationships were correlation, linear regression, analysis of variance (ANOVA) and analysis of covariance (ANCOVA). The hypotheses under test were: season will have marked effects; mussel pH on Day 0 should reflect pH at Day 4, pH should be static from Day 4 after the fermentation nominally stops; and the proportion of males should have a marked effect on colour.

Results

pH of *Perna* in different trials over one year

Season did not appear to have any clear effect on pH at Day 0 and later at Day 4 (Figure 14).

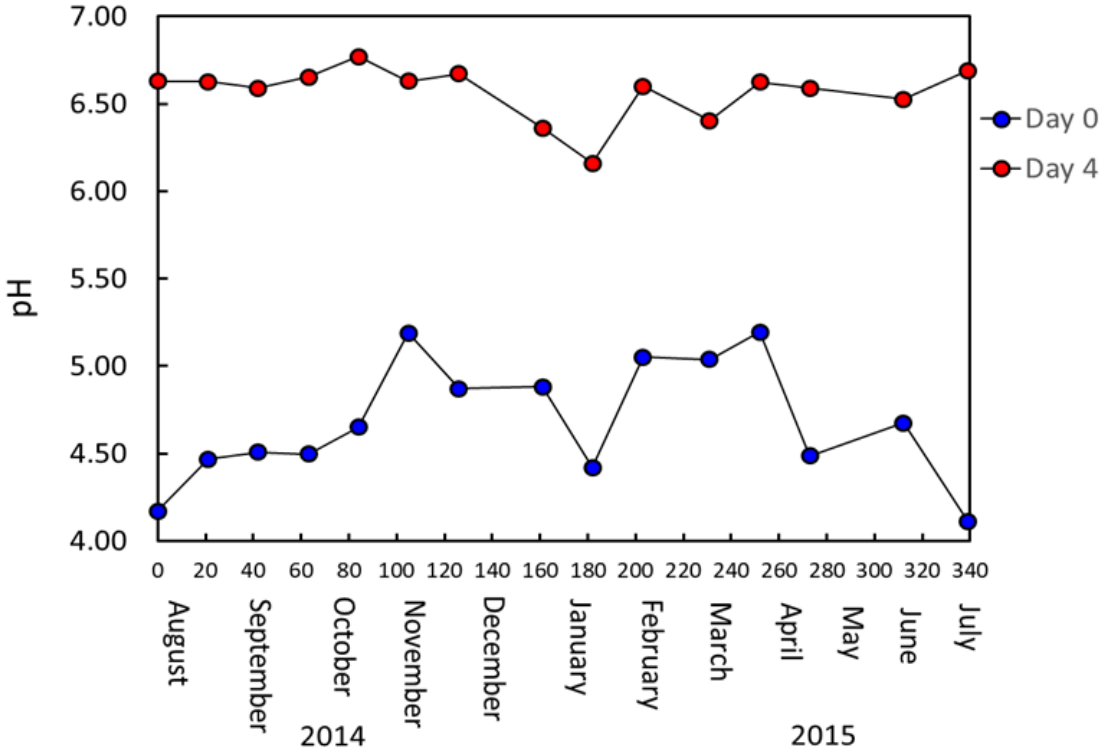


Figure 14 pH differences between Day 0 and Day 4 for 15 trials made throughout a year.

The trial with the lowest Day 4 pH was Trial 15 – July 2015 – pH 4.11 and was also the trial with the greatest pH difference between Day 0 and 4, 2.58 pH units. The trials with the highest Day 4 pH were Trials 6 – November 2014 and 12 – March/April 2015 – 5.19. The trial with the least pH difference was Trial 11. By inspection it can be concluded that season has no obvious effect on pH differences. The season hypothesis was not proven.

From inspection of Figure 15, the relationship between Day 0 and Day 4 pH was likely to be random, and this was confirmed by the correlation results (Figure 15, where the correlation was -0.061, P = 0.69. The hypothesis about pH on Day 0 and Day 4 was also not proven.

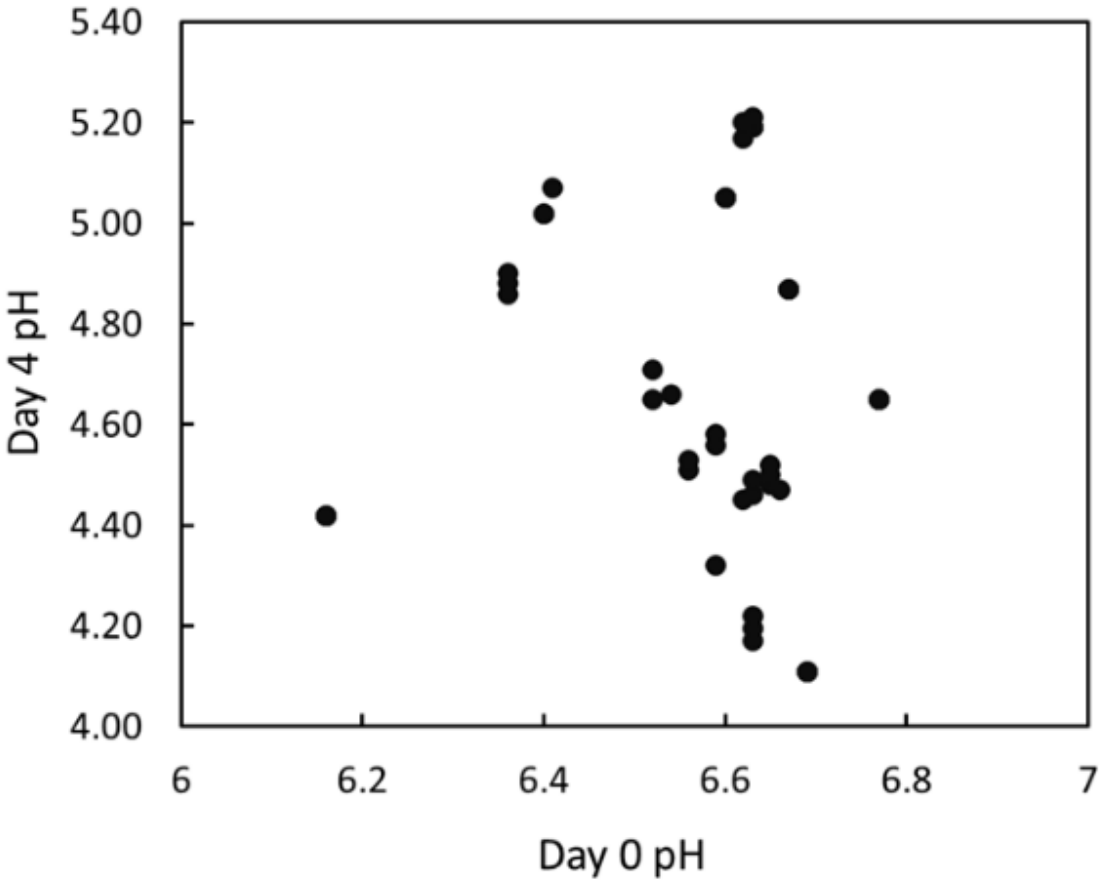


Figure 15 The scatter plot of correlation between pH at Day 0 and 4 showed the data were randomly scattered.

For all 15 preparations during the year of study, pH declined within the 96 hours of incubation at 30°C (Figure 16) then appeared to remain rather stable during 46 days of chill storage.

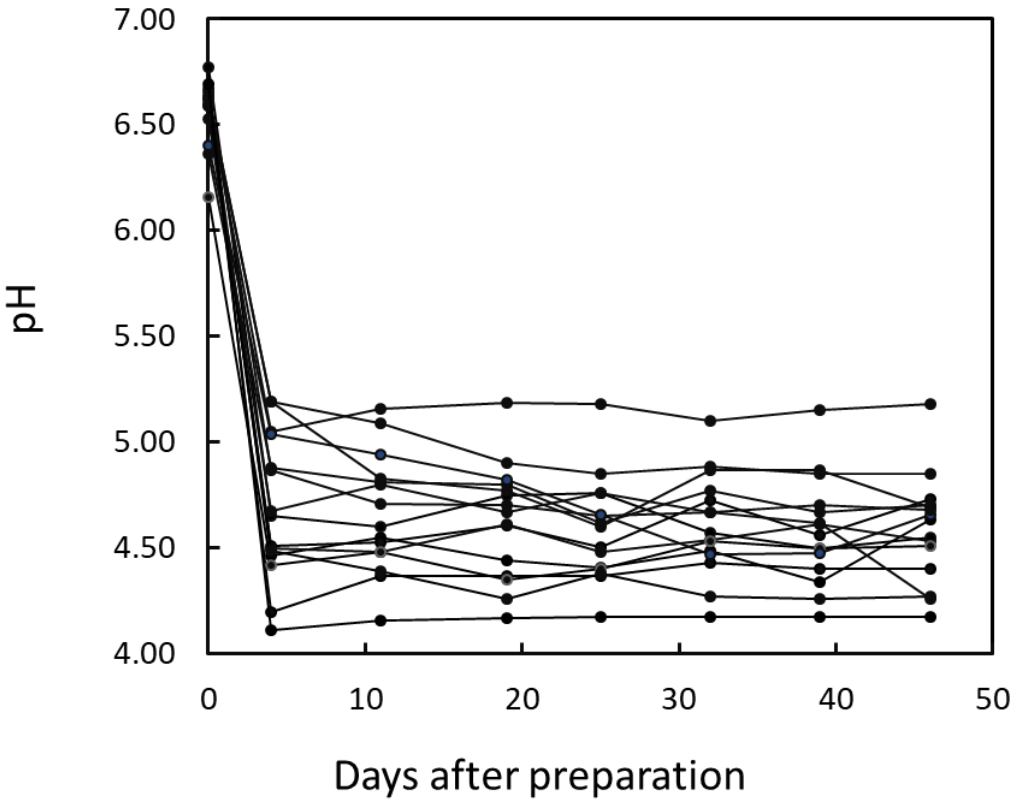


Figure 16 Change of pH in *Perna* preparations to 46 days in 15 trials. Indvidual trials are not identified.

Although inspection of Figure 16 suggests that pH is static over all trials from Day 4 onward, this was not the case when data were pooled. Linear regression (Figure 17) showed a subtle but steady significant decline in pH ($R^2 = 0.334$, $P = 0.033$) between the pH on Day 4 and Day 46. The static pH hypothesis was also not proven.

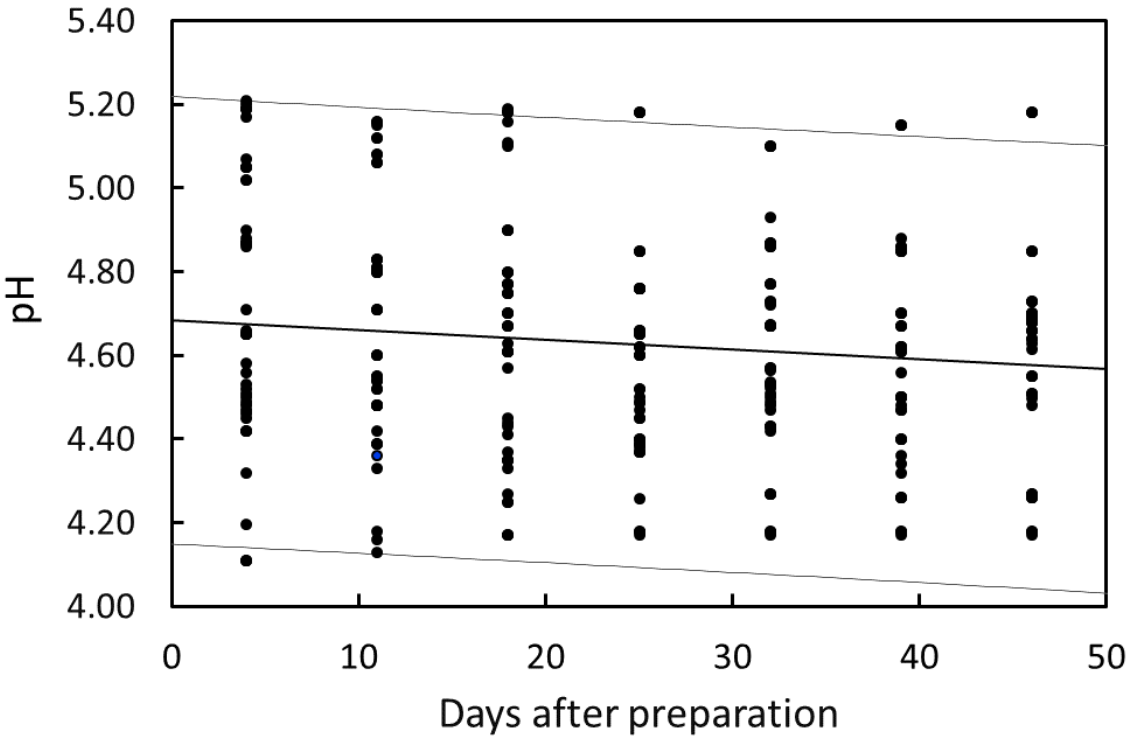


Figure 17 Linear regression model of pH excluding Day 0 pH data. The linear equation was pH = 4.68 - 0.00234 x Days after preparation. The 95% confidence limits were the upper and lower feint lines.

Determination of *Perna* colour

The proportion of males by weight in the 15 trials had no effect on pH but had several miner effects on colour at Days 0 and 4, and later to Day 46 (Figures 18, 19 and 20).

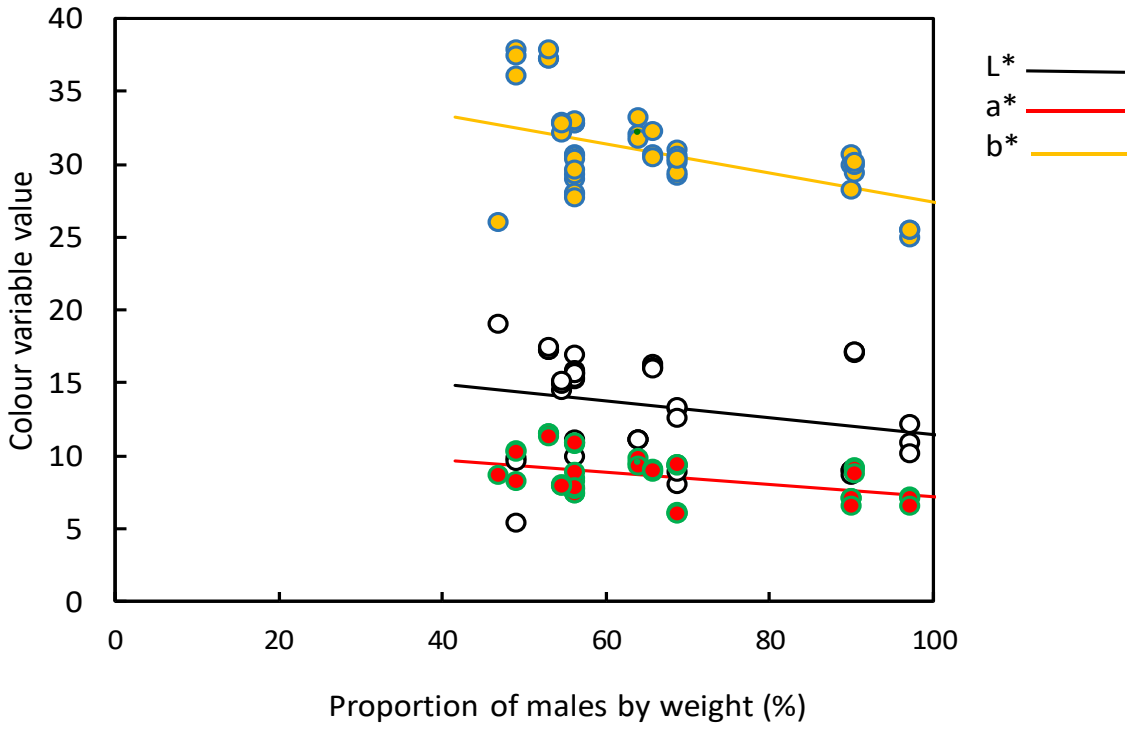


Figure 18 Linear regression of colour variables L*, a* and b* on proportion of males by weight Day 0.

For Day 0 regressions (Figure 18) the P values for the plots were 0.081 for L* (not significant), 0.003 for a* and 0.001 for b*. By Day 4 (Figure 19) the respective equivalent values were 0.11, 0.02 and 0.45. Colour differences due to gender were clearly reduced by fermentation.

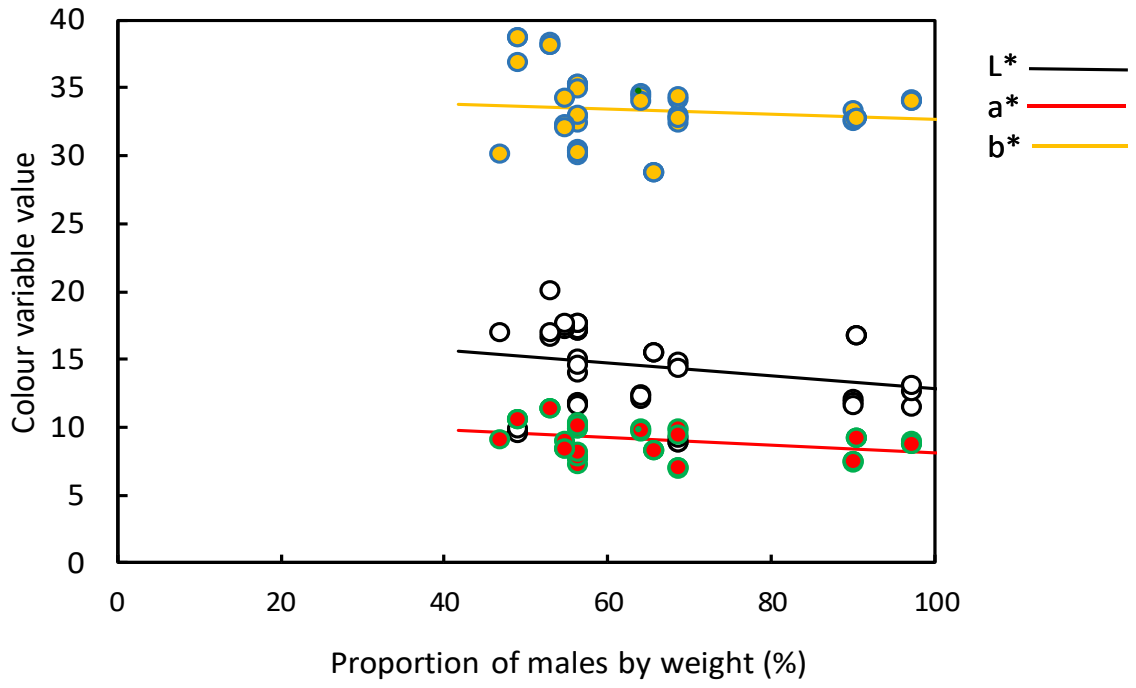


Figure 19 Linear regression of colour variables L*, a* and b* on proportion of males by weight Day 4.

However, taking a broader view of Days 4 to 46 pooled, where 315[7] data points were available for each colour variable, colour was obviously affected by proportion of males by weight Figure 20, and significantly so for each variable (all $P < 0.001$).

The colour hypothesis proposed at the start of the chapter was that the proportion of males should have a marked effect on colour. It had an effect, but not a marked effect.

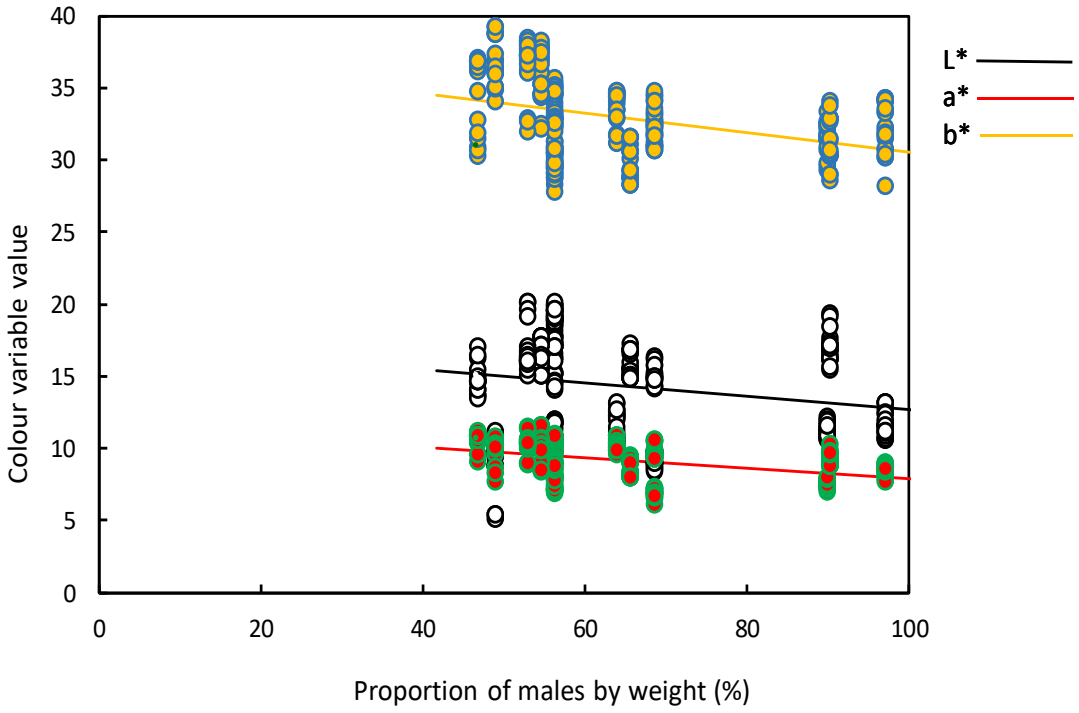


Figure 20 Linear regression of colour variables L*, a* and b* on proportion of males by weight for 15 trials over 7 time points from Day 4 to Day 46.

[7] 15 trials x 7 days x 3 replicates = 315 data points

Storage at 4°C had no effect on colour of *Perna*. Days 4 to 46 were pooled for 315 data points for each colour variable (Figure 21), showed that colour was not significantly affected by storage time (P values for L* = 0.94, R^2=0.009; a* = 0.85, R^2=0.007; b* = 0.906 R^2=0.006).

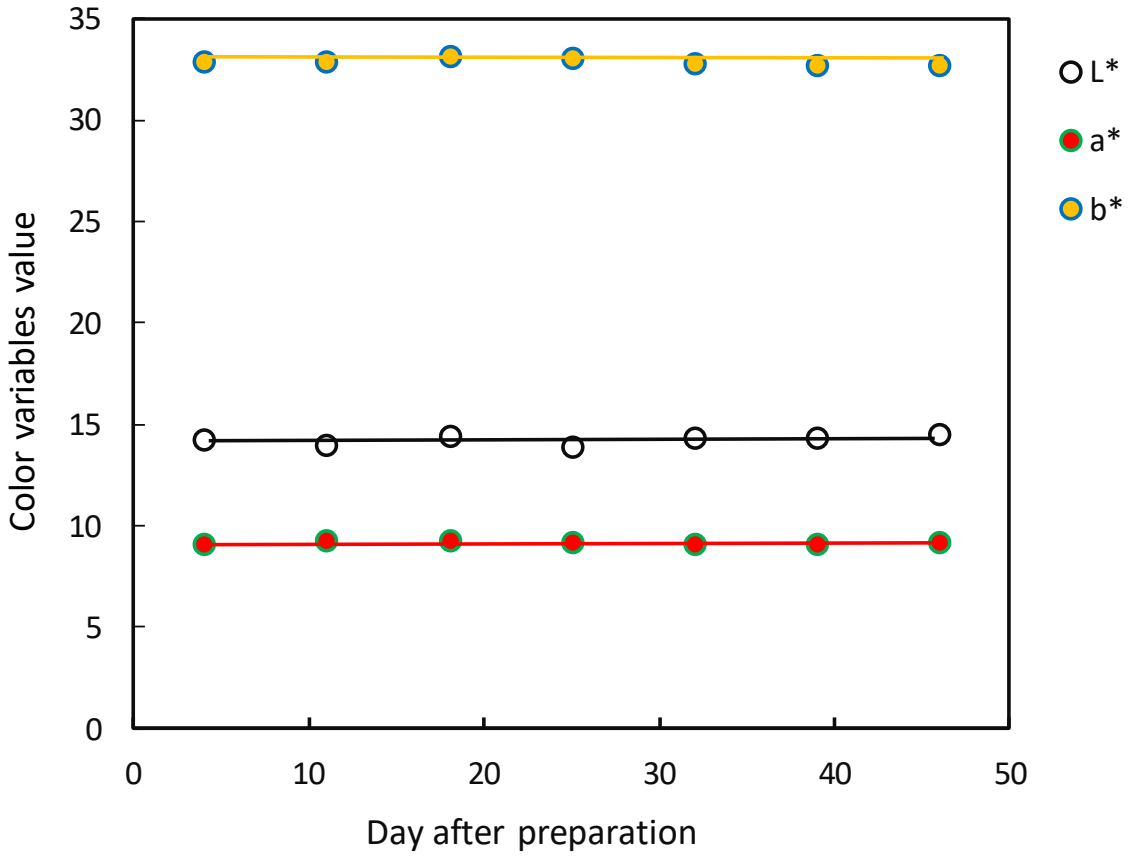


Figure 21 Colour variables values for L*, a* and b* during storage at 4°C over 7 time points from Day 4 averaged over 15 trials. The fitted lines are linear regressions.

As described in Materials and Methods, colour was routinely measured for many trials. Although not a part of the seasonal trial, colour due to storage temperature was recorded for a fatty acid stability experiment described later (pages 100, 101 and 102), where two preparations were examined. *Perna* samples prepared in the standard way were stored at 4°C, ambient temperature, and at 35°C for 46 days after preparation. Colour values are shown in Table 5. All values had variously changed by Day 46, and there appeared to be other effects in response to storage temperature, but these were not analysed except by inspection.

Table 5 Mean of L*, a*, b* for *Perna* samples stored at 4°C, ambient temperature, and at 35°C for 46 days after preparation.

		Preparation August 2015			Preparation September 2015		
		L*	a*	b*	L*	a*	b*
Day	Temperature of storage (°C)						
0	Not applicable	18.56	8.81	29.53	18.70	11.24	35.32
4	Not applicable	18.54	7.92	27.33	17.91	10.59	34.34
46	4	16.88	9.55	30.79	17.41	10.71	38.99
46	Ambient (~ 22)	15.50	10.63	35.14	15.14	12.61	40.48
46	35	14.71	9.55	36.73	13.81	9.99	31.58
Effect of storage between Days 4 and 46 (P value)		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The colour data presented above were all for *Perna* stored to 46 days. Much longer storage times resulted in darkening of product held at ambient temperature (Figure 22) compared to that stored at 4°C (Figure 23). This confirmed that chill storage temperature has minimal effect on colour of *Perna* compared to storage at room temperature.

Figure 22 *Perna* stored at room temperature for three years (L* = -0.62, a* = 8.51, b* = 18.09). The value -0.62 for L* is technically impossible because L* is on a 0 to 100 scale, but can be attributed to the blank as described in text.

It is likely that at low reflectance values the blank subtraction is not completely reliable. The fact remains that this example of Perna is extremely dark. This sample can be compared with that in Figure 23 which was stored at 4°C. It was much lighter.

Figure 23 *Perna* stored at 4°C for three years (L*=15.81, a*=7.47, b*=30.17).

This sample can also be compared with that in Figure 24 prepared from whole mussels, which was stored at room temperature.

Figure 24 Non-comminuted mussel stored at room temperature for two years (L*=5.65, a*=7.44, b*=18.77).

Table 6 pH and microbial profile for three trials of *Perna* prepared from May to July 2015 from day 0, 4 and 11.

	Preparation May 2015			Preparation June 2015			Preparation July 2015		
		Log_{10} cfu g^{-1}			Log_{10} cfu g^{-1}			Log_{10} cfu g^{-1}	
Day	pH	APC	LAB	pH	APC	LAB	pH	APC	LAB
0	6.59 ± 0.00	7.25 ± 0.00	7.01 ± 0.00	6.59 ± 0.00	7.58 ± 0.19	No data	6.69 ± 0.00	7.20 ± 0.07	7.26 ± 0.05
4	4.49 ± 0.14	8.47 ± 0.05	8.47 ± 0.01	4.67 ± 0.01	8.52 ± 0.55	8.30 ± 0.32	4.11 ± 0.00	8.68 ± 0.00	8.78 ± 0.00
11	4.39 ± 0.00	8.47 ± 0.01	8.49 ± 0.06	4.80 ± 0.03	8.83 ± 0.01	8.90 ± 0.05	4.16 ± 0.03	8.85 ± 0.02	9.47 ± 0.03

Microbial analysis of three of the storage trials over the first 11 days

Measured as aerobic plate count (APC) and lactic acid bacteria counts (LAB) the microbiological characteristics of the three trials of *Perna* made in May, June and July 2015 were slightly different from one another before fermentation (Table 6). These data were recorded after culture addition and may reflect subtle addition differences on the day of preparation. After the four days of fermentation, the LAB number was slightly higher for July trial compared to trials prepared in May and June. This indicated that, there was more growth of LAB for July trial. This was perhaps responsible for the lowest pH in July (Table 6), but other factors cannot be ruled out as will be discussed next. LAB are facultative anaerobes and the similarity of the APC and LAB data within each day shows that LAB dominate the microflora in freshly made *Perna*. The longer-term effects of *Perna* storage on microbial profile are examined in the later Chapter 7.

Discussion

pH of *Perna*

Inspection of at Day 0 shows variation from pH 6.15 to 6.75 which, in the context of the logarithmic scales is a significant variation, but one that shows no obvious pattern with season. It is known that the live green shell mussels are highly variable in pH. Mateparae (2003), showed that live mussel pH in a population from Nelson, New Zealand, was variable ranging from about 6.65 to 7.05 with no discernible difference between August and October. The current work spanned an entire year and importantly the mussels were cooked before pH determination. Rapid temperature increase during cooking causes an increased rate of rigor development, and a consequent drop in pH (Abril et al., 2001; Honkavaara, Rintasalo, Ylönen, & Pudas, 2003; Kreikemeier, Unruh, & Eck, 1998; Mateparae, 2003). This is due to lactic acid accumulation, but there is likely to be a trade-off between acceleration of glycolysis (pH falls strongly) and glycolytic enzyme denaturation (pH does not fall). It is proposed that the pH levels encountered after cooking that were obtained here, 6.15 to 6.75 reflect the onset of rigor. However, other factors might be involved, realising that enzymes are not equally denatured by heating at the same rate. Other variables adding to the complexity of these results are that the mussels' source was unknown, the sea conditions were unknown, and the time between harvest and sale was also unknown. There is a considerable literature on the variations of the proximate composition of green shell mussels (Miller & Tian, 2018; Newell, Hilbish, Koehn, & Newell, 1982; Snodden & Roberts, 1997), but none of those data could be available for the mussels used here in product development.

Given that all 15 preparations had the same concentration of added glucose it might have been expected that pH at Day 4 would be highly correlated with pH at Day 0, or that pH values at Day 4 would be close to constant. This was clearly not the case (Figure 15). It is shown elsewhere in the thesis (pages 118 and 137) that 2% glucose as used here is capable to causing a pH fall as low as 4. Therefore, glucose is not limiting, and besides, residual mussel glycogen also has the potential to release more fermentable glucose arising from slow acid hydrolysis and/or amylases secreted by the added microflora. But as noted above, the glycogen status of the mussels used could not be known. One possible reason for the failure of many preparations is variation in the culture used, probably due to culture handling. The intended use of these cultures is to open the pack and add to 200 kg of unfermented salami mixture. This was not possible because of cost and availability. Over the year, the strategy

was to subdivide several batches of culture and to repack them under a vacuum in lots suitable for each preparation. The pack as received were all freeze dried, because that is the best method of preservation among the several dying options (Santivarangkna, Kulozik, & Foerst, 2008). Despite the improved stability of freeze-dried bacteria cultures obtained during storage in the dried state, the bacteria cultures are not permanently stable and significant loss of viability is often unavoidable. The factors that affect viability are variously storage temperature, water activity and molecular mobility, and storage atmosphere (Kurtmann, Carlsen, Risbo, & Skibsted, 2009; Tymczyszyn et al., 2012). Temperature is the most critical of these (Połomska, Wojtatowicz, Zarowska, Szołtysik, & Chrzanowska, 2012). Culture subdivision may have been a cause of the Day 4 variation. Although every effort was made to subdivide BFL-F02 as quickly as possible, this bench top action may have led to different starting inoculum activities.

A counter argument is that because numbers of bacterial increase markedly over the four days (Table 6) it seems reasonable that the bacteria could overcome the problem of a poor start. That this was not the case as shown on page 142 where final numbers of bacteria could vary by an order of magnitude.

Turning now to the kinetics of pH fall after Day 4, it is clear from Figure 16 that pH continued to fall in some preparations but not in others. Summed over 15 trials, there was a small but steady decline at these refrigeration temperatures (Figure 17). Elsewhere in this thesis it was shown that storage at higher temperatures does result in a much more obvious pH decline after Day 4 (Figure 30). This is not surprising given that a typical salami fermentation temperature is 30°C and above.

Colour of *Perna*

As described in Materials and Methods (Chapter 2) males were determined by colour alone. The female gonads are orange/apricot and the male are creamy white due difference in carotenoid content in female and male gametes (Alfaro et al., 2001; Buchanan, 2001). Female and male mussels have a consistent cycle of gamete development, discharge, and redevelopment, where discharge (spawning) results in a depleted, dark-coloured mussel with inferior appearance. It has been shown that the gonad is the major site of fat accumulation in green shell mussel and it has been linked to reproductive status and food supply (Bayne & Bayne, 1976; Mclean & Bulling, 2005). It has been reported that the amount of fat in mussel increases before spawning and decreases to its lowest level immediately after spawning as a

result of lipid loss during reproduction. Generally, gonad development starts in December, with the early stage becoming increasingly dominant until around February, followed by a later stage of development in March. This continues through to May. Spawning begins in June and July, and spawning continues to December but with differences due to region and water temperature (Alfaro et al., 2001, 2003).The point is that colour is not only affected by gender but also by spawning. Therefore, the following discussion must be read with this uncertainty about sex identification.

The analysis (Figure 19 and Figure 20) on colour regressed on the proportion of males by weight at Day 0 (Figure 19) and Day 4 (Figure 20), showed any effect of proportion on colour was nearly erased with fermentation. Slopes became flatter. But in general, when data of Day 4 to Day 46 for all trials were plotted (Figure 20), some effect of male proportion was still evident. Overall, it shows all trials were slightly affected by proportion of males. This indicates that on mincing, the effect of gonads colour is largely swamped by the colour of other mussel compounds. The orange/apricot for female and creamy white for male, so obvious in the whole mussel, got lost as the viscera were blended with the gonads.

Storage in chill temperature to 46 days did not affect the colour of *Perna* (Figure 21). However, comparison of *Perna* colour in different storage temperatures for long periods markedly affected colour, as seen in Figure 22, Figure 23 and Figure 24 Although these informal outcomes were not replicated, it seems obvious that storage temperature is the dominant factor governing stability. The *Perna* in Figure 23 had been stored three years at chill temperatures and was visually and instrumentally similar to the colour shown in Figure 21 to Day 46 at chill temperature. The likely cause of darkening at higher temperatures is probably the Maillard reaction with residual glucose reacting with amino acids (Maillard, 1912). The Maillard reaction occurs at a wide range of temperatures, but goes more rapidly at higher temperatures, as is common for chemical reactions. The effect of pH of food on Maillard reaction have been reported to depend on type of amino acids composition of the foods. In the Maillard reaction, the D-glucose and glycine Maillard reaction system showed that the degradation of N-(1-deoxy-D-fructose-1-yl)-glycine at lower pH is favored by 1,2-enolization, which subsequently generates 3-deoxyglucosone. 3-Deoxyglucosone has been reported to be the key intermediate Maillard reaction product for the generation of coloured compounds in a model system at an acidic pH, 5.5 (Davies, Wedzicha, & Gillard, 1997; Leong & Wedzicha, 2000). Perhaps significantly *Perna* contains a high content of free glycine as shown in Figure 30 in the next chapter.

Basic microbiology of *Perna*

LAB usually increased during four days of fermentation and they remained almost constant during storage. LAB growth has been often correlated with the decrease in pH during fermentation (Cocolin, Manzano, Cantoni, & Comi, 2001). The growth of LAB in fermenting foods can be affected by many factors, particularly nutrients, water activity, oxygen concentration, temperature, and pH. During the early phase of fermentation, which is associated with the lag phase of microbial growth, existing microorganisms on materials, utensils and from the environment slowly increase and compete for nutrients with LAB from starter cultures to produce metabolites different from lactic acid. This competition may not be a problem in salami processing because the bacteria population on raw meat will be far less diverse and lower in absolute numbers. Mussels are filter feeders and are known to be highly associated with marine microorganisms in addition to their gut biome. This difference between raw meat and raw mussel is well illustrated in Figure 8, where fermentation of raw mussel was attempted with an added LAB culture and 2% each of salt and glucose. Whereas successful fermentation of raw meat can easily be achieved relying on only of LAB on hands and equipment, the added culture was swamped by the endogenous biome of the raw mussel. The outcome was a stinking sulphurous slurry (Figure 8). Bover-Cid, Izquierdo-Pulido, and Vidal-Carou (2000), stated that the effectiveness of the starter culture depends on the hygienic quality of the raw material used, as is confirmed in Figure 8.

The solution to the problems in Figure 8 was obvious: cooking before fermentation to minimize the mussel biome microflora (but not spores) and denature the hydrolytic enzymes. (Moreover, the mussel was much easier to shuck.) However, the cooking of mussels can be affected by mussel size, degree of opening in response to heat and water content within mussels. There is likely to be a complex reduction of microorganisms to each individual mussel, possibly leading to variable competition with added BFL-F02 culture. Although the three *Perna* preparations in Table 6 had a rather similar APC and LAB profile at Day 0, the details of the biome remain unknown.

The production of lactic acid is undoubtedly the determining factor on which the shelf life and the safety of the product depends. The inhibition of pathogenic and spoilage microflora is dependent on a rapid and adequate formation of lactic acid. Moreover, it has been reported that a rapid decrease in pH caused by amine-negative starter cultures can largely prevent biogenic amine accumulation in sausages (Maijala, Eerola, Aho, & Hirn, 1993). The rate of *Perna* fermentation by BFL-F02 and four other cultures is examined in

detail in Chapter 7.

Chapter 4

Proteins, peptides and amino acids

Introduction

At all phases of *Perna* preparation, the profile of proteinaceous matter is likely to undergo changes. In the live mussel this matter will be a mixture of amino acids, peptides and proteins from the body of the mussel, as well as from the contents of its digestive system. After boiling, the subsequent profile is unlikely to be greatly affected by the enteric microflora, because that will be largely destroyed by the heat. The added culture, especially, and other microorganisms on equipment will dominate the fate of proteinaceous matter as fermentation progresses.

In meat fermentation, biochemical reactions occur, involving proteins, carbohydrates, and fats which determine the ultimate characteristics of the product (Hierro, de la Hoz, & Ordóñez, 1999). The carbohydrate fermentation is mainly achieved by lactic acid bacteria resulting in a pH drop in fermented meats (Flores, 2018), while coagulase-negative staphylococci are involved in the bacterial formation of aroma compounds (Ravyts et al., 2010). Meat proteins are known to undergo hydrolysis, first to polypeptides by endogenous muscle enzymes, such as cathepsins and calpains (Molly et al., 1997; Toldrá, Rico, & Flores, 1992) and then further to smaller peptides by the action of peptidases. Free amino acid generation from peptides by aminopeptidases is the final step in proteolysis process and has been attributed to protease enzymes generated by microorganisms as well as endogenous enzymes of the meat itself (Casaburi et al., 2007; Casaburi et al., 2008; Hughes et al., 2002). Low-molecular-weight peptides and free amino acids are major components of the non-protein nitrogen fraction in fermented meats, contributing to generation of volatile and non-volatile flavor compounds (Montel, Masson, & Talon, 1998). Fats are hydrolysed by various lipases, generating free fatty acids, which themselves have flavour and are substrates for oxidative changes that are responsible for an array of flavour compounds (Stahnke, 1995; Verplaetse, 1994).

Material and methods

Amino nitrogen, UV absorbance and soluble protein

Proteins, peptides and amino acids can be measured in several ways. Kjeldahl nitrogen is useful for determining crude protein but would be of no use here because what nitrogen from these sources goes into the fermentation mixture at Day 0 stays there with no change. Of more interest is the progression of hydrolysis, which can be measured in several ways. The methods used here were amino nitrogen, soluble protein, ultraviolet (UV) absorbance, and amino acid profile. Of these, UV absorbance is the perhaps the least useful but was quick and easy to do.

Amino nitrogen is an old method described by Wadsworth and Pangborn (1936) as more recently defined by Li and Seong-Gook (2014)[8]. To summarize the method described in Chapter 2, a 2-g dispersal of *Perna* in water was crudely filtered through cheese cloth, mixed with formaldehyde adjusted to pH 8.4. The aldehyde reacts with free amino groups and lowered the pH. Mixture was titrated NaOH solution and titer used to determine the mass of amino groups, which have a 1:1 stoichiometric reaction with NaOH.

Unlike the amino nitrogen method, which was applied to all *Perna* matter except that which was retained on cheese cloth, the following methods applied only to matter that was in nominally true solution. To achieve this the dispersates were strongly centrifuged and finely filtered as described in Chapter 2. All filtrates were optically clear.

The UV spectra shows absorbances due to aromatic amino acids, nucleic acids and unknowns, pigments for example, that absorb in this range. Nucleic acids show a strong absorbance in the region of 240 to 275 nm. Proteins usually show absorption maxima between 275 and 280 nm, due to the absorbance of the two aromatic amino acids tryptophan and tyrosine and, to a small extent, by the absorbance of cysteine (i.e. of disulphide bonds) (Schmid, 2001). Protein unfolding transitions can be measured by following the absorbance changes at 287 to 292 nm as a function of temperature or denaturant concentration (Schmid, 2001). According to Schmid, UV absorbance between at 275 to 292 nm will give the best indication of soluble proteins, peptides and amino acids. However, to get a wider picture of UV absorbance the region between 200 and 400 nm was also monitored.

[8] Their formulation calculations were found to be wrong and have been modified to give the correct answer.

The bicinchoninic acid assay (BCA), which measures the concentration of peptide bonds, was used to measure soluble proteins and peptides, but obviously not free amino acids. Although the method has some limitations with peptides smaller than 2,000 Da and peptides rich in cysteine, cysteine, tryptophan and tyrosine Thermo Scientific Pierce Protein Assay Technical Handbook (2009) it is rapid and sensitive over a wide dynamic range.

Amino acid analysis

Effect of storage temperature

It is first useful to describe more experimental detail than was given in Chapter 2. Three almost completely independent experimental batches were started on a single day in December 2015, each with subsequent storage at 4°C, ambient and 35°C, to Day 74. The mechanics of preparation were as follows. All mussels were cooked at once and shucked. One third of the mussel meat was minced, followed by the second and the third. The first third was processed (other ingredients, mixed, distributed and vacuum packed) followed by the second and final the third fraction. The entire process took a day, and – as will be shown – may have had an interesting effect on some results. Each experimental batch had a minimum of two, usually three, replicate barrier bags for each time point and temperature.

Amino acid analysis was done by using liquid chromatography with mass spectrometry (LCMS). The method, based on 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatisation (Accutag), was calibrated with a standard amino acid of 37 stable amino acids (Sigma A9906) and two unstable amino acids asparagine and glutamine, as well as the biogenic amines tryptamine, tyramine and histamine. D4-Alanine was the internal standard. Extracts were injected in triplicate in LCMS. The means of injection replicates were averaged across the replicate barrier bags to yield one of three means for each storage treatment. Those three values were usually[9] the basis of mean and standard deviation calculations.

Effect of seasonal change

It is again useful to briefly describe more experimental detail than was given in Chapter 2. At the time points shown in Table 7 Chapter 3, a single mussel selection was processed and ultimately distributed among three replicate barrier bags each used for different assays

[9] In Figure E, the basis for calculation was the three replicate bags within batch.

e.g. free amino acids, fatty acids, amino nitrogen, soluble protein etc. For free amino acids in this seasonal trial, water extracts were made one barrier bag from which three replicate injections were analysed as a minimum. Data are the means of these replicates.

Effect of starter culture

During fermentation, glucose is metabolised to produce lactic acid leading to a lowering of pH. Many pathogenic bacteria do not grow at low pH but LAB and some *Staphylococcus* species can remain viable. These bacteria hydrolyse the protein into smaller chemical species, shown to be dominated by free amino acids, as shown in the previous section. To provide for different market preferences, Chr-Hansen and other culture producers offer a range of starter cultures comprising different combinations of bacteria.

Results

Amino nitrogen, UV absorbance and soluble protein

Amino nitrogen, measuring total amino groups, was determined in samples during fermentation and storage to 74 days at 4°C, ambient temperatures and 35°C (Figure 25). Before fermentation at Day 0 the mean of amino nitrogen for five *Perna* trials exhibited the numerically lowest amount of amino nitrogen, with a slight and non-significant increase observed by Day 4. *Perna* stored at different temperatures showed increase of amino nitrogen with temperature and storage time to Day 18. Exactly what happened between Day 18 and Day 46 cannot be known, but by Day 46 the values had dropped to around 2 mg g^{-1} and 4°C and ambient temperature. (Measurements at 35°C were not done beyond Day 18 because the *Perna* had greatly deteriorated as judged by colour (see later), and samples were needed for amino acid analysis, described later.) The amino nitrogen at 4°C and ambient then remained stable to Day 74.

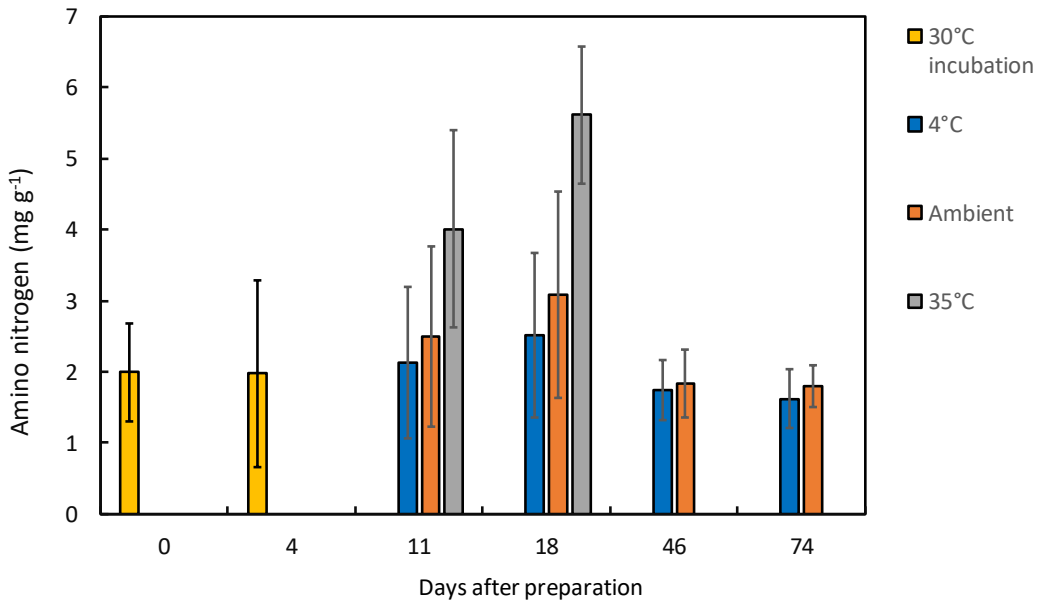


Figure 25 Mean amino nitrogen values during fermentation at 30°C and storage at 4°C, ambient and 35°C over 74 days averaged over five trials. Data are presented as category data in a histogram. Vertical lines are standard deviations. The differences between Days 11 and 18 were significant for temperature (P = 0.017) but day was not significant (P = 0.224). Pooled Days 11 and 18 were different from pooled Days 46 and 74 (P = 0.03).

The mean UV absorbance of trials between 275 to 292 nm (Figure 26) showed a small decrease from Day 0 to Day 39 and a subsequent increase to Day 130. However, these trends were not marked. The mean of UV absorbance between 200 and 400 nm of the same trials showed a similar trend, with rather similar values and variation (Figure 27).

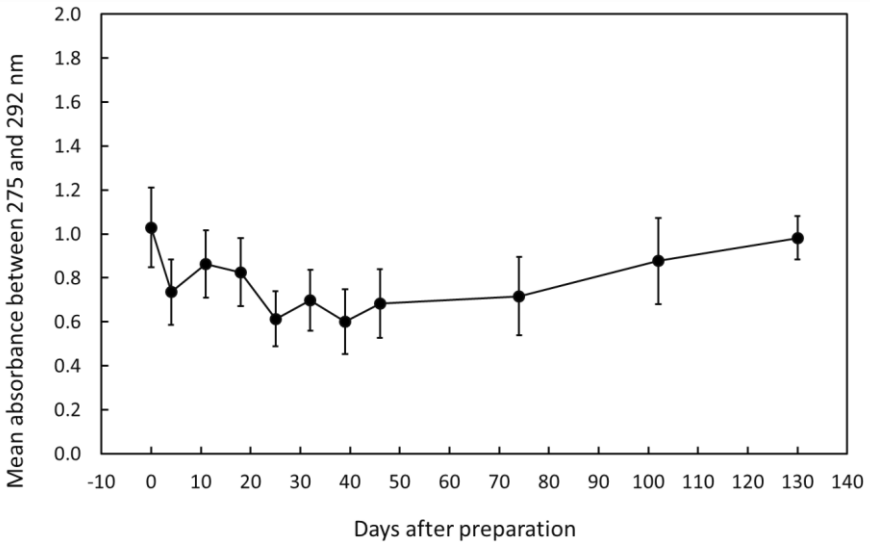


Figure 26 UV absorbance values between 275 and 292 nm during storage at 4°C over 130 days averaged over 15 trials from Days 0 to 18 and at Day 46, and over two to six trials on all other days.

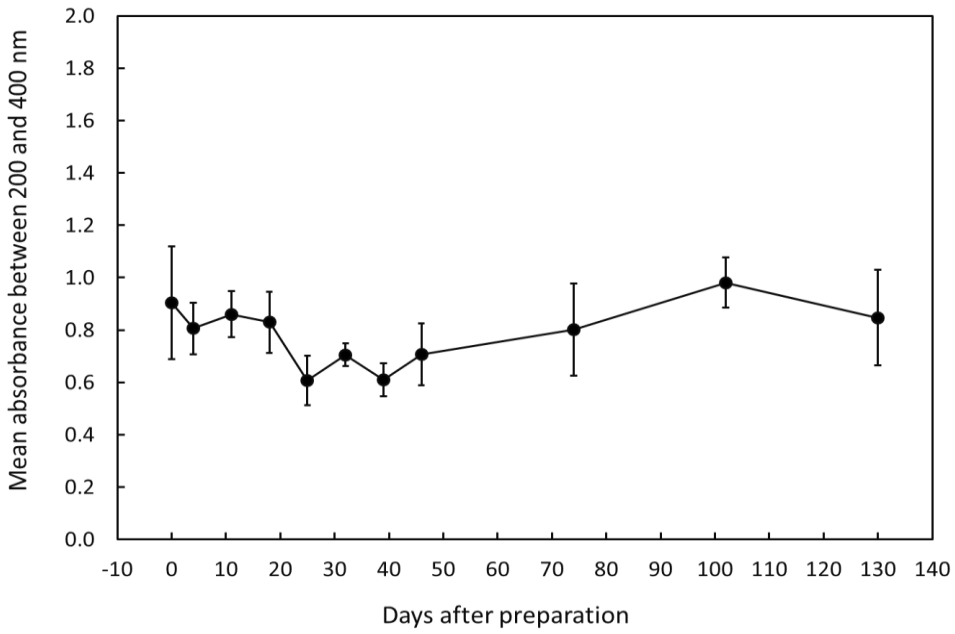


Figure 27 UV absorbance values between 200 and 400 nm during storage at 4°C over 130 days averaged over 15 trials from Days 0 to 18 and a Day 46, and over two to six trials on all other days.

The soluble protein as measured by the BCA assay showed a clear decrease during fermentation and storage at 4°C for 130 days (Figure 28). There was a rapid decrease was from Day 0 to Day 18, with evidence of stability to Day 46 then a further decline to Day 130.

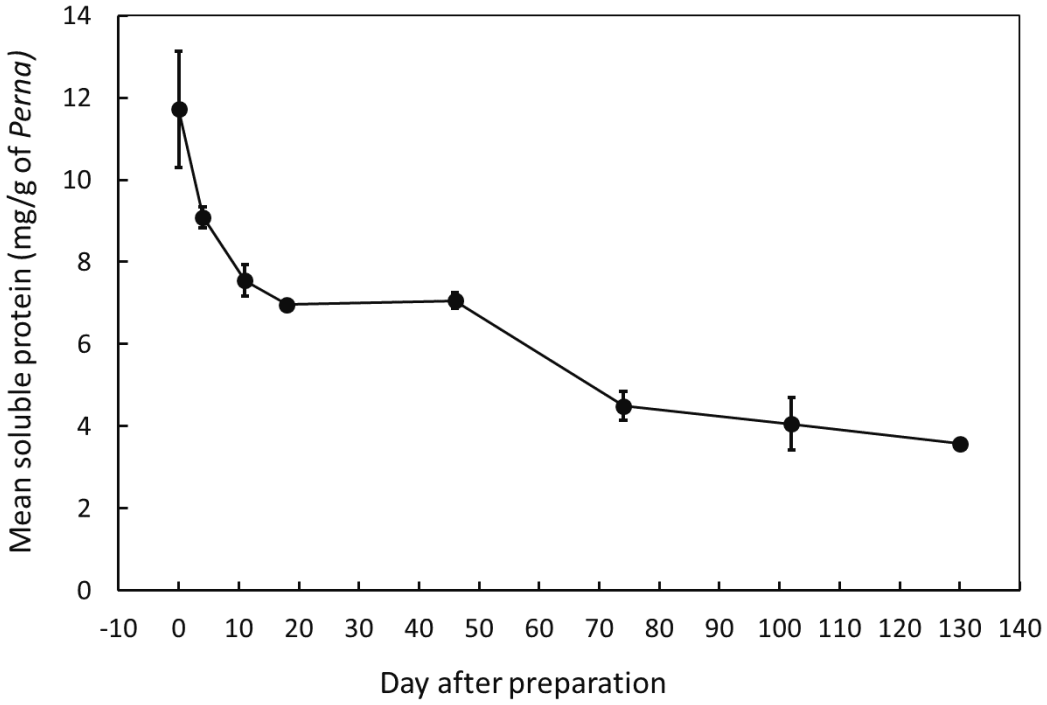


Figure 28 Soluble protein values during storage at 4°C over 130 days averaged over 7 to 13 trials from Days 0 to 74, and over two to six trials on other days.

Amino acid analysis

Effect of storage temperature Figure 29 shows the means for each independent batch where data are expressed as μmol g^{-1} of *Perna*. Up to 23 amino acids were identified along with three biogenic amines, tryptamine, tyramine and histamine. Some amino acids were not resolved (e.g. Hyp, Leu, Ile) and in these cases the combined calibration curve, which was also not resolved, was the basis for quantitation. All peaks in the chromatograms were assigned to an amino acid(s), with no unknown peaks or small peptides.

Day 0 was dominated by arginine, then glycine, glutamic, aspartic and alanine (Figure

29). The data are presented in batch order as made, Batch 1, 2 then 3 on the same day. Inspection shows a progressive loss of many amino acids over order of preparation. Arginine showed the greatest absolute loss over preparation time, and the progressive losses were significant for at least arginine, aspartic and serine (0.63, 0.12 and 0.09 μmol g^{-1}difference respectively, between Batches 1 and 3). (For clarity only amino acids present in higher concentrations bear standard deviation bars.) Summed over all amino acids, the mean concentration in μmol g^{-1} was about 0.16 μmol g^{-1}.

By Day 4 the situation had changed (Figure 30). The mean concentration was about 0.10 μmol g^{-1}, with nearly all amino acids reducing, with some evidence of three increasing. Arginine, fell by about 90% overall, while losses of others were usually much lower except where concentrations at Day 0 were low to begin with. The standout amino acid was undoubtedly arginine.

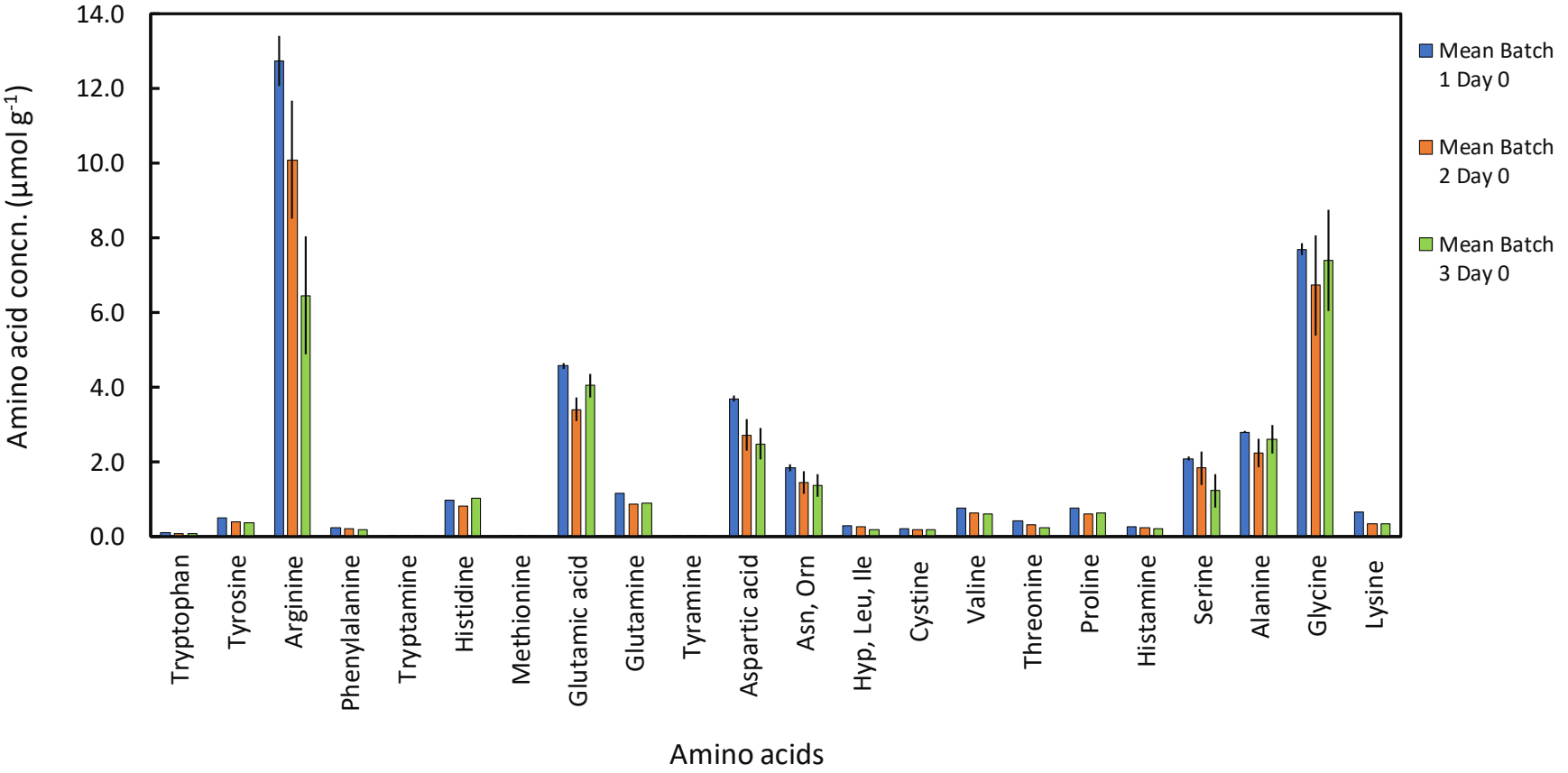


Figure 29 Amino acid concentration before fermentation, Day 0. Data are concentration values with standard deviation bars for amino acids present in higher concentrations. Preparation in December 2015.

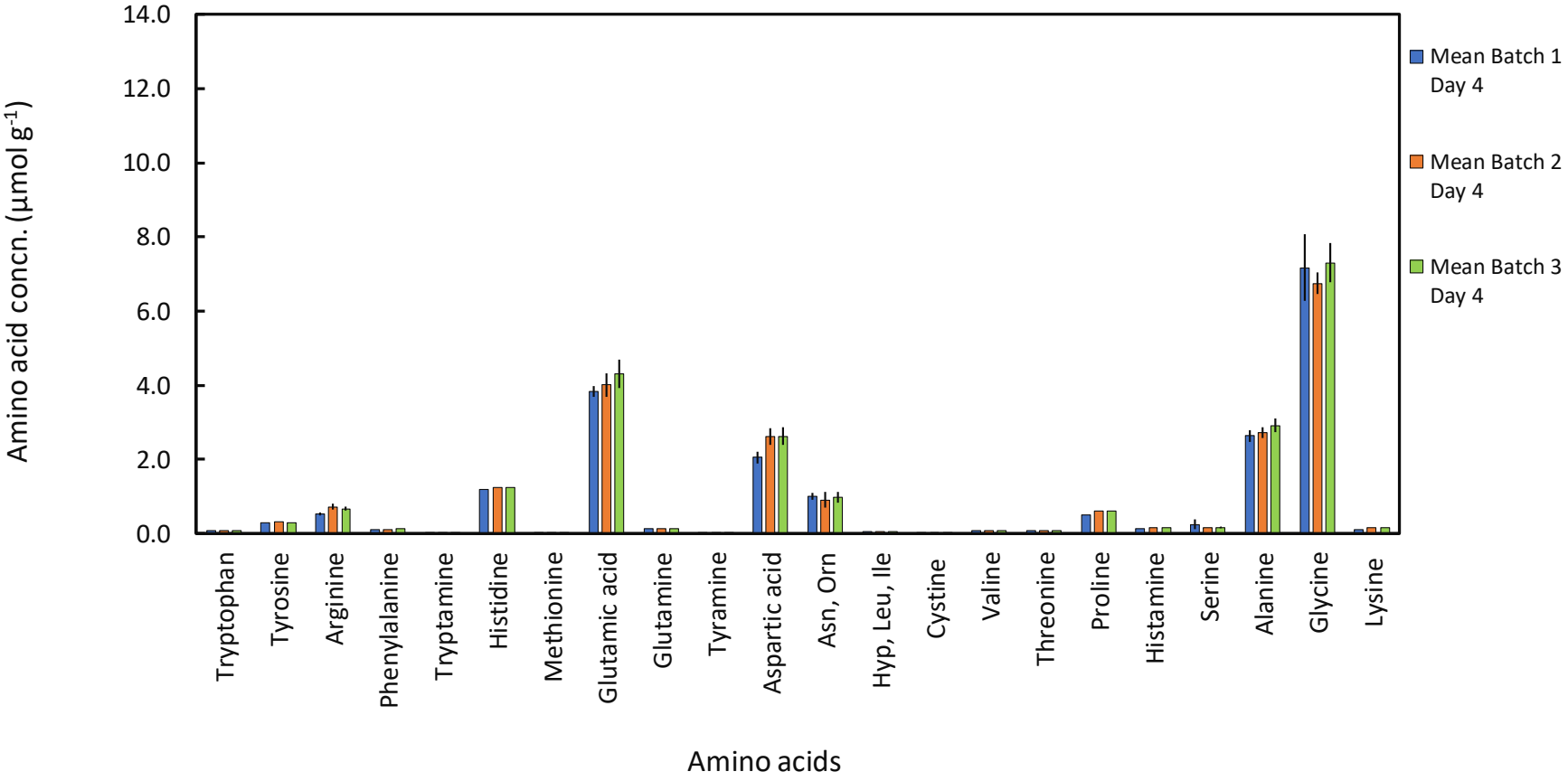


Figure 30 Amino acid profile after fermentation, Day 4. Data are molar percent with standard deviation bars for amino acids present in higher concentrations in Day 0 (Figure 29). Preparation in December 2015.

In contrast to Figures 29 and 30, the data are expressed as absolute concentration which would be important for example in identifying flavor compounds truly active in the eating experience. However, in Figure 31, 32, 33, 39 and 40 are in amino acid profile because as the ratio between different amino acids is easier to see than when expressed as absolute concentrations. Figure 31 shows that arginine was the most dominant before fermentation (> 25 %) and fell markedly by Day 4 as also shown in Figure 29 versus Figure 30. Amino acids that assumed more relative importance by Day 4 were histidine, glutamic and aspartic acids, proline, alanine and glycine. Many of these differences were highly significant ($P < 0.01$, at least). The changes in amino acids shown between Day 0 and Day 4 were much greater than on subsequent storage, and for reasons of clarity, standard deviations are seldom shown in figures than come later. Differences are usually obvious by inspection

.

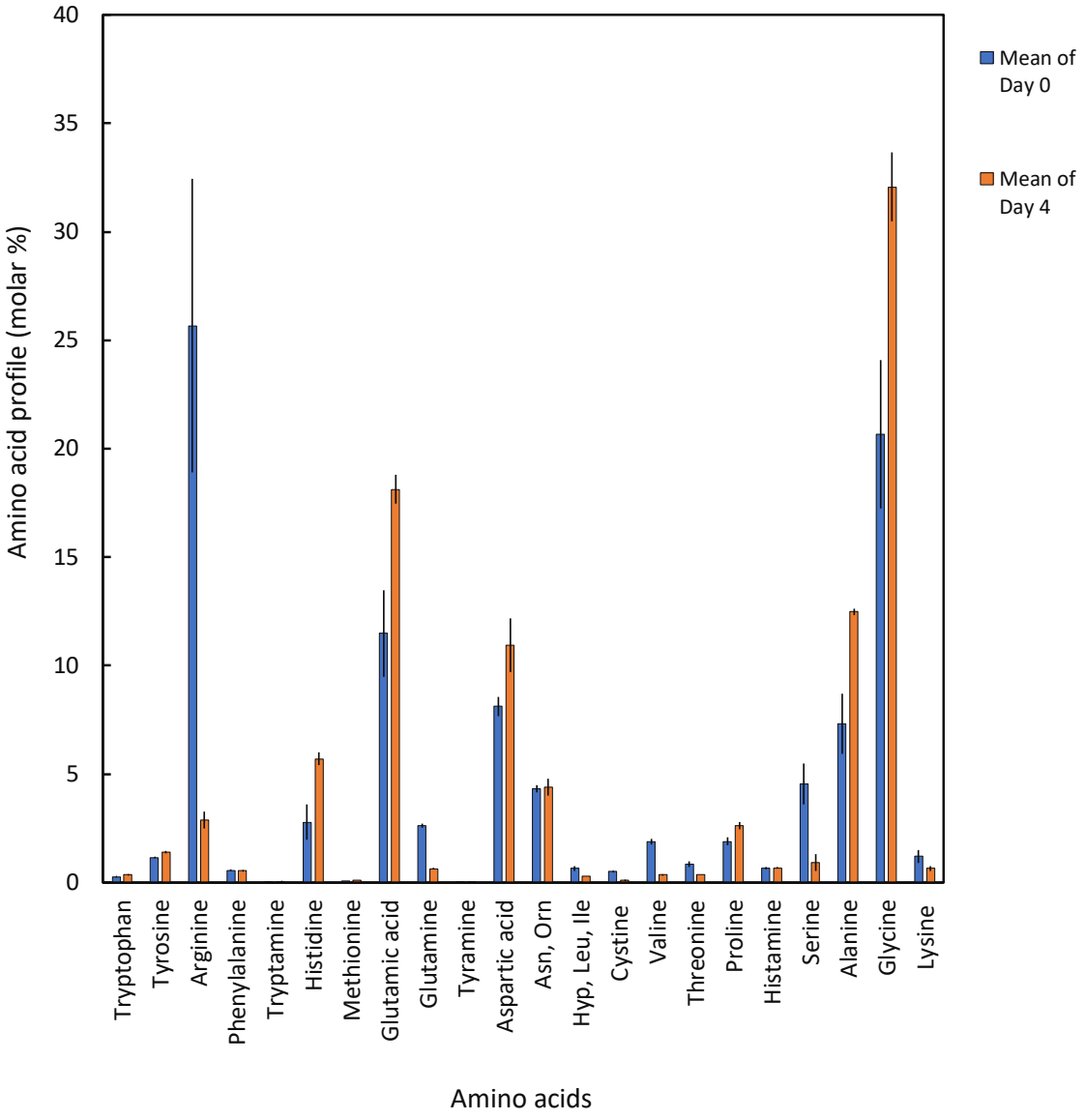


Figure 31 Molar percent amino acid profile before fermentation, Day 0, and after fermentation, Day 4. Data are molar percent with standard deviation bars. Preparation in December 2015.

Storage at different temperatures after fermentation (4°C, ambient, and 35°C) had no effect on *Perna*'s soluble amino acid profile to Day 46 (data shown in Appendix III and Day 74 (Figure 32).

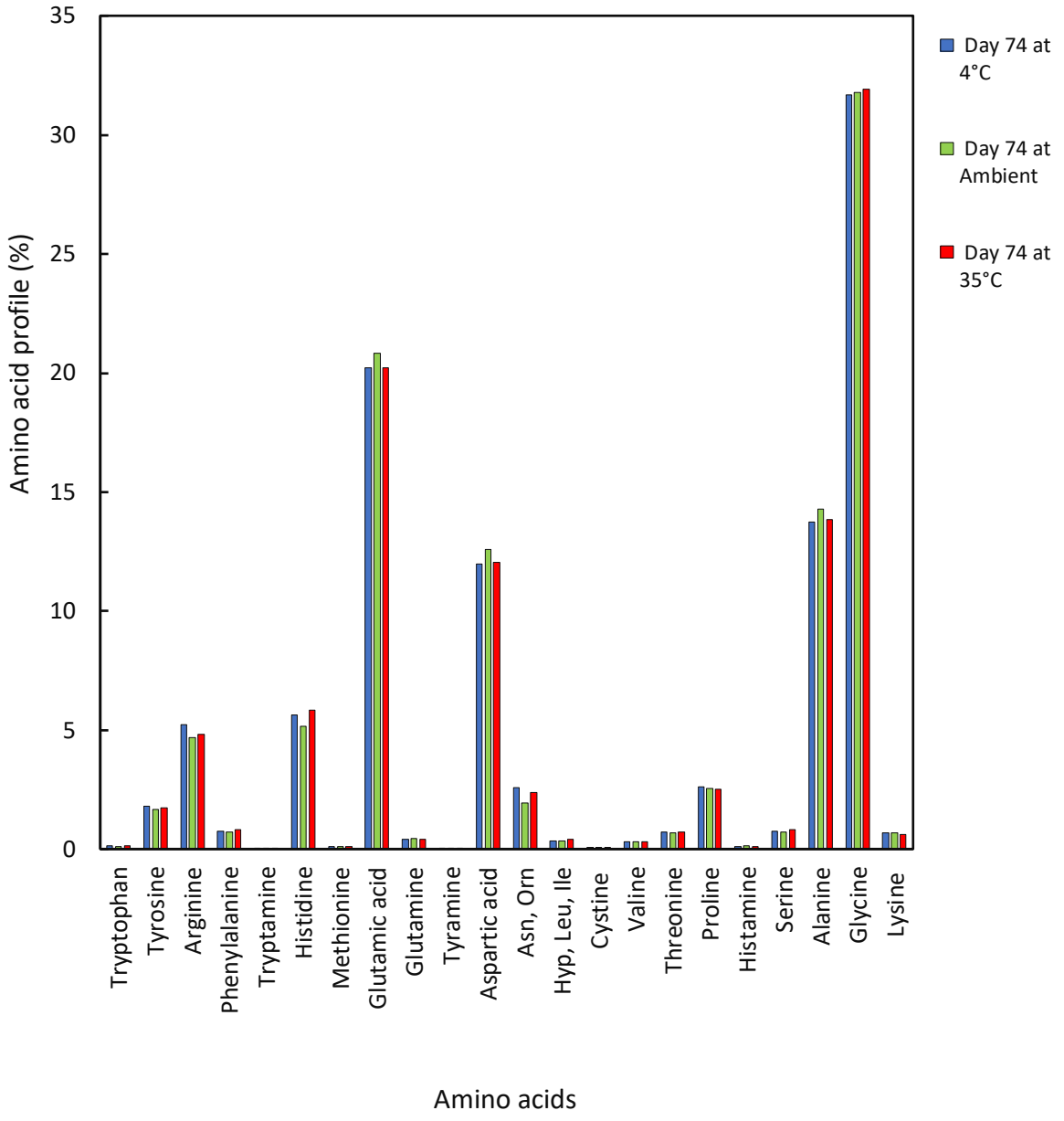


Figure 32 Molar percent amino acid profile at Day 74, stored at 4°C, ambient, and 35°C. For each temperature the data are means of the three experimental batches. Standard deviation bars are not shown for clarity. Preparation in December 2015.

At this point it was useful to pull all the storage information together to summarize the effect of storage time to Day 74. Figure 33, for ambient storage, is representative of the time/temperature effects. (The two other equivalent graphs (4°C and 35°C) are shown in Appendix III and Appendix IV but show the same result.) In Figure 33, the big change was from Day 0 to Day 4, as previously described. At later time points, Days 4, 46 and 74 as a group, many amino acids became relatively more important. These increases were at the expense of other amino acids, notably arginine, glutamine, valine and serine; amino acids remained static.

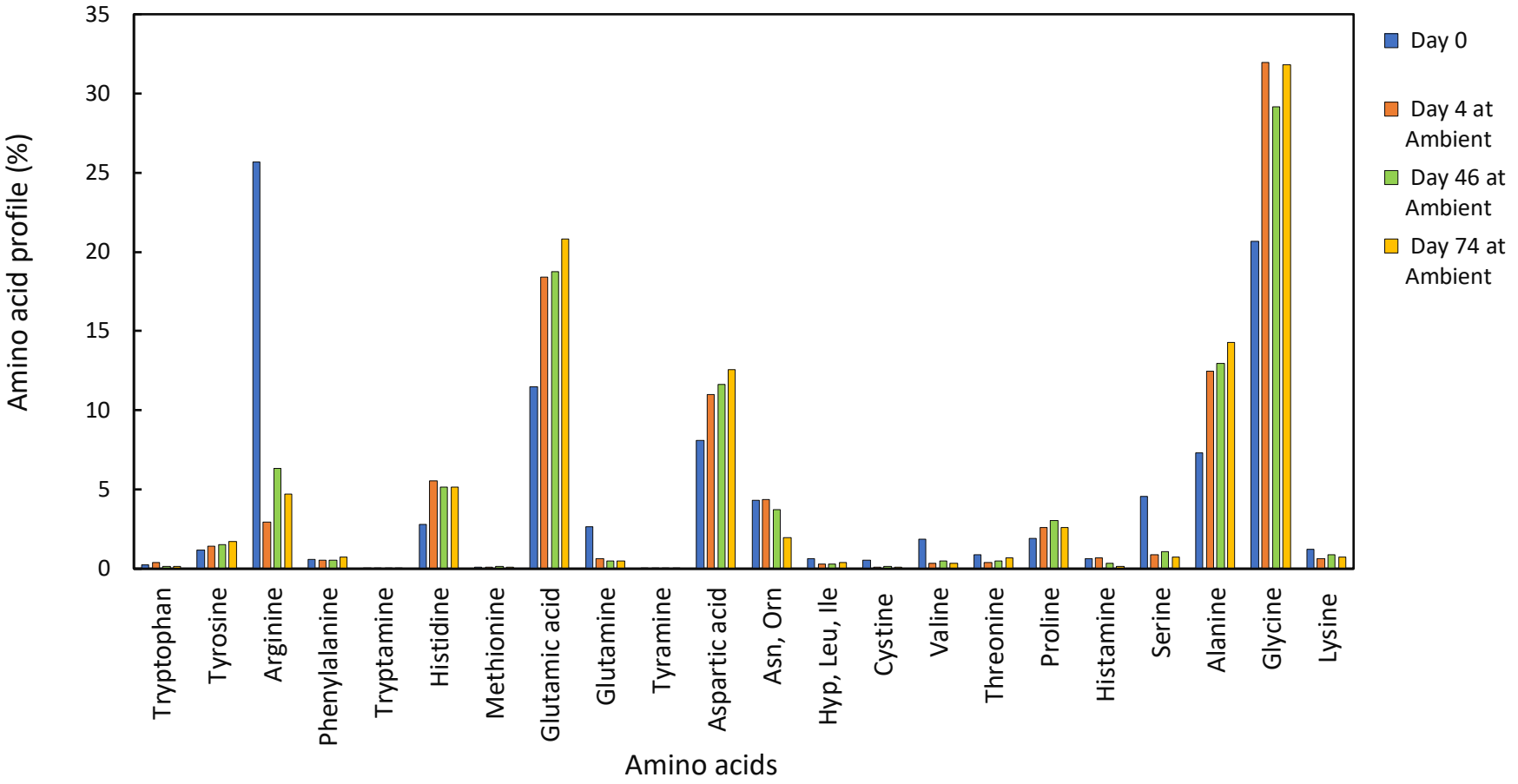


Figure 33 Molar percent amino acid profile before fermentation at Day 0 and after fermentation at Days 4, 46 and 74, at ambient storage. For each day the data are means of the three experimental batches. Standard deviation bars are not shown for clarity. Preparation in December 2015.

Effect of seasonal change

Results in the previous section on storage temperature were from a single triplicated preparation made from mussels sourced in December 2015. Looking back to those results, e.g. Figure 31, the proportion of arginine was high at Day 0 and fell markedly by Day 4. That December 2015 pattern – designated Pattern 2 as will be shown below – was in marked contrast to a pattern (Pattern 1) observed in the two previous months in the previous year 2014, and to a third pattern (Pattern 3) observed in July 2015 (Table 7).

Table 7 Patterns observed in amino acid profile in a seasonal study of *Perna* production. These represent the eight trials where free amino acids were determined.

Pattern 1 (2 trials)		Pattern 2 (5 trials)		Pattern 3 (1 trial)	
Dates	Shown in	Dates	Shown in	Date	Shown in
Oct 2014 Nov 2014 (Spring)	Figure 34	Mar 2015 Apr 2015 Jun 2015 Dec 2015 Jan 2016 (Summer, autumn, early winter)	Figure 35	Jul 2015 (Winter)	Figure 36

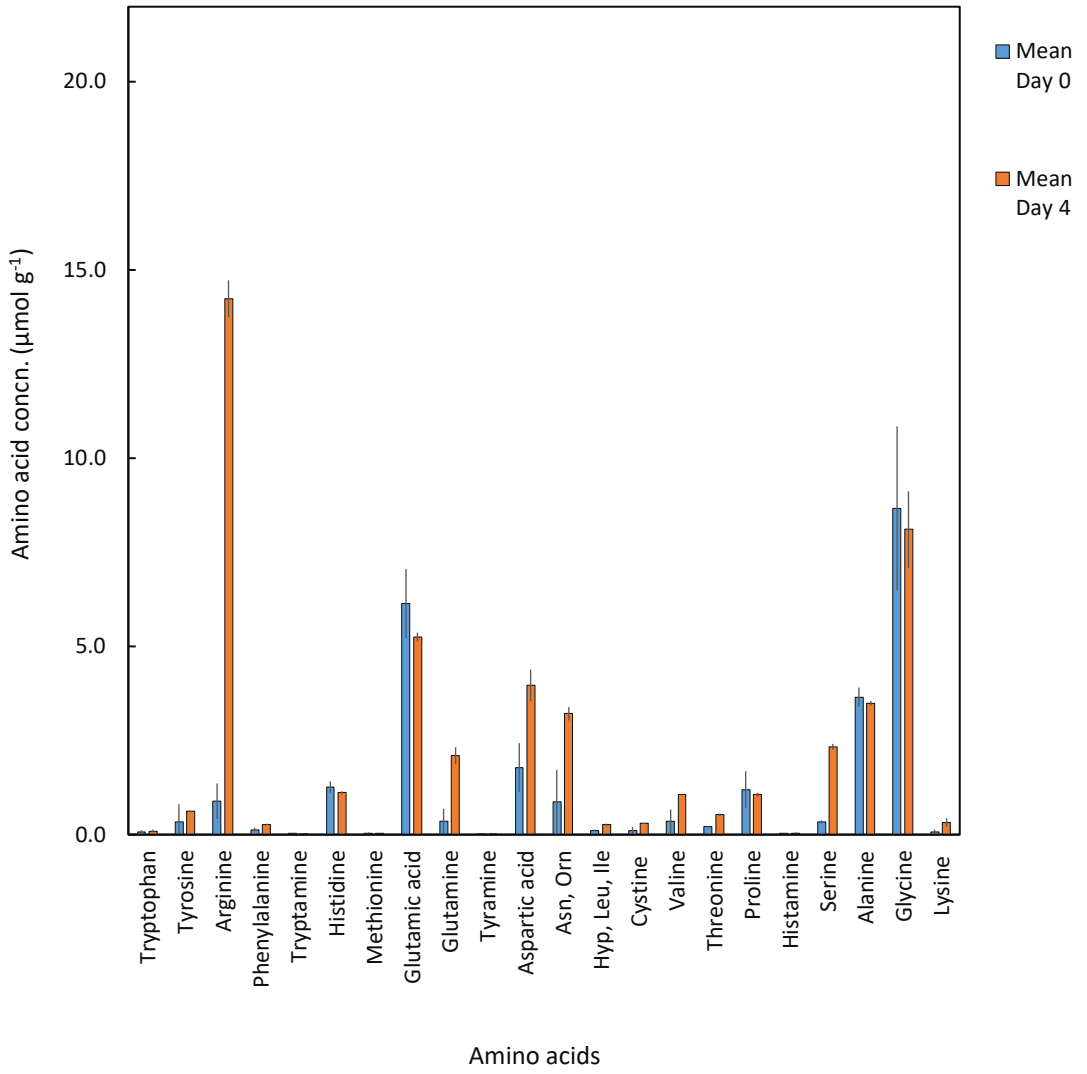


Figure 34 Mean amino acid concentration for Pattern 1 trials before fermentation at Day 0 and after fermentation at Day 4. In this pattern the arginine proportion was low on Day 0 but high on Day 4. Error bars are standard deviations.

In Pattern 1, the concentration of arginine was low on Day 0 and high on Day 4 (Figure 34). In Pattern 2, the reverse was true (Figure 35).

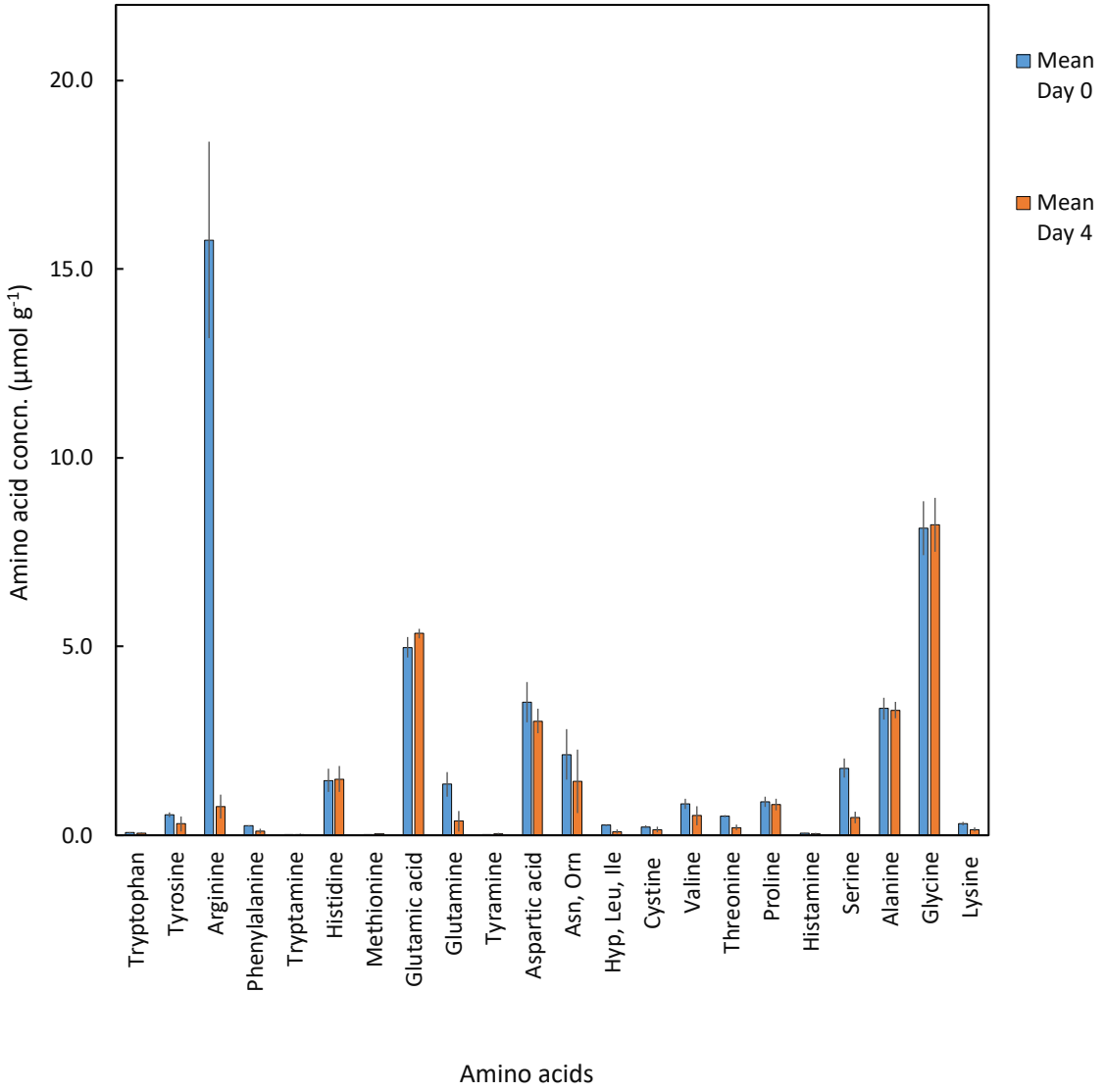


Figure 35 Mean amino acid concentration for Pattern 2 trials before fermentation at Day 0 and after fermentation at Day 4. In this pattern the arginine proportion was high on Day 0, but low on Day 4. Error bars are standard deviations.

Although Pattern 3 had only example, from July 2015, the arginine was different again: arginine was low on Days 0 and 4 (Figure 36).

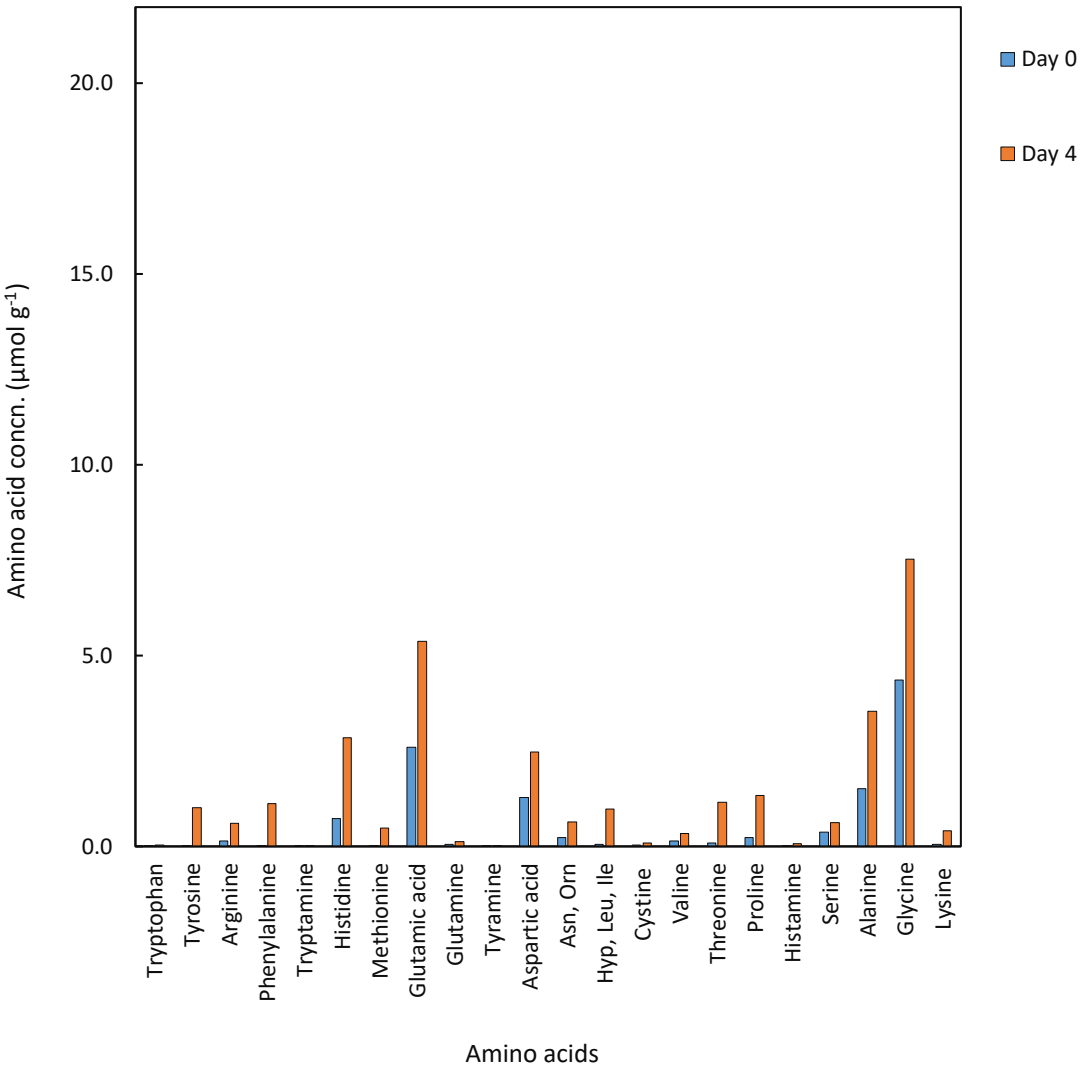


Figure 36 Amino acid concentration for Pattern 3 before fermentation at Day 0 and after fermentation at Day 4. In this pattern the arginine concentration was low on Day 0 and also low on Day 4.

A direct comparison of these three patterns are shown in Figure 37 and Figure 38, where the means of absolute concentrations are plotted together, but without standard deviations shown to improve clarity. (The values are also shown in Appendix V.)

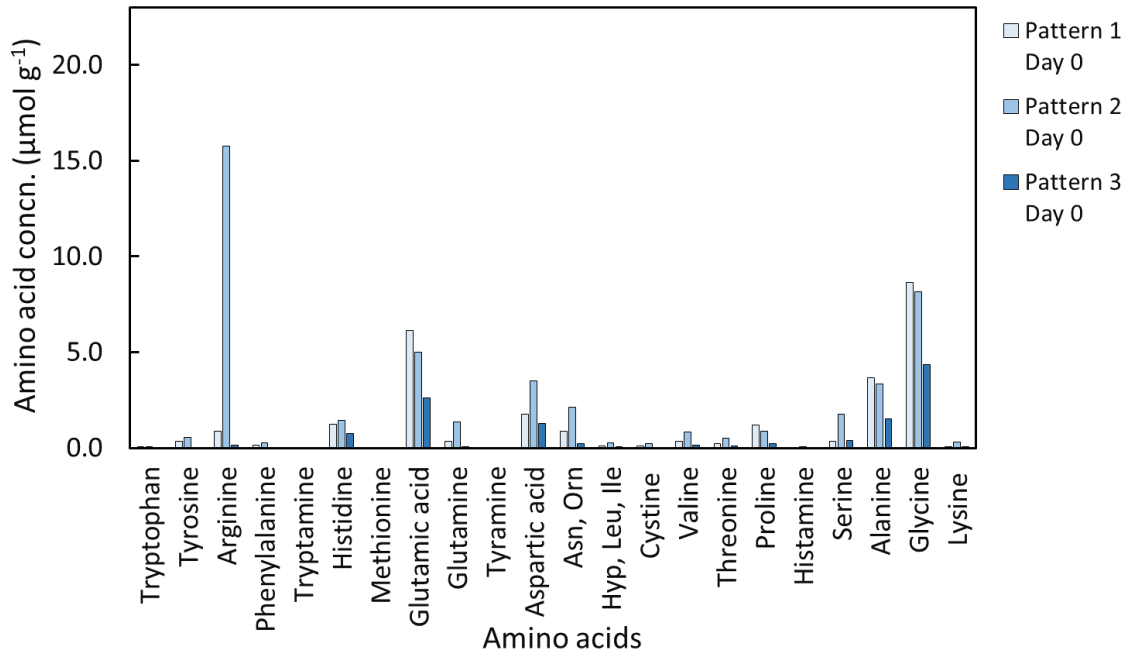


Figure 37 Comparison of amino acid concentration for the three patterns before fermentation at Day 0.

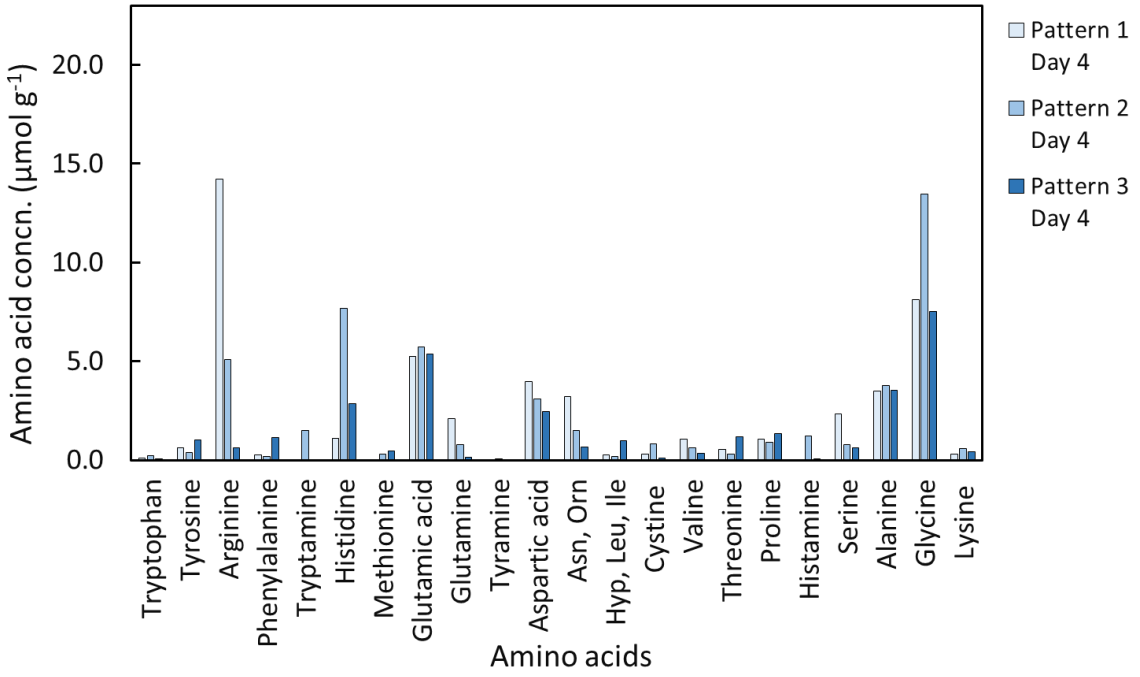


Figure 38 Comparison of amino acid concentration for the three patterns after fermentation at Day 4.

Effect of starter culture

It was of interest to see how five Chr-Hansen cultures (Table 4 and Appendix VI) performed for three of *Perna*'s measurable qualities, soluble amino acids reported here, volatiles by headspace sampling (Chapter 7), and *Listeria monocytoenes* survival (Chapter 7). The motive for amino acid analysis was that soluble amino acids contribute to flavour.

The experiment was performed on single date – which was in the proposed Pattern 2 time frame – and the amino acid analysis was independent of the analyses in the previous section. Although the boiling and shucking took several hours the subsequent steps were expediated by assistants such that all treatments and replicates (5 cultures x 3 days of storage x 3 vacuum packs) were prepared within one hour. Thus, this experiment did not suffer from the effect of mixing delays that were responsible for the interesting loss phenomenon in Figure 34. Figure 39 shows a profile typical of Pattern 2 in respect of arginine, with a higher proportion on Day 0 but lower on Day 4, but there were clear differences from one culture to another. By chance and not design, the proportion of arginine (and tryptamine) fell in the sequence 1 to 5, and was reciprocal to increases in the proportions of glutamic acid, aspartic acid, proline and alanine. The dominant amino acid, glycine, was static at Day 4 as was the case for several other amino acids.

The effect of different starter culture was observed only from Day 0 to Day 4 (during fermentation). By Day 46 (Figure 40) – and likely sooner than that – the amino acid profiles were essentially identical. A very similar result was obtained at Day 74, data not shown.

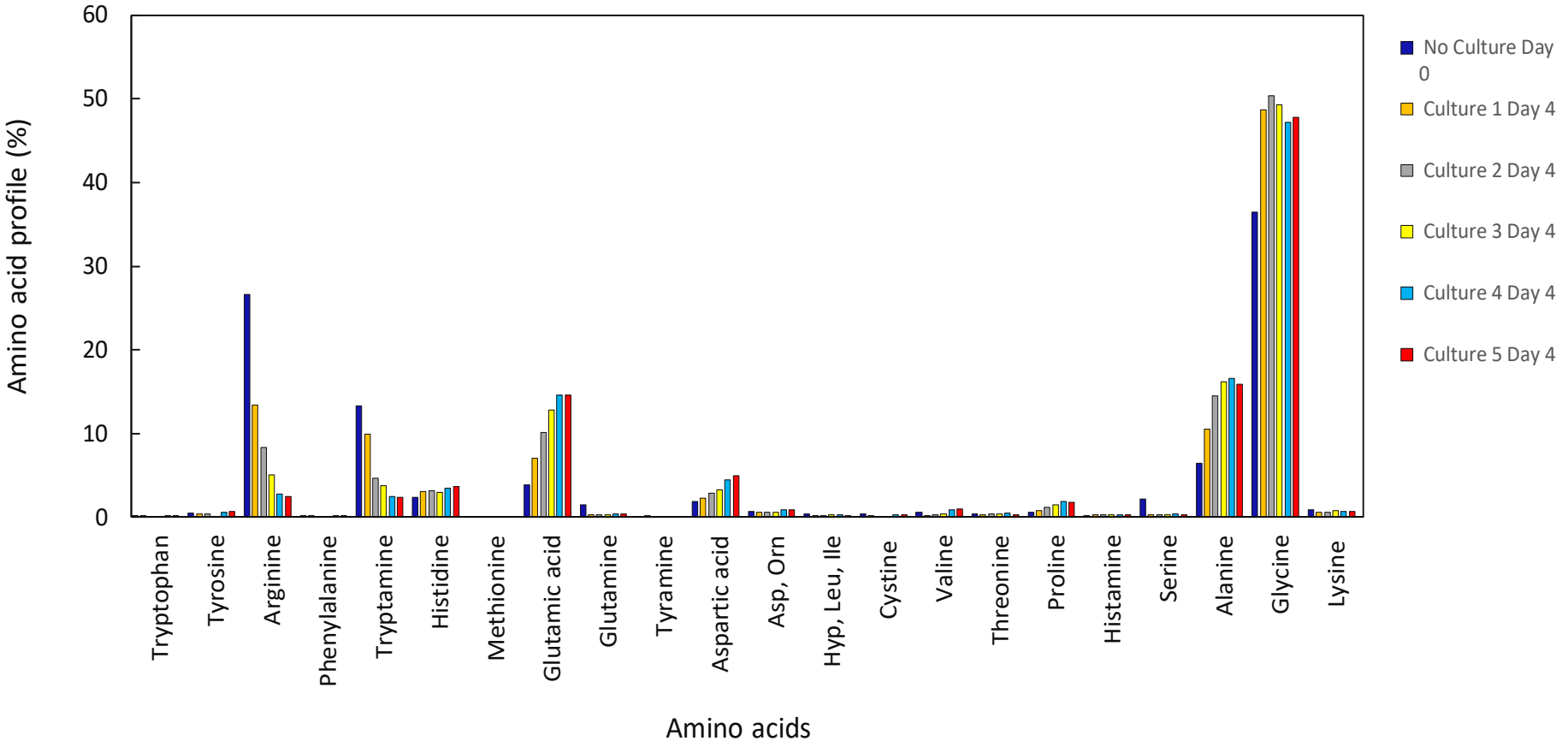


Figure 39 Molar percent amino acid profile before fermentation at Day 0 and after fermentation at Day 4. Bars are means of three replicate fermentation bags each with multiple injections, showing the effect of different starter cultures indicated in Table 4. Standard deviations are not shown for clarity.

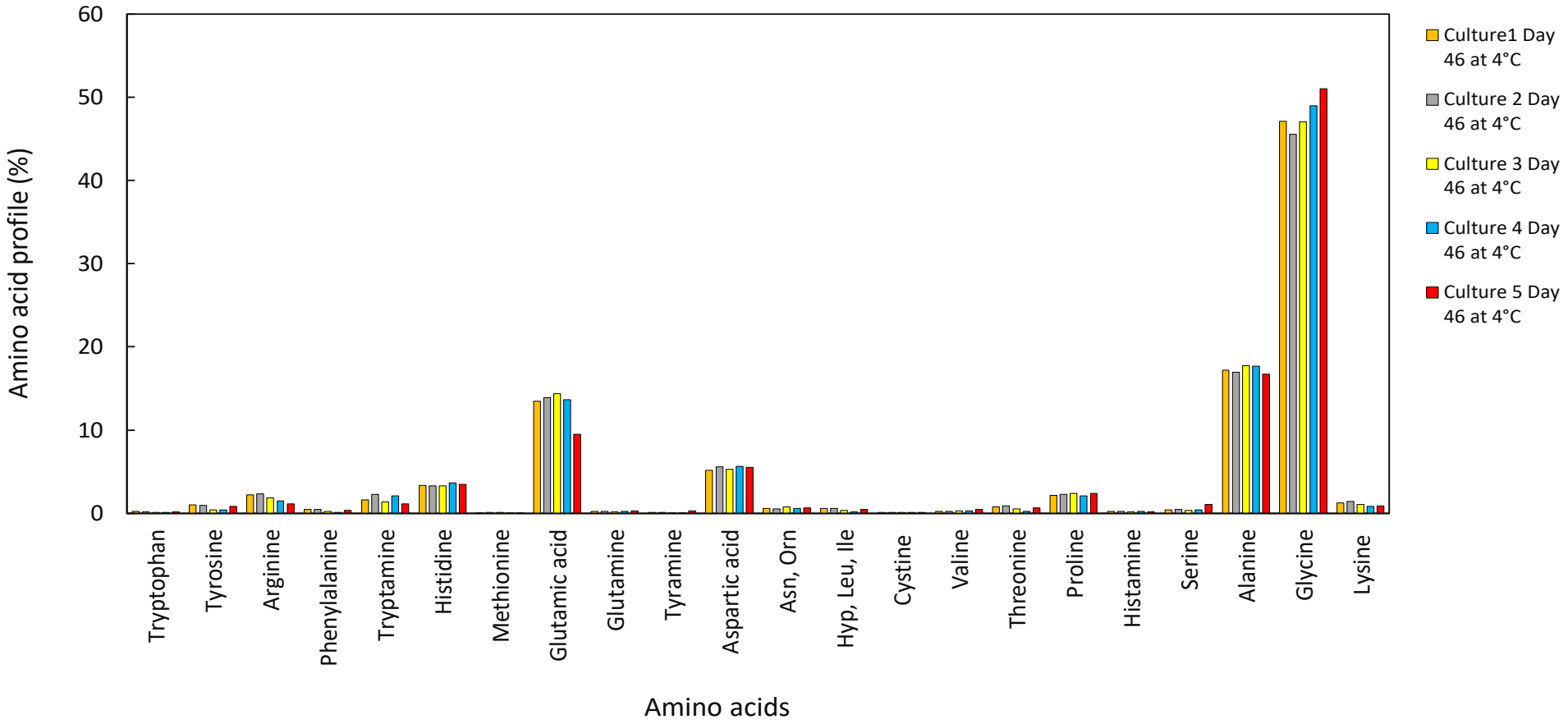


Figure 40 Molar percent amino acid profile at Day 46. Bars are means of three replicate fermentation bags each with multiple injections, showing the effect of different starter cultures indicated in Table 4.

Discussion

Amino nitrogen, UV absorbance and soluble protein

Amino nitrogen was used in this study as measure of amino groups that were chemically available. During fermentation protein tends to hydrolyse to peptides and amino acids, but that is no guarantee that these will remains as stable entities due to reactions like the Maillard reaction (Peralta et al., 2008), and as a substrate for the microbiota whether from culture or contaminating microorganisms. The outcomes are not known but will be diverse. Figure 25 showed that the rate of hydrolysis was positively affected by temperature between Day 11 and 18, particularly when stored at 35°C. Although the microorganisms involved in fish sauce manufacturing will be different from those used to ferment *Perna*[10], temperature has clear effects on protein hydrolysis and amino nitrogen content of fish sauce increasing with temperature (Jiang, Zeng, Zhu, & Zhang, 2007; Tungkawachara, Park, & Choi, 2003; Xu, Xia, Yang, & Nie, 2010). This could possibly be due to growth of fermenting microorganisms having the optimum growth temperature of around 35°C. In lactic fermented beef using a commercial culture, Zhou et al. (2017), showed that protein hydrolysis proceeded strongly at 35°C, probably due to the continuing viability or growth of microorganisms and the resulting secreted proteases.

For the two lower storage experiments, the amino nitrogen subsequently fell by Days 46 and 74, but the fall was not marked. When ambient and 4°C data were pooled for Days 11 and 18 (designated early) and compared with equivalent late data (Days 46 and 74), the difference was significant ($P < 0.03$). It is proposed that the drop represents the unspecified chemical changes noted in the previous paragraph. A decrease of amino nitrogen after 25 days of Antarctic krill fermentation (18% salt) at 35°C was observed by Fan et al. (2017). They suggested this could be due to microorganisms consuming the readily available amino nitrogen produced earlier in the fermentation process. Extending this argument, the amino nitrogen might remain stable from Day 46 to 74 because there would be no more growth of microorganisms and no further consumption of amino nitrogen. However, this argument is unproven.

[10] The salt content of typical fish sauce ferments is more than 10%; lactobacilli cannot grow above about 9% salt (Xiong et al., 2016).

The mean UV absorbance of trials between 275 to 292 nm (Figure 26) and 200 to 400 nm (Figure 27) both showed a trend of an initial decline to Day 40 and a subsequent slow increase to Day 130. Fewer data were available for these later days, so the increase may or may not be meaningful. Overall however, the capacity to absorb UV light should largely unaffected with time as long as the aromatic rings of tyrosine and tryptophan remained intact. Thus it is not easy to explain the initial decline to Day 40. The similarity in trend and absolute values between 275 to 292 nm (Figure 26) and 200 to 400 nm (Figure 27) indicates that proteinaceous matter was dominant in the water-soluble portion of *Perna* rather than nucleic acids. Nucleic acids absorb strongly in the region of 240 to 275 nm, with a maximum at 260 nm. If these acids were present in large quantities, Figure 26 and Figure 27 would be markedly different. They were not. The sources of nucleic acids will be the mussel itself, the biota of its digestive tract and the culture organisms. Extraction of nucleic acids requires aggressive solvents like phenol-chloroform. Heating is likely to be insufficient and cellular matter from the mussel and microorganisms would be lost from the filtrate by intense centrifugation, and filtration of dead or alive but intact cells through the 0.2 μm filters. A 0.20 or 0.22 μm filter is a standard method for bacterial (but not viral) sterility (FDA, 2004).

Figure 28 shows soluble protein values up to 130 days at 4°C. The bicinchoninic acid assay measures proteins and peptides, but peptides smaller than 2,000 Da cannot be quantified for reasons described earlier in this chapter. Several effects could be at work here. Many investigators have reported that acidic pH can increase the activity of proteolytic enzymes, especially cathepsins (Bover-Cid et al., 2000; Molly et al., 1997; Riebroy, Benjakul, Visessanguan, Erikson, & Rustad, 2008), perhaps accounting in part for the decrease as peptides below 2,000 Da might accumulate. Another effect might be that protein denaturation under acidic conditions generates secondary/tertiary protein structures where hydrophobic acids become exposed in otherwise water-soluble proteins (Frias, Song, Martínez-Villaluenga, De Mejia, & Vidal-Valverde, 2007; Meinlschmidt, Ueberham, Lehmann, Schweiggert-Weisz, & Eisner, 2016; Rui et al., 2019; Song, Kim, & Kim, 2008).This leads to precipitation in aqueous environments. During fermentation of soya beans, acids produced by LAB reported to interrupt soluble protein complexes, causing formation protein aggregates with reduced solubility (Rui et al., 2019). Fermentation of soy resulted in a drop in soluble protein ranging between 54 and 93% (Rui et al., 2019). Zhou et al. (2017), also observed insoluble proteins to increase at the expense of water-soluble proteins as lactic fermentation progressed in beef, but that study was limited to fermentation

to 32 hours only. In my study, there was certainly a substantial drop between Day 0 and Day 18, and that continued at a slower rate to 130 days. Overall, the loss in soluble protein is clear (Figure 28), but the cause(s) is not proven.

How can this loss in soluble be reconciled with the amino nitrogen results for 4°C and ambient (25) and UV absorbance (Figure 26), where losses are either minimal (Figure 25) or small gains are observed to Day 130 (Figure 26)?

The answer may be that as peptides below 2,000 Da accumulate with time, they would no longer be measurable by the BCA assay but would remain measurable by amino nitrogen (with the potential of some Maillard reaction losses) and UV absorbance. UV absorbance would be the least affected by proteolysis because absorbance due to aromatic amino acids would occur whether peptide-polymerized or not.

Amino acid analysis from fermentation with culture BFL-F02

There is little knowledge in the scientific literature on the protein and on the seasonal variation in protein. Compositional research has concentrated on fat content and fatty acid profile in New Zealand green shell mussels. One qualitative study on protein in mussels by Scotti, Dearing, Greenwood, and Newcomb (2001) reported that green shell mussel haemolymph is dominated by glycoprotein termed pernin, which is rich in histidine and aspartic acid.

Miller and Tian (2018), investigated seasonal changes over a year in proximate composition in green shell mussel. They looked at changes in the proximate composition of mussels over a year from April 2016 through to March 2017. There were clear differences between seasons, the most marked being a low concentration of protein and carbohydrate in July 2016 (12 % w/w for protein). While the proximate composition of green shell mussel related well with temperature data, Miller and Tian (2018) suggested that the condition was related to the abundance of food in the region (i.e., increased phytoplankton growth) and the role of temperature as a driver for conditioning and spawning.

Vlieg (1988), found that green shell mussels proximate protein content composition of 11.9 (w/w), which is in general agreement with comprehensive data in Appendix VII. The New Zealand database reports 10.7% (w/w) for raw mussels (Sivakumaran, 2017).

There are still fewer studies on protein in greenshell mussel at a more detailed level. Mateparae (2003), found that the adductor muscle, which comprises only 10% of mussels mass, contained only 1.4% collagen, based on its low hydroxyproline content (1.59%),

determined after acid hydrolysis.

Seasonal changes related to the sexual cycle occurs in mussels (Wisely, 1981) and these affect the protein content with season. Zandee, Kluytmans, Zurburg, and Pieters (1980), analysed the mussel *Mytilus edulis* from Europe's North Sea and found little seasonal variations found in the total free amino acid concentrations from both the total tissues and different organs. However, the individual amino acids showed clear seasonal changes that will be discussed later in all tissues, some amino acids showed an inverse relation to each other.

In addition to the Zandee study, there two comprehensive free amino acid studies on a European mussel *Mytilus galloprovincialis* sourced from Spain by Babarro, Reiriz, Garrido, and Labarta (2006), and Fuentes, Fernández-Segovia, Escriche, and Serra (2009). Their data were based on aggressive extraction methods (200 mM perchloric acid and 100 mM HCl, respectively) on whole raw mussels, whereas the extraction used here was on cooked mussels using water only. As pointed out earlier water was the solvent chosen because in food as consumed, water is the solvent of interest. Moreover, the heat involved in cooking will have denatured endogenous proteases, which could continue to generate free amino acids, and destroy most of the mussel enteric microorganisms. Those that survive, as well as the culture bacteria, would be removed from the final extract by the filtration step at 0.1 μm.

Table 8 compares the free amino acid data from the present study, expressed as μmol g^{-1} wet weight after cooking, compared with data from raw *Mytilus galloprovincialis* where data are expressed as μmol g^{-1} dry weight[11]. Moisture content was not determined in the present study, but assuming a 80% moisture content in *Perna*, the free amino acid expressed on a dry weight basis is comparable to the other data in Table 8. Differences can arise from the different species, different extraction methods and the fact that *Perna* was prepared from cooked mussels immersed in water that is discarded. Moreover, the heating associated with cooking accelerates the Maillard reaction that occurs most readily with amino acids in the free form.

[11] Data in Fuentes et al. (2009) were initially expressed as g per 100 g dry weight but were converted to a mole basis.

Table 8 Comparison of free amino acids (FAA) in mussels for current study and literature.

Free amino acids[1]	Mean FAA for current study, averaged over whole year µmol g^{-1} wet weight	Mean FAA by Fuentes et al. (2009), sampled in summer µmol g^{-1} dry weight	Mean FAA by Babarro et al. (2006), sampled in winter µmol g^{-1} dry weight
Tryptophan	0.062	4.03	0.62
Tyrosine	0.426	3.57	3.06
Arginine	10.10	23.19	5.39
Phenylalanine	0.187	1.31	0.27
Histidine	1.31	4.38	0.90
Methionine	0.025	2.97	0.61
Glutamic acid	4.97	11.47	8.01
Glutamine	0.937	0.00	5.87
Aspartic acid	2.80	8.29	16.40
Asn, Orn	1.58	4.49	2.10
Hyp, Leu, Ile	0.196	6.14	1.58
Cystine	0.165	0.00	0.37
Valine	0.619	2.32	0.94
Threonine	0.380	2.97	6.81
Proline	0.881	8.40	2.95
Serine	1.24	8.69	4.26
Alanine	3.20	25.18	21.89
Glycine	7.79	66.87	91.33
Lysine	0.216	8.21	1.07

[1] Data in Table 8 have been grouped to match the profile as presented in the present study where not all amino acids were resolved.

Data from Fuentes et al. (2009) and Babarro et al. (2006) were significantly correlated with data in the present study correlation (r = 0.748, $P < 0.001$ and r = 0.579, p = 0.009, respectively. When the Fuentes and Babarro data were arranged in ascending order of abundance, three of the four amino acids were present in all three data sets.

When the top four were removed from the data (Table 9 and the correlations recalculated, the high correlations were lost (r = 0.428, P = 0.112 and r = 0.425, P =0.114 respectively). Therefore, most of the correlation was driven by the four highest values for amino acids that dominated the profiles. In the study by Zandee et al. (1980) on *Mytilus edulis*, glycine was the most dominant free amino acid, followed by alanine, serine, glutamine/glutamic acid and aspartic acid.

Importantly free amino acids constitute an important fraction of nonprotein nitrogenous compounds in shellfish muscle. The free amino acids alanine, glutamic acid and glycine are greatly responsible for the flavor of cooked shellfish. Cha, Kim, and Jang (1998), report that alanine and glycine contribute to sweet tastes, arginine to bitter tastes, and glutamic acid to the 'umami' taste. The data sets presented here collectively suggest that these amino acids are important definers of mussel flavour.

Table 9 The four dominant free amino acids (FAA) arranged in ascending order of abundance based on Fuentes et al. (2009) and Babarro et al. (2006) each compared with the present data.

Names of free amino acids	Mean FAA by Fuentes et al. (2009) µmol g^{-1} dry weight	Mean FAA for current study µmol g^{-1} wet weight	Names of free amino acids	Mean FAA by Babarro et al. (2006) µmol g^{-1} dry weight	Mean FAA for current study µmol g^{-1} wet weight
Glutamic acid	11.47	4.97	Glutamic acid	8.01	4.97
Arginine	23.19	10.10	Aspartic acid	16.40	2.80
Alanine	25.18	3.20	Alanine	21.89	3.20
Glycine	66.87	7.79	Glycine	91.33	7.79

Taurine is a sulphur-containing amino acid which is not incorporated into protein, but rather occurs as the free amino acid or in simple peptides. It is known to be highly concentrated in seafoods especially those derived from invertebrates such as molluscs and

crustaceans (Dragnes, Larsen, Ernstsen, Mæhre, & Elvevoll, 2009) but has no marked flavour on its own. In the current study a taurine external standard was not included in the calibration mixture, so while almost certainly present, it was not identified.

As discussed earlier there was no control over mussel source given that this project is primarily about food, not mussel physiology. However, there appears to be a link with mussel spawning and associated energy requirements. From the results for the effect of season on concentration of free amino acids (Table 7, Figure 34, 35 and 36) three patterns were established. In Pattern 1 (Table 7) the overall free amino acid concentration was high, but the arginine proportion was low (spring); in Pattern 2 (summer, autumn and early winter) the overall free amino acid concentration was intermediate to high, and the proportion of arginine was very high; in Pattern 3 the amino acid concentrations were low and the proportion of arginine was very low.

Many studies have reported the environmental condition that may affect the variant of free amino acid such as salinity changes (Blank, Bastrop, Röhner, & Jürss, 2004; Matsushima & Hayashi, 1992; Paynter, Pierce, & Burreson, 1995; Pierce, Rowland-Faux, & O'Brien, 1992), environmental and developmental changes (Powell, Kasschau, Chen, Koenig, & Pecon, 1982; Zurburg & De Zwaan, 1981), food supply (Welborn & Manahan, 1995), parasitism (Paynter et al., 1995), reproduction (Kasschau & McCommas, 1982) and pollutants (Hummel et al., 1996). These factors acting synergistically and antagonistically lead to specific qualitative and quantitative free amino acid compositions (Kube, Gerber, Jansen, & Schiedek, 2006; Zurburg, Hummel, Bogaards, De Wolf, & Ravestein, 1989). Kube, Postel, Honnef, and Augustin (2007), worked with populations of Baltic clam and *Mytilus* mussel species in Europe distribution showed the highest free fatty acid concentrations summer and lowest in winter, paralleling results found here. In temperate climates daylight hours and light intensity is lowest in winter, so the food supply for mussels is lowest.

Ogilvie, Ross, and Schiel (2000), reported that within Marlborough Sounds farms in New Zealand, the depletion of phytoplankton is most likely to occur in winter. This is likely to occur at other sites too in New Zealand simply on account of daylight hours and light intensity. R. W. Macdonald et al. (2000), found that site and season had the most influence on raw green shell mussel composition, probably due to differences in feed availability with site and time of year. Therefore, low food availability combined with spawning is a likely reason for the low free amino acid concentrations in Pattern 3 (Figure 36). At this time of the year

the mussels can be described as ‘exhausted’. That the proportion of arginine was very low cannot be explained by the fact phospho-L-arginine is one of the compounds that serve as rapidly available store of phosphate bond energy in invertebrates (Bies & Newsholme, 1975; Grieshaber, Hardewig, Kreutzer, & Pörtner, 1993), because after the phosphate group phosphorylates ADP to form ATP, the arginine remains. The explanation may simply be that arginine with its four nitrogen atoms on the side chain is preferentially metabolized as a nitrogen source. Glycine has been reported to fluctuate with reproduction cycle in marine invertebrate (Kasschau & McCommas, 1982; Zurburg et al., 1989), also possibly serving as energy source. The proportions of arginine are not so easy to explain for Patterns 1 and 2, but this model is proposed: In spring, Pattern 2, the amino acids are recovering but nitrogen is still limiting, and glycogen levels are low. Thus, any excess nitrogen in arginine is metabolized to other amino acids by transamination reactions, and with low glycogen levels the total proximate profile is dominated by proteins, peptides and amino acids. Thus, on a wet weight basis the concentrations of free amino acids are highest in Pattern 1. By summer the food supply is no longer limiting, glycogen is abundant, so the free amino acid concentration is slightly lower. At the same time arginine can accumulate as an energy reserve.

It must be emphasized however, that with no control over the mussel supply and limited replication for particularly Pattern 3, the model presented is somewhat speculative. Figure 38 summarized the fate of the free amino acids for Pattern 1, 2 and 3 at Day 4, by which time the effects of the fermentation microorganisms is expected to swamp any effect due to season. Comparing the Day 0 profile Figure 37 with that in Figure 38 shows a marginal increase in the concentrations of several amino acids, but a marked increase in arginine concentration for Pattern 1.

There is no obvious explanation for this, and no further data for later days are available. Moreover, the results in Figure 29, 30 and 31 show that arginine is a fermentation target of microorganism(s), presumably because of its high nitrogen content. This was particularly clear Figure 29 where there was a delay between batch inoculation and vacuum packing. The proportion of arginine decreased with time. Thus, the high value for arginine in Figure 29 remains unexplained. In the following discussion on the outcomes of the five different cultures, some further insights on arginine will be revealed in respect of longer storage.

Amino acid analysis from fermentation with five fermentation cultures

Textbook descriptions of metabolic pathways between amino acids like arginine, proline, ornithine, histidine and glutamic acid, show that depending on physiological conditions, metabolic flux is reversible (Ochs, 2014). That particular textbook is generic to organisms, while more specific individual research papers dealing with microorganisms give more detail relevant to the results reported here. Thus, work by Ough, Crowell, and Gutlove (1988) and Derman, Soderholm, Lindstrom, and Korkeala (2015) on yeasts demonstrates a catabolic pathway from arginine to glutamic acid and proline. Both LAB and coagulase-negative staphylococci – of which *Staphylococcus carnosus* is an example – are able to catabolize arginine, which is an obvious nitrogen source (Figure 41).

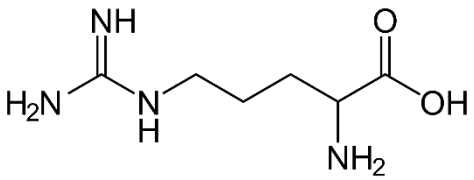


Figure 41 Structure of arginine showing the nitrogen-rich side chain.

Gaenzle (2015), reported that low pH in fermentation medium shifts lactic acid bacteria metabolism to from hexose fermentation utilization of amino acids. Glutamine, glutamate, and arginine play a major role in pH homeostasis and stationary phase survival of lactic acid bacteria Konings (2002), a situation that might be expected by Day 4 of fermentation. Inspection of Figure 40 shows that, depending on the culture used, there was a reciprocal relationship between arginine and glutamic acid. Where arginine was low, glutamic acid was high. Similarly, proline appeared to increase. Alanine and aspartic acid also increased although the links to amino acid metabolism are not clear from the literature. Importantly loss in arginine appears to be the main driver of these changes, but it is also interesting to note that the biogenic amine, tryptamine decreases in concert with arginine. Tryptamine too is a possible source of nitrogen.

It is suspected that at Day 4 the culture microorganisms were in the act of establishing an equilibrium to suit their survival in a steady state situation where lactic acid has accumulated creating an acidic environment. In respect of the individual cultures, this model

would suggest that Culture 4 and 5 were the fastest metabolizers[12].

Now, inspection of Figure 40 at Day 46 shows that the arginine and tryptamine proportions were all low, all glutamic acid proportions were high and similar, and proline proportions were all similar. The implication is sometime between Day 4 and Day 46, a very similar and stable metabolic environment was established for all five cultures. The results also suggest that *Perna* flavour due to amino acids would be the same for all the cultures.

Biogenic amines

Biogenic amine are organic molecules with low molecular weight, biologically active and normally formed by bacterial decarboxylation of their precursor amino acids. Thus, decarboxylation of amino acids other than glutamic acid generates biogenic amines with adverse implications for human health when consumed in concentrations above certain levels (Lee et al., 2016). The production of biogenic amines requires the presence of microorganisms possessing amino acid decarboxylating activity (Santos, 1996). Some of Enterobacteriaceae, LAB and staphylococci have been reported to contribute to biogenic amine formation in dry-cured sausages, and the same is likely to be true in *Perna*.

The important biogenic amines in seafoods are histamine, tyramine, tryptamine, putrescine (from ornithine), and cadaverine (from lysine) (Biji, Ravishankar, Venkateswarlu, Mohan, & Gopal, 2016). The physiological role of biogenic amine synthesis mainly seems to be related to defense by bacteria to withstand acidic environments (Lamberti et al., 2011; Spano et al., 2010).

Food safety is regulated by limiting the amount of histamine permitted in fish flesh with the Australasian standard requiring less than 200 mg kg^{-1} histamine (FSANZ, (2010) while other administrations require as low as 50 mg kg^{-1}, the upper limit for my *Perna*.

[12] It is restated that the 1 to 5 regular pattern in Figure 39 was by chance alone.

Table 10 Concentration of biogenic amines before fermentation and after fermentation with different starter cultures.

Culture	Day after preparation	Tryptamine		Tyramine		Histamine	
		μmol g^{-1}	mg kg^{-1}	μmol g^{-1}	mg kg^{-1}	μmol g^{-1}	mg kg^{-1}
None	0	8.63	1380	0.009	1.00	0.16	1.83
1	4	3.67	588	0.064	8.80	0.10	1.14
2	4	1.67	267	0.010	1.40	0.09	0.95
3	4	1.04	166	0.005	0.70	0.07	0.79
4	4	0.71	110	0.003	0.40	0.07	0.83
5	4	0.78	120	0.004	0.60	0.08	0.86
1	46	All below detection limit					
2	46						
3	46						
4	46						
5	46						

The cooking step in *Perna* production may not have destroyed all Enterobacteriaceae, which are well known for histamine formation, but because they have high sensitivity to acidity (Lücke, 1986) they are unlikely to be present in fermented *Perna* to produce the amines, if any. What biogenic amine formation might occur must happen during the hours involved in cooking, mincing etc. and/or in the early stages of fermentation Table 10 shows the concentration of these compounds on Days 0, 4 and 46.

The initially high concentrations of tryptamine is a concern but the regulations ignore that amine. The reason for the high concentration, particularly at Day 0 is unknown. In the previous seasonal experiments the concentrations of tryptamine – and other biogenic amines – were negligible. Any of these *Perna* preparations would have passed biogenic amine limit criteria.

In Table 10 it is clear that biogenic amines were not formed on fermentation and indeed the concentration of tryptamine declined with time (Table 10) to zero by Day 46. The fermented meat literature reports the ability of culture bacteria to variously inhibit biogenic

amine formation (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2001; Petäjä, Eerola, & Petäjä, 2000) and amine oxidizing ability(Dapkevicius, Nout, Rombouts, Houben, & Wymenga, 2000; Mah & Hwang, 2009).

Culture 5 has been shown to be a standout in terms of changing the amino acid profile quickly, as is clear in Figure 39 for example. In later Chapter 8, Culture 5 is shown to be a standout in other respects too. In the meantime, fatty acid (Chapter 5) and other analyses (Chapters 6 and 7) will be described.

Chapter 5

Fatty acids in *Perna*

Introduction

Many nutritional theories are focused on the health implications of the fatty acid profile of the diet. The central dogma of fatty acid profiles and health are that saturated fats are considered the least desirable, whereas omega-6 and omega-3 fatty acids are more desirable, particularly the latter. Both omega-3 and omega-6 fatty acids are essential in the human diet and have well described positive metabolic functions (see e.g.(Alexander, Miller, Van Elswyk, Kuratko, & Bylsma, 2017; Mazereeuw et al., 2017; Simopoulos, 2011). The polyunsaturated fatty acids (PUFA) dominate the literature of health effects. The main dietary PUFAs are linoleic (18:2n6) and arachidonic (20:4n6) acids for the omega-6 class, and alpha-linolenic (18:3n3), eicosapentaenoic (20:5n3; EPA) and docosahexaenoic acids (22:6n-3, DHA) for the omega-3 class.

Perna canaliculus contains around 2% fat, of which a remarkably high one-third comprises EPA and DHA (M. Miller et al., 2014; K. J. Murphy et al., 2002). Polyunsaturated fatty acids are particularly unstable to oxidation and it was of interest to know the fate of the fate of fatty acid in the *Perna* process, comprising, cook, comminute, ferment under vacuum and store under vacuum.

Materials and methods

The gas chromatographic procedure has been described in detail in Chapter 2, but in outline given here. The Bligh and Dyer (1959) method was used for fat extraction. The fatty acids in that fat were trans esterified as methyl esters before gas chromatography. Methyl esters were separated on a Phenomenex FAME-WAX capillary and detected by flame ionization. A Supelco® 37 Component FAME Mix was the qualitative standard used to identify fatty acid methyl esters in mussel lipid extracts. Data are presented as percent of total by weight.

Results

Thirty-two fatty acids were identified in unfermented and fermented *Perna* stored at three temperatures (4°C, ambient and 35°C) to Day 74. They comprised 13 saturated fatty

acids and 19 unsaturated fatty acids (Appendix VII). The fatty acids were dominated by palmitic acid (C16:0), docosahexaenoic (DHA) (C22:6n3) and eicosapentaenoic acid (EPA, C20:5n3 Figure 42, 43 and 44. Other fatty acids present between 10 and 2% of the profile were palmitoleic, stearic, linoleic, pentadecanoic, cis-11,14-eicosadienoic (C20:2n6), henicosanoic (C21:0) and linolelaidic (C18:2n6). All other fatty acids are reported in Appendix VII.

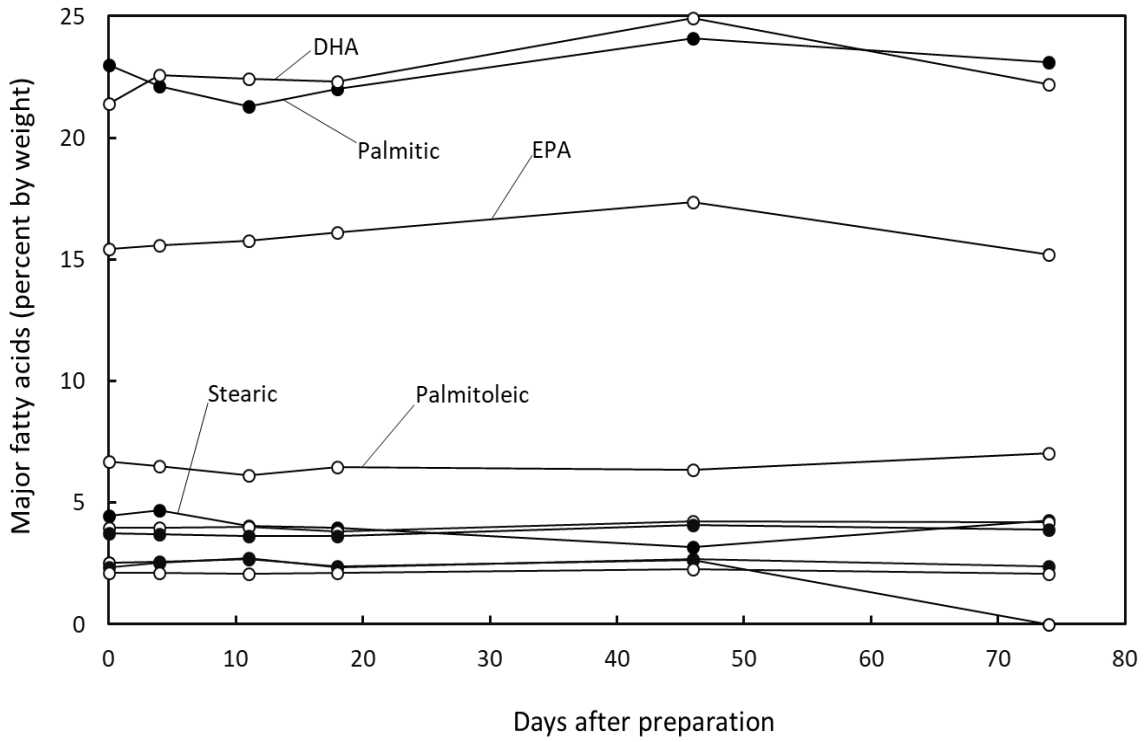


Figure 42 Fatty acid profile percent by weight for *Perna* from day of preparation, followed by fermentation for four days and then storage at 4°C to Day 74. The filled circles represent saturated fatty acids and unfilled represent unsaturated fatty acids. Other fatty acids with proportions lower than stearic acid were, in decreasing order, linoleic, pentadecanoic, cis-11,14-eicosadienoic, henicosanoic and linolelaidic.

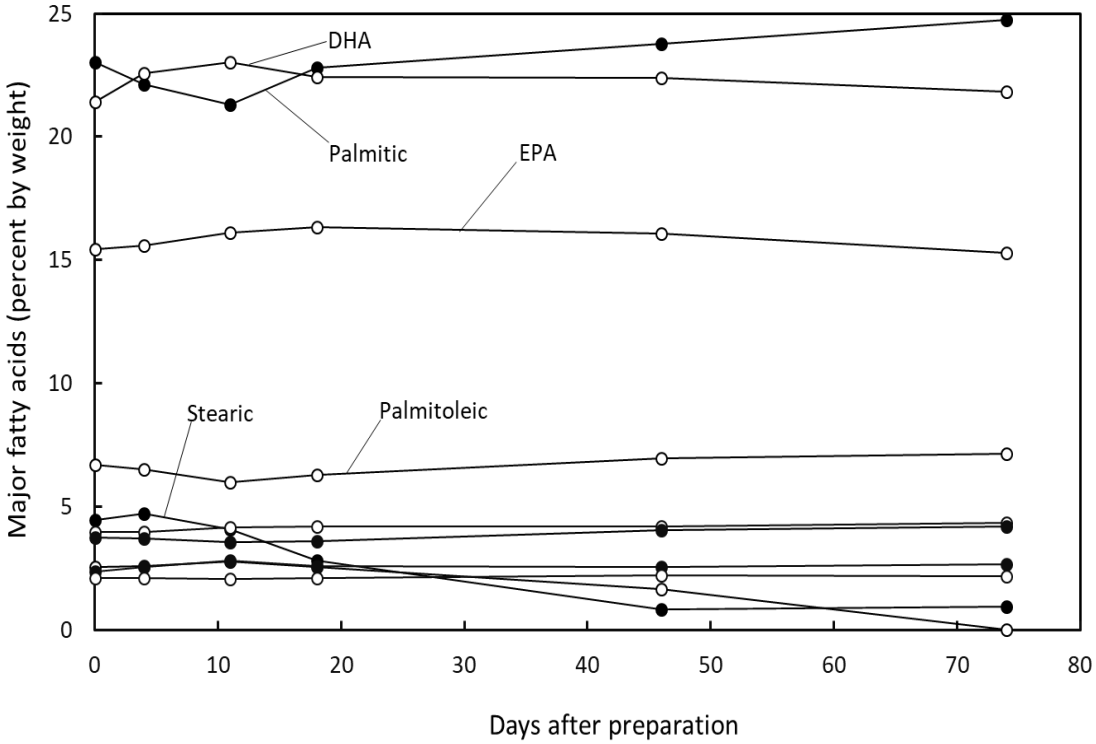


Figure 43 Fatty acid profile percent by weight for *Perna* from day of preparation, followed by fermentation for four days and then storage at ambient temperature to Day 74. The filled circles represent saturated fatty acids and unfilled represent unsaturated fatty acids. Other fatty acids with proportions lower than stearic acid were, in decreasing order, linoleic, pentadecanoic, cis-11,14-eicosadienoic, henicosanoic and linolelaidic.

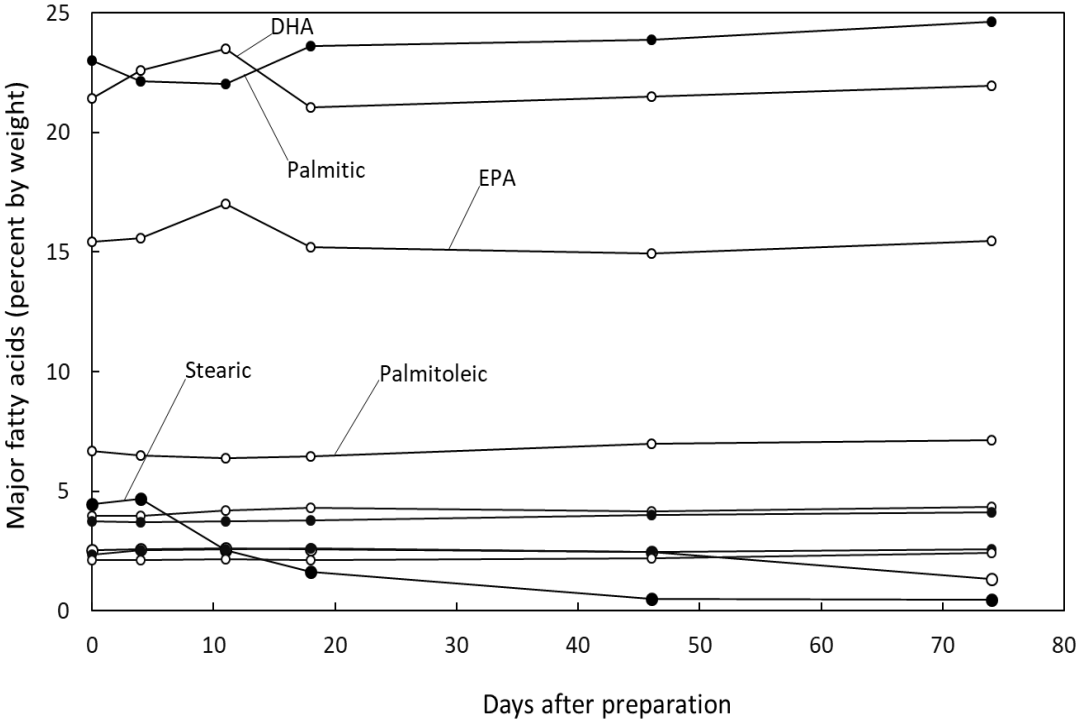


Figure 44 Fatty acid profile percent by weight for *Perna* from day of preparation, followed by fermentation for four days and then storage at 35°C to Day 74. The filled circles represent saturated fatty acids and unfilled represent unsaturated fatty acids. Other fatty acids with proportions lower than stearic acid were, in decreasing order, linoleic, pentadecanoic, cis-11,14-eicosadienoic, henicosanoic and linolelaidic.

Inspection shows that the fatty acids are stable in *Perna* to 74 days under all conditions. Other experiments to 102 days confirmed this enduring stability. The only fatty acid that appeared to change was stearic acid at ambient and 35°C.

Discussion

To date the fatty acid profile of mussels has focused on the polyunsaturated omega-3 fatty acids, particularly DHA and EPA. This is understandable given their good reputation in human health, particularly relating to arthritic and cardiovascular conditions (Filion et al., 2010; Halpern, 2006; M. Miller et al., 2014).

Numbers of products derived from *Perna canaliculus* fat extracts are available over the counter, each with their health claims (such as Seatone, Biolane, Lyprinol, and GlycOmega PLUS among others). Processing mussels to prepare these products will always carry the risk of fatty acid oxidation, because polyunsaturated fatty acids are very unstable. This risk will be compounded by the iron content of mussels (up to 280 µg g^{-1},(Nielsen & Nathan, 1975). Iron as ions is a well-known catalyst of fat oxidation (Ochs, 2014).

Although *Perna* is not an extract, the anaerobic fermentation step and subsequent storage under vacuum has proved to be a very good method of maintaining fatty acid stability. The loss of stearic acid at ambient and 35°C has no obvious explanation, but the fact that it was more stable at 4°C (4.2% at Day 0 and 4.3 % at Day 74) may reflect stearic acid's high melting point (69°C), which would affect molecular mobility of any triacylglycerol containing that fatty acid.

As to the origin of *Perna canaliculus* fatty acids, the obvious source is their diet gained by filter feeding microplankton (Safi & Hayden, 2010). Chuecas and Riley (1969), did a comprehensive study of the fatty acid profiles of a very wide range of marine phytoplankton, presumably sourced from the northern hemisphere. They showed that palmitic acid was the dominant saturated fatty acid in nearly all 27 species of phytoplankton, representative of eight classes. The diet of the mussels used here is unknown, but it seems likely that palmitic acid is sourced directly from their diet. The same applies to DHA and EPA. The ratio of DHA:EPA in mussels has previously been used as an indication of their diet (Budge & Parrish, 1998). Authors like M. Miller et al. (2014) and A. G. Taylor and Savage (2006) have shown varying ratios of EPA and DHA in *Perna canaliculus*, reflecting the abundance of different microplankton species in their diet in different locations and seasons. M. Miller et al. (2014) found an average ratio of 0.7, while A. G. Taylor and Savage (2006) found a ratio of 1.7. The ratio in the present study was 1.4. Overall the fatty acid profile presented no surprises, but importantly the fatty acids are very stable in the *Perna* process. The likely cause of this stability is the absence of oxygen due to vacuum packing from Day 0 onward.

Chapter 6

Volatiles compounds and the effect of glucose concentration

Introduction

SPME technique

SPME is simple, effective adsorption/desorption technique which eliminates the need for solvents or complicated apparatus for concentrating volatile or non-volatile compounds in liquid samples or headspace. A 10-mm coated fibre is exposed for a period to the headspace above a food then desorbed onto a gas chromatographic column for analysis. Highly specific coatings have been developed with a high affinity for target analyte and an excellent separation efficiency, making methods highly sensitive despite the small quantities of sorbent used in SPME. SPME is a non-exhaustive extraction technique in which only a small portion of the target analyte is removed from the sample matrix.

Methods

These have been described in detail in Chapter 2 but summarised below.

Choice of fibre

SPME fibers need to be coated with specific sorbents with a high affinity for target analyte. The polarity and volatility characteristics of the target compound are the primary issues addressed before selecting a fibre coating. The coating type of SPME fibers is primarily responsible for the extraction of analyte. The rule ‘like dissolves like’ applies in the selection of the appropriate SPME fibre for extraction. Thus, nonpolar analyte are most effectively extracted with a nonpolar fibre coating and polar analyte are most effectively extracted with a polar coating.

Volatiles were monitored by the headspace SPME method according to Tuckey et al. (2013) with minor modifications after optimization trials with dedicated divinylbenzene (DVB), carboxen (CAR), polydimethylsiloxane (PDMS) fibre coatings, ultimately settling on a combination DVB/CAR/PDMS fibre coating. A DVB/CAR/PDMS fibre coating absorbed the greatest number of volatile, with high responses. This is a common choice where the volatiles are a mixture of polar and non-polar materials (Boyce & Spickett, 2002).

Perna samples were weighed to 2.0 ± 0.1 g on base of 10 mL flat bottom vials fitted

with a septum. No internal standard was included because of the heterogeneity inherent in *Perna*. The fibre was exposed to the sample headspace for 10 minutes. The fibre was subsequently inserted into the Agilent GC injection port for analysis as described in Chapter 2. An Agilent Mass Hunter Workstation software instrument was used for data acquisition and analysis.

Data analysis

Data presentation presented as percent of mass spectral response, with no attempt to confirm identities with external standards. Patterns were of more interest.

To resolve differences due to culture and days of storage, the classical discriminant analysis routine in XLSTAT could not readily be applied to the data set because the quantitative variables were many (69) but the observations (five cultures, five days of storage) were fewer. For discriminant analysis, the reverse is required, fewer variables and more observations. To identify what compounds tended to characterise different cultures and days, the partial least squares (PLS) regression routine was used, resulting in equations that included all variables, the coefficients of which were positive, negative or close to zero. In the event, most were close to zero for all cultures and days and therefore did not contribute to discrimination. Strongly positive and negative variables were of interest and could easily be identified by sorting and by visualisation in correlation plots. The correlation plots in PLS plot explanatory variables – volatile compounds in this case – against the culture and day.

As will be seen, the volatiles' names are frequently loaded very closely together and very difficult to tease out. For this reason, in the figures and tables that follow from Table 11, compounds are number coded, e.g. C-7 (where C had to be included to satisfy the software). They occurred in groups that associated with different cultures and days, so it was possible to see how one culture was similar to another or otherwise. The numbers are decoded in the discussion.

As a mathematical technique, correlation does not depend on concentration, only relationships between values, so flavour compounds identified as being characteristic of, say, one culture are not necessarily present in high concentrations although they might be.

Results

Effect of culture, storage day and temperature on SPME volatiles

The concentrations of the volatile compounds obtained by GCMS showed in Figure 45

and 46 for storage at 4°C, presented in order of elution. Equivalent graphic results for ambient temperature storage are shown in the Appendix VIII the patterns observed were like those at 4°C. However, they have been selectively statistically analysed as is done for storage at 4°C (Table 11).

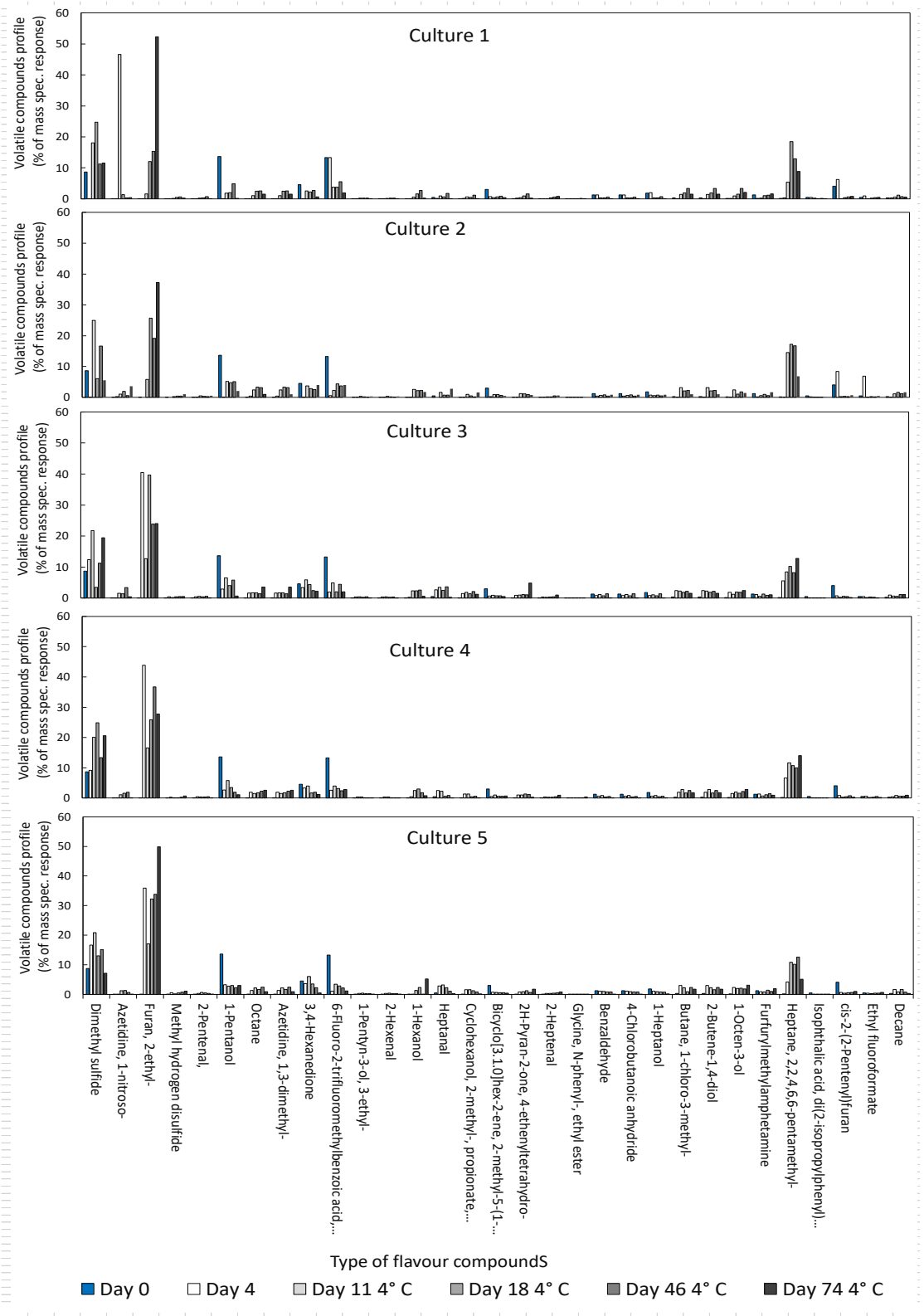


Figure 45 Volatile compound (1 to 31) of *Perna* at Day 0 and during storage for 74 days at 4°C with five cultures. Compounds are shown in order of elution. Day 0 is blue, with increasing intesity of grey from Dys 4 to 74, left to right.

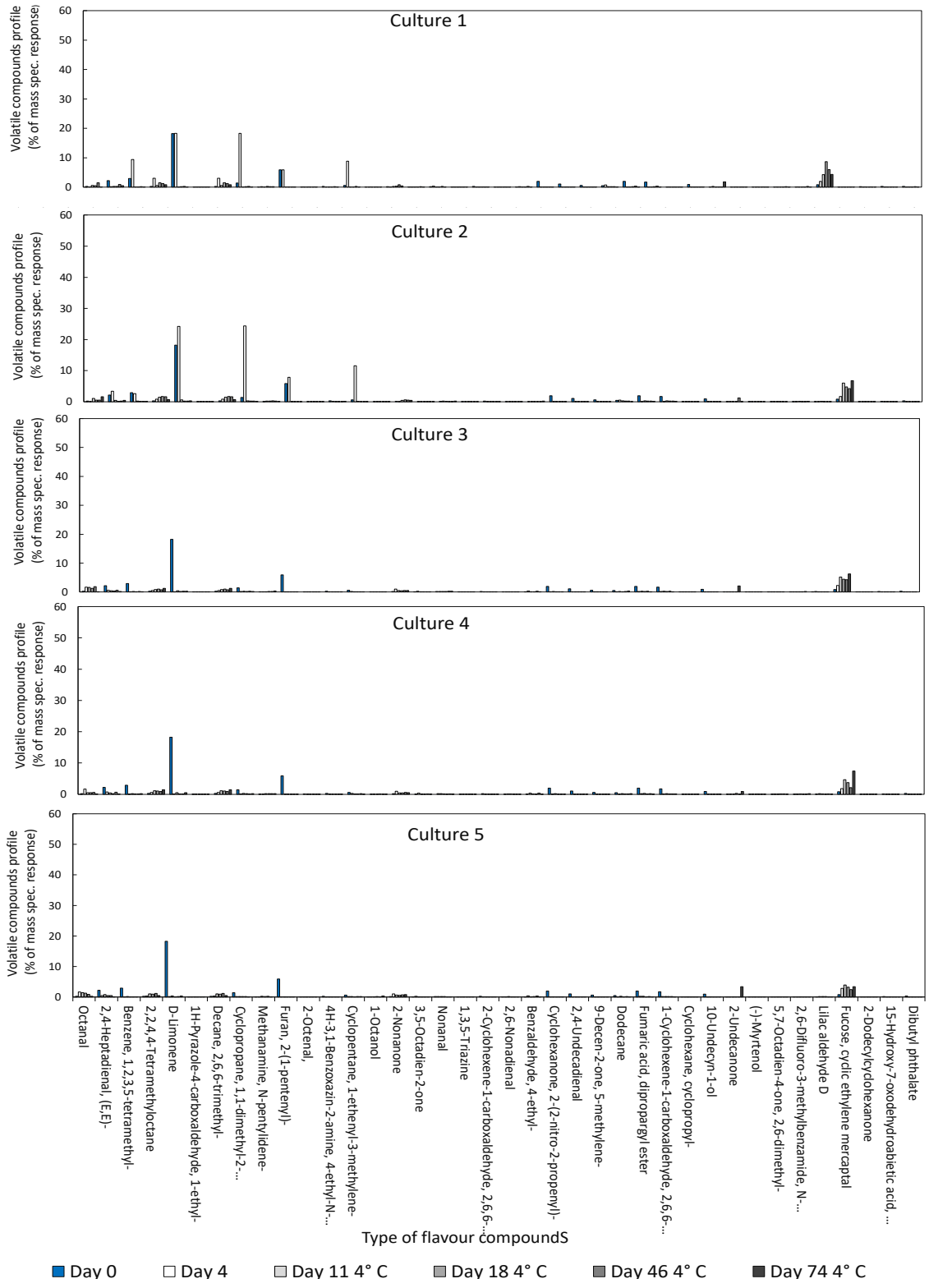


Figure 46 Volatile compound (32 to 69) of *Perna* at Day 0 and during storage for 74 days at 4°C with five cultures. Compounds are shown in order of elution. Compounds are shown in order of elution. Day 0 is blue, with increasing intesity of grey from Dys 4 to 74, left to right.

A total of 69 volatile compounds were tentatively identified by comparison to the NIST 14 database (NIST, 2014), 14 hydrocarbons, 10 alcohols, 15 aldehydes, 9 ketones, 4 amines, 7 esters and 11 other compounds, including sulphides and nitrogen compounds (Appendix VIII).

All cultures showed a big difference between Day 0 (no fermentation) and post fermentation days. After fermentation, the volatile pattern changed markedly, with some compounds relatively increasing, some decreasing and some static. A marked change does not necessarily mean that a compound is organoleptically important because the odour values (Varlet & Fernandez, 2010) are unknown in the context of the *Perna* matrix. Moreover, as noted above, volatiles bind to SPME fibres in a selective way. Some odorous compounds may not bind at all, but that is a limitation applying to all SPME work. Table 11 summarises six comparisons of the many possible. In outline, Day 0 is compared only with Day 4; cultures are compared only after fermentation; and post fermentation days are analysed Days 4 to 74. In the figures and tables that follow from Table 11, compounds are number coded according to likely chemical class (hydrocarbons, aldehydes etc.) that is not the same as the numerical order of elution. A final summary will show which of these compounds dominate the variation.

Table 11 A non-exhaustive analysis of SPME data to show some differences between cultures, days of storage and storage temperature.

	Shown in	
Comparisons of interest	Figure	Table
Day 0 versus Day 4 all cultures (storage temperature not relevant)	Figure 47	Table 12
Day 4 comparing cultures (storage temperature not relevant)	Figure 48	Table 13
Day 74 comparing cultures (storage at 4°C)	Figure 49	Table 14
Day 74 comparing cultures (storage at ambient)	Figure 50	Table 15
Days 4 to 74 pooled comparing cultures (storage at 4°C)	Figure 51	Table 16
Days 4 to 74 pooled comparing cultures (storage at ambient)	Figure 52	Table 17

Day 0 versus Day 4 all cultures (storage temperature not relevant)

The PLS correlation axis t2 (Figure 47) was able to resolve the effect of fermentation, such that the top compounds associated with fermentation were as shown in Table 12, at the relative expense of other compounds. Fermentation clearly made a difference. 2-Ethylfuran (C-7) and a heavily substituted cyclopropane, 1,1-dimethyl-2-(3-methyl-1,3-butadienyl)-cyclopropane (C-45), were dominant after fermentation.

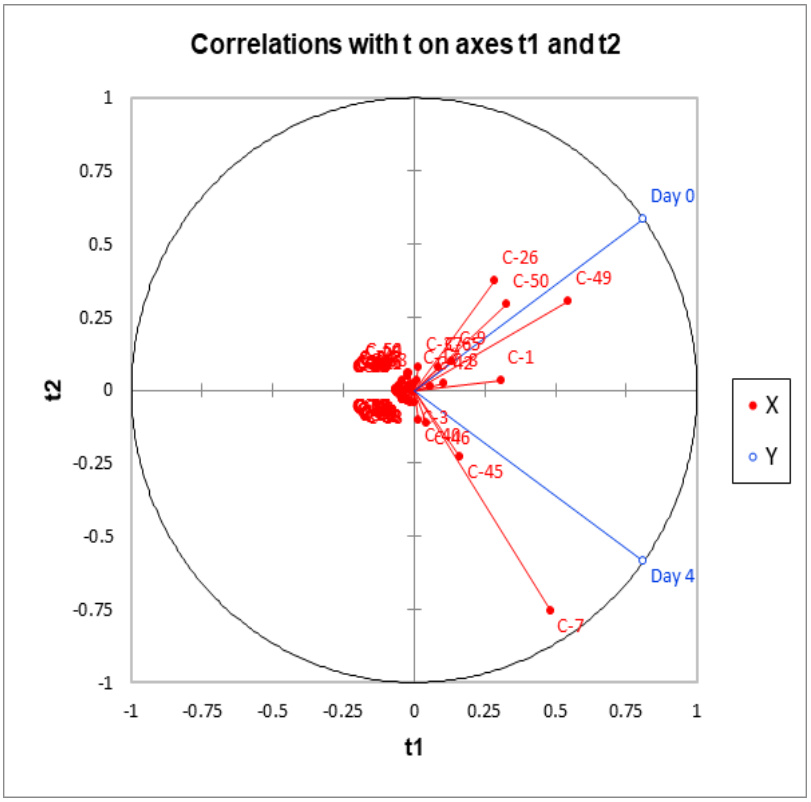


Figure 47 PLS correlation matrix comparing Day 0 with Day 4 for all cultures pooled

Table 12 Important identifiers of *Perna* fermentation, in descending of relative dominance.

Compounds high at Day 0	Compounds high at Day 4
C-26	C-7
C-49	C-45
C-50	

Day 4 comparing cultures (storage temperature not relevant)

Cultures 1 and 2 (BC1 and 2) (Figure 48) were clearly differentiated from Cultures 3, 4 and 5. Within group the volatiles profile was nearly identical, so one axis was sufficient to explain the data. The standout compound was C-7, 2-ethylfuran, almost absent in Cultures 1 and 2, likewise C1, dimethylsulfide (Table 13).

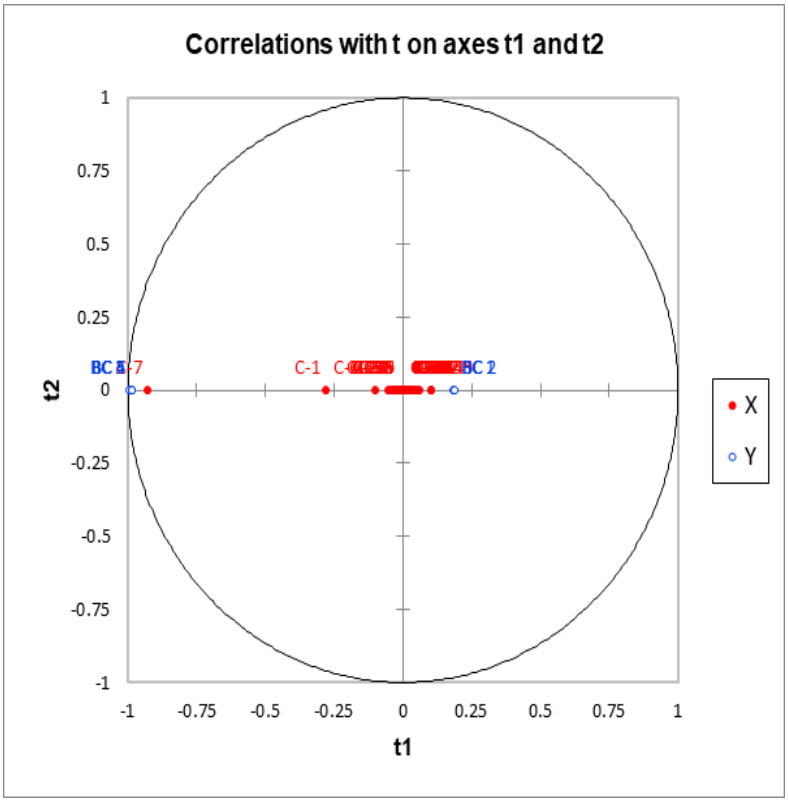


Figure 48 PLS correlation matrix comparing cultures on Day 4.

Table 13	Important identifiers of *Perna* culture at Day 4, in descending of relative dominance.
Compounds high for Cultures 1 and 2	Compounds high for Cultures 3, 4 and 5
Unresolvable	C-7 C-1

Day 74 comparing cultures stored at 4°C

Cultures 1, 2 and 5 (BC1, 2 and 5) were clearly differentiated from Cultures 3 and 4. 2-Ethylfuran (C-7) and dimethyl sulphide (C-1) (Table 14) were again highly differentiating. The alkane 2,2,4,6,6-pentamethylheptane (C-40 in Figure 49) was a feature of Cultures 3 and 4.

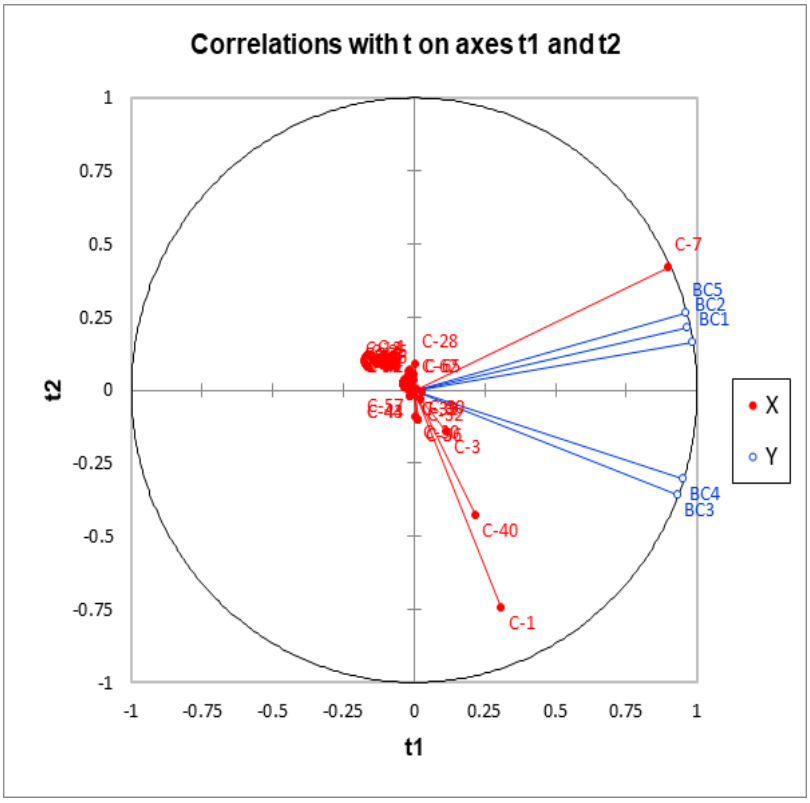


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Figure 49 PLS correlation matrix comparing cultures on Day 74, stored at 4°C

Table 14 Important identifiers of Perna culture at Day 74, 4°C storage, in descending of relative dominance.

Compounds high for Cultures 3 and 4	Compounds high for Cultures 1, 2 and 5
C-1 C-40	C-7

Day 74 comparing cultures (storage at ambient)

Whereas two culture groups were clearly identified at Day 74, 4°C storage, the equivalent ambient storage was more complicated. The standout cultures were 1 and 5 (Figure 50), characterized by dimethyl sulphide (C-1), 1-octen-3-ol (C-32) – a fat oxidation product, and 2,2,4,6,6-pentamethylheptane (C-40) (Culture 1), and 2-ethylfuran (C-7) (Culture 5) (Table 15). Other cultures (2, 3 and 4) were not characterized by these compounds but they were present.

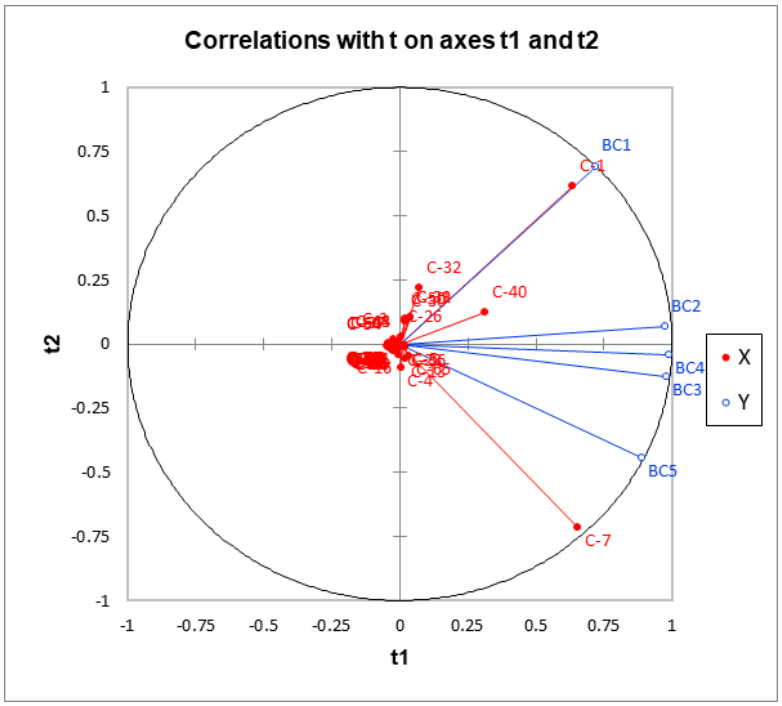

Figure 50 PLS correlation matrix comparing all cultures at Day 74, stored at ambient temperature.

Table 15 Important identifiers of *Perna* culture at Day 74, ambient storage, in descending of relative dominance.

Compounds high for Culture 1	Compounds high for Culture 5
C-1 C-32 C-40	C-7

Days 4 to 74 pooled comparing cultures (storage at 4°C)

Day 74 comparing cultures, two culture groups were clearly identified in pooled Days 4 to 74 at 4°C storage, the difference between these two groups were less comparing to equivalent 4°C storage (Figure 51). The standout cultures were 1 and 5, characterized by methyl hydrogen disulphide (C-2), 1-chloro-3-methylbutane (39), octane (36), 3-ethyl-1-pentyn-3-ol (27), lilac aldehyde (10), and 1-(1,3-dithiolan-2-yl) pentane-1,2,3,4-tetrol (3) in Culture 5 (Table 16). Other cultures (2, 3 and 4) were not characterized by these compounds but they were present.

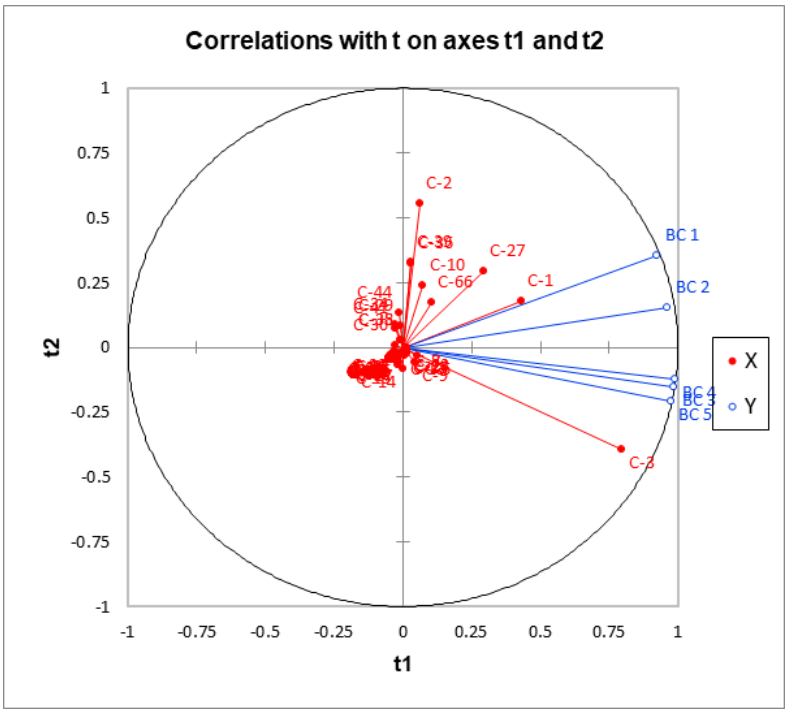

Figure 51 PLS correlation matrix comparing all cultures at Day 4 to Day 74, stored at 4°C temperature.

Table 16 PLS correlation matrix comparing all cultures from Day 4 to 74, stored at 4°C in descending of relative dominance.

Compounds high for Cultures 1 and 2	Compounds high for Cultures 3, 4 and 5
C-2	C-3
C-39	
C-36	
C-27	
C-10	

.

Days 4 to 74 pooled comparing cultures (storage at ambient)

Two culture groups were again identified in pooled Days 4 to 74 at ambient storage, but the difference between these two groups was slightly wider compared to equivalent 4°C storage (Figure 52). The standout cultures were 1 and 3, characterized by dimethyl sulphide (C-1), 1-chloro-3-methylbutane (39) and octane (36). Cultures 3, 4 and 5 were characterized 2-ethylfuran (7) and 1-(1,3-dithiolan-2-yl) pentane-1,2,3,4-tetrol (3) (Table 17).

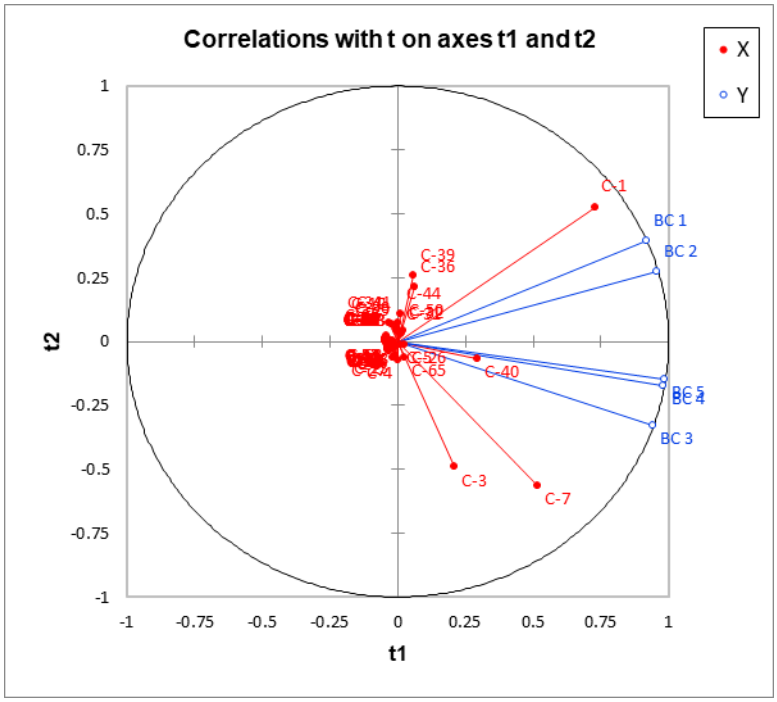

Figure 52 PLS correlation matrix comparing all cultures from Day 4 to 74, stored at ambient.

Table 17	Important identifiers of *Perna* culture between Days 4 and 74, ambient temperature, in descending of relative dominance.
Compounds high for Cultures 1 and 2	Compounds high for Cultures 3, 4 and 5
C-1 C-39 C-36	C-7 C-3

Effect glucose concentration on *Perna* pH and SPME volatiles using one culture only

Glucose concentration affected the pH of *Perna* after fermentation and storage (Figure 55). After 11 days, the pH was rather stable with storage days at each glucose concentration and temperature. The pH fell with increase in sugar concentration to Day 4, in the sequence: 0.25% = 5.98, 5.50, 5.12, 4.76, 2.0% = 4.76 (Table 18). The last value was on the high side of *Perna* pH values throughout the thesis where 2% glucose was routinely used, possibly because the culture was old[13].

Except for the lowest glucose concentration (0.25%), the pH values remained somewhat static with time within each storage temperature, 4°C and ambient. Moreover, the ambient-treatment pH values were always lower than the equivalent 4°C-treatment pH values, with the exception of the lowest glucose concentration (Figure 55).

The simplest explanation of the usual lower pH values for ambient storage is that microflora continues to metabolize the available glucose to lactic acid at ambient temperature, but not in the cold. As for the anomalous result for 0.25%, it is proposed that with insufficient glucose, the microflora metabolizes other substrates instead at ambient temperature, the most likely being amino acids. Deamination of these liberates ammonia, which is alkaline, and is likely to be the cause of the pH increase on ambient storage shown in the top graph of Figure 55.

These differences are particularly important because the appearance of the lower glucose treatments was not typical of *Perna* made with 2% glucose (Figure 53). They were much darker and smelt strongly when opened for pH measurement and SPME sampling. The smell was not attractive. The effect was more obvious with ambient temperature storage than with 4°C storage (Figure 54 and 55).

[13] The valuable culture packs were suited to 200 kg of meat, and therefore were opened, subdivided, vacuum packed and refrozen awaiting periodic use. This practice is not ideal for reliable bacterial viability.

Figure 53 Colour of treatments fermented with different concentrations of glucose (0.25, 0.5, 0.75, 1.0 and 2.0%) at Day 11 with ambient temperature storage.

Figure 54 Colour of treatments fermented with different concentrations of glucose (0.25, 0.5, 0.75, 1.0 and 2.0%) at Day 11 with storage at 4°C.

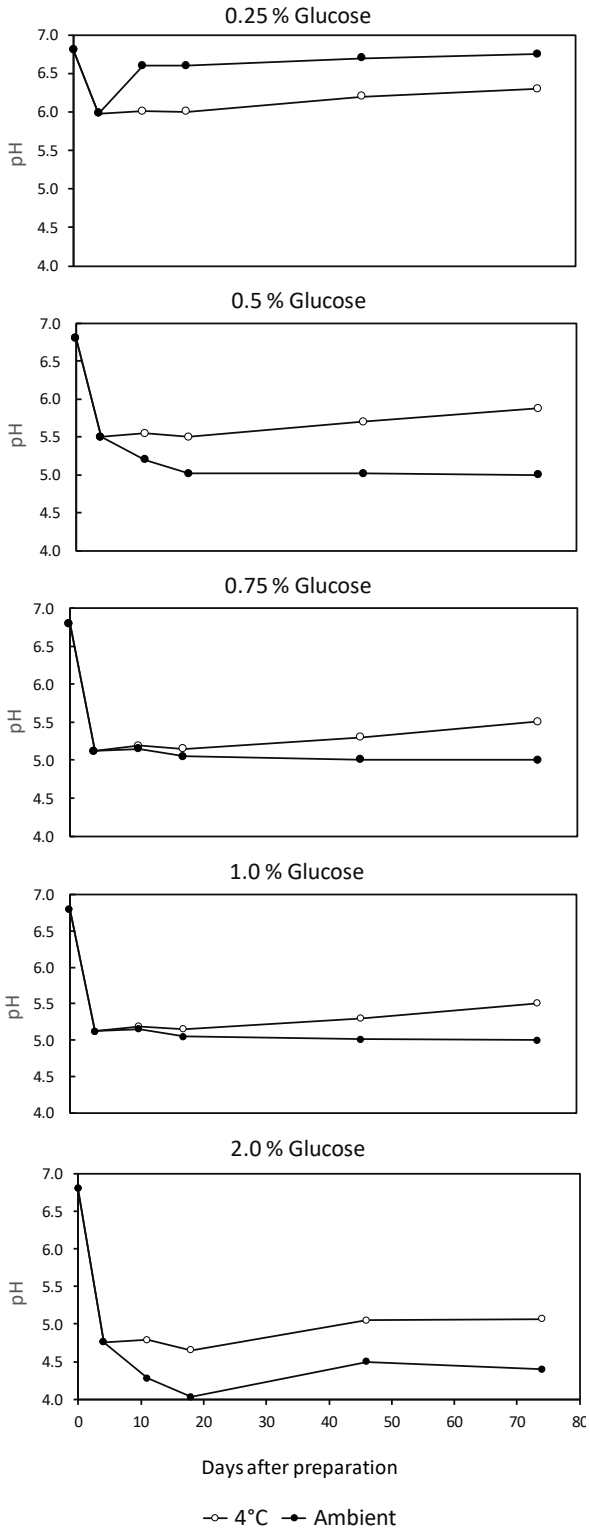

Figure 55 Change of pH for *Perna* fermented with different concentrations of glucose (0.25, 0.5, 0.75, 1.0 and 2.0%) and stored for 74 days at 4°C and ambient temperature.

Table 18 Change of pH for *Perna* fermented with different concentrations of glucose at Days 4 and 74, stored at 4°C and ambient temperature.

Glucose concn. (%)	Day 4	Day 74 4°C	Day 74 Ambient
0.25	5.98	6.30	6.75
0.50	5.50	5.88	5.00
0.75	5.12	5.51	5.00
1.00	4.76	4.80	4.70
2.00	4.76	4.70	4.40

The concentrations of the volatile compounds obtained by SPME-GCMS are shown in Figure 56 and 57 for storage at 4°C, presented in order of elution. Glucose concentration affected the profile of volatile compounds after fermentation and storage (Figure 56 and 57). As a simple overview by inspection, a few compounds like 2-ethylfuran, 1-octen-3-ol and others showed increases with increasing concentration of glucose. In contrast, most volatiles showed a decrease in relative concentration with increasing glucose. As with the different culture trial above, there were also changes unique to the transition from Day 0 to Day 74. Table 19 is analogous to Table 12, aiming to explore the main features due to glucose concentration.

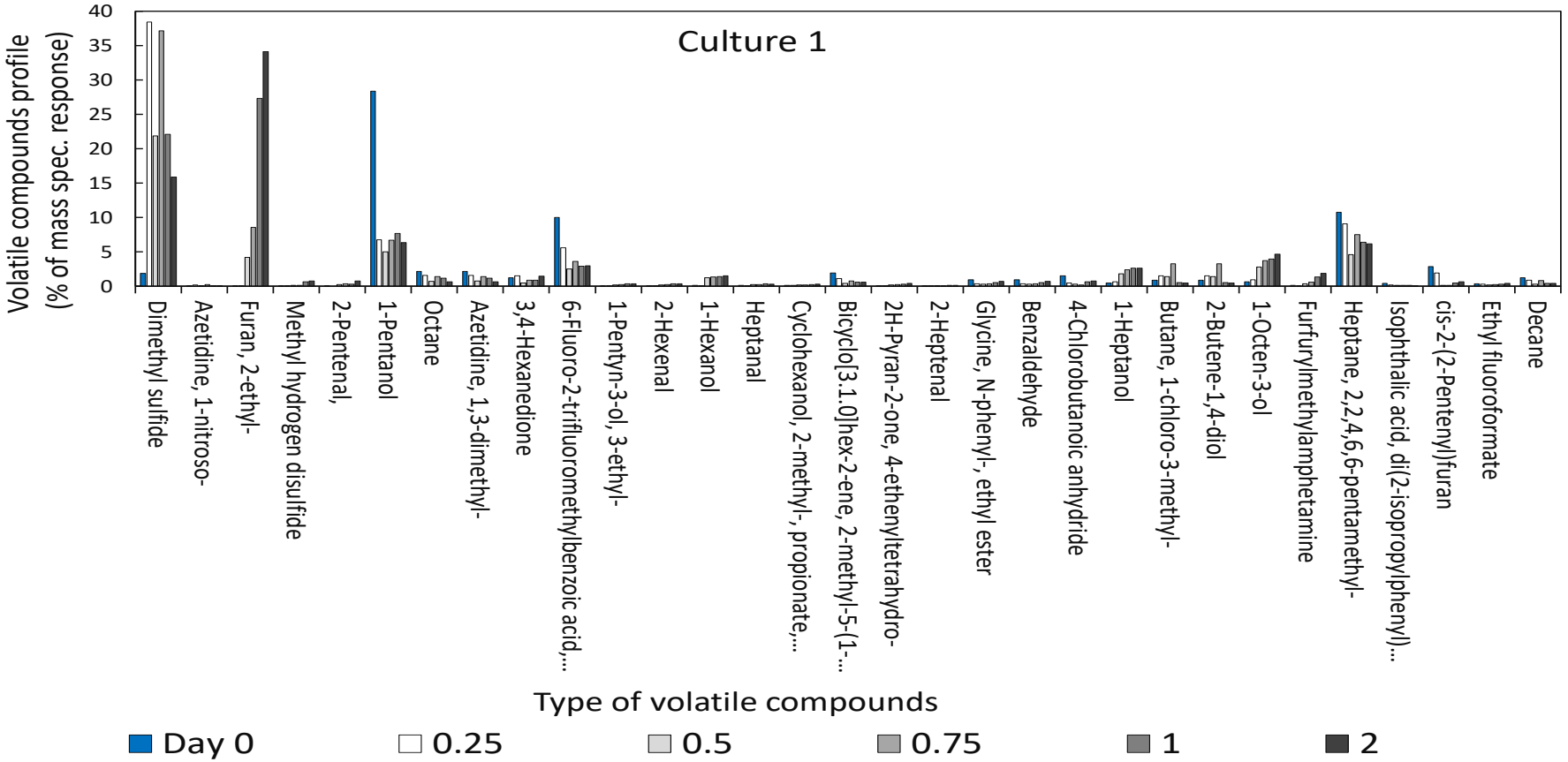

Figure 56 Volatile compounds (1 to 31) of *Perna* at Day 4 for glucose concentrations of 0.25, 0.5, 0.75, 1.0 and 2.0%, using Culture 1 only. There was no 0% glucose treatment but a Day 0 treatment is included. Compounds are shown in order of elution. Compounds are shown in order of elution. Day 0 is blue, with increasing intesity of grey from Days 4 to 74, left to right.

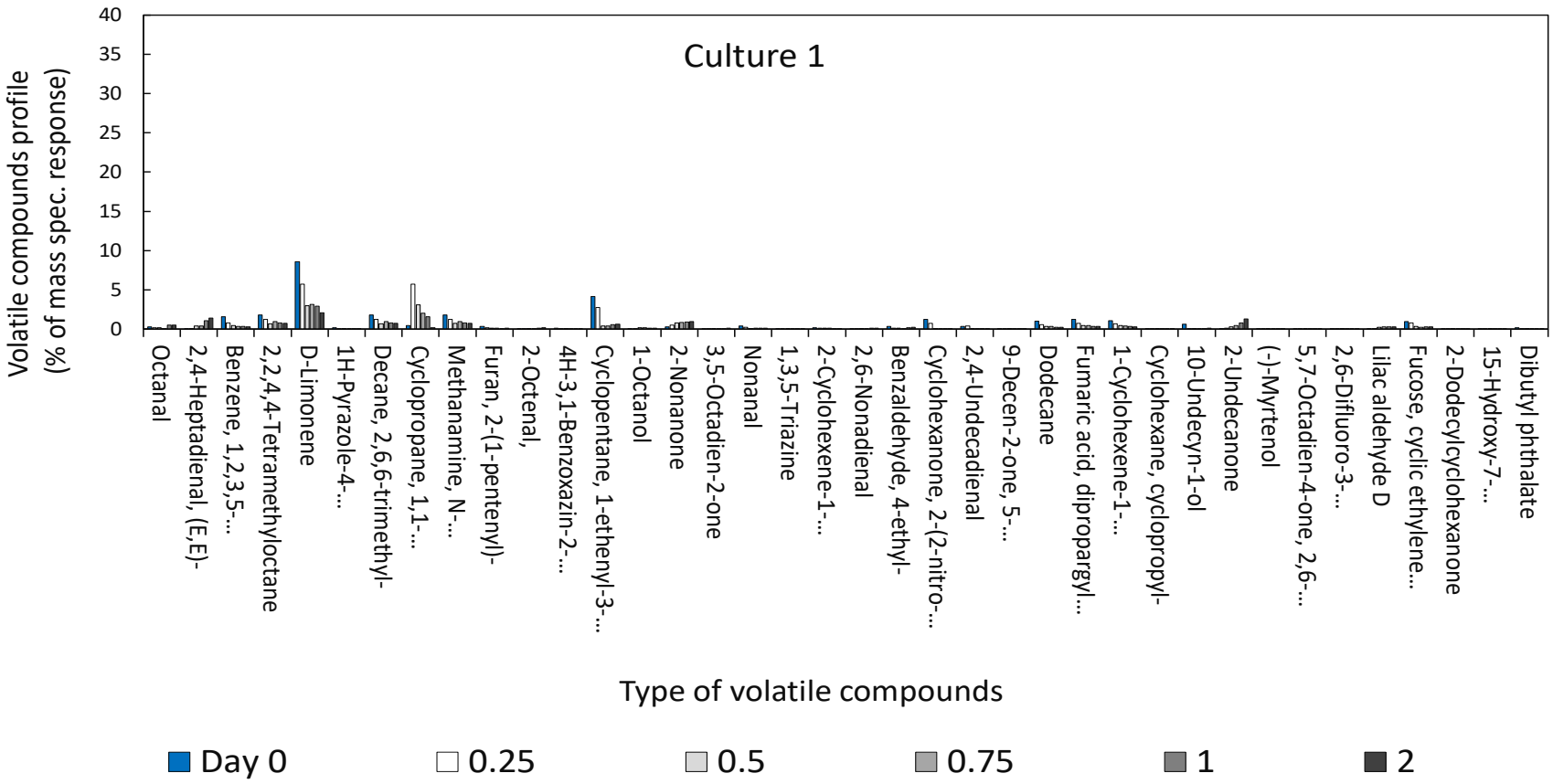

Figure 57 Volatile compounds (32 to 69) of *Perna* at Day 4 for glucose concentrations of 0.25, 0.5, 0.75, 1.0 and 2.0%, using Culture 1 only. There was no 0% glucose treatment but a Day 0 treatment is included. Compounds are shown in order of elution. Compounds are shown in order of elution. Day 0 is blue, with increasing intesity of grey from Days 4 to 74, left to right.

Table 19 A non-exhaustive analysis of SPME data to show some differences due to added glucose concentration.

Comparisons of interest	Shown in	
	Figure	Table
Day 4 all glucose concentration (storage temperature not relevant)	Figure 58	Table 20
Concentration 0.25 versus concentration 2 at 74 (4°C storage)	Figure 59	Table 21
Concentration 0.25 versus concentration 2 at 74 (ambient storage)	Figure 60	Table 22

Day 4 all glucose concentration (storage temperature not relevant)

At Day 4 two culture groups were clearly identified, one with 0.25, 0.5, 0.75% and another group with 1 and 2% (Figure 58). The standout compounds were respectively dimethyl sulphide (C-1) and 1-1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3) (Table 20). It is obvious that below 1% glucose the profile changed, prompting an analysis of the differences between 0.25 and 2% glucose after long storage to 74 days at two storage temperatures. These comparisons are shown in the next two subsections.

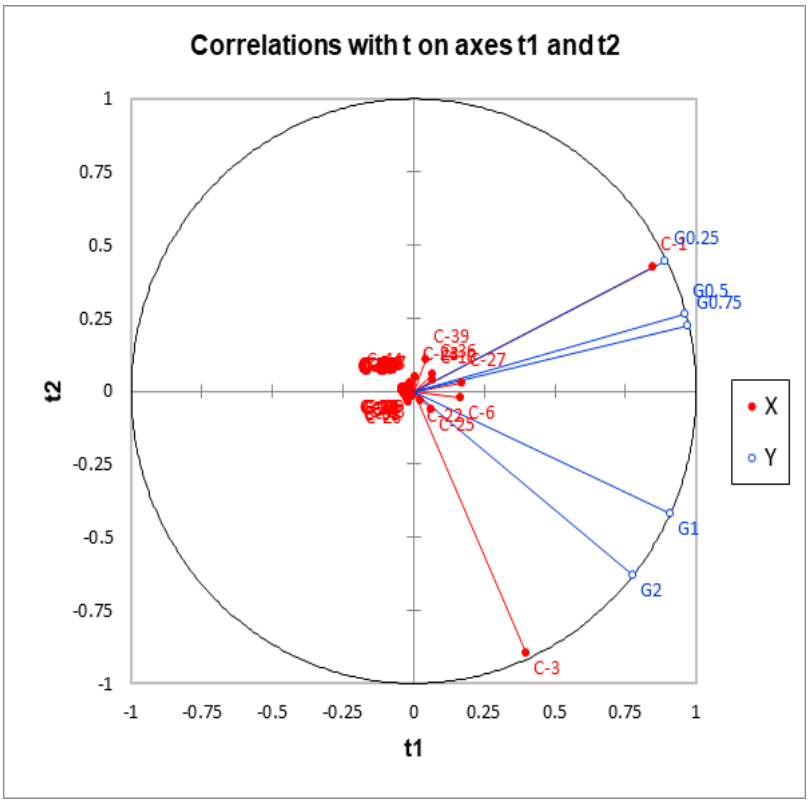

Figure 58 PLS correlation matrix comparing glucose concentrations at Day 4.

Table 20	Important identifiers of Perna at Day 4 in descending of relative dominance at five glucose concentrations.
Compounds high for glucose concentrations 0.25, 0.5 and 0.75 %	Compounds high for glucose concentrations 1 and 2%
C-1	C-3

Concentration 0.25 versus concentration 2 at Day 74 (4°C storage)

At Day 74, 4°C storage, concentrations 0.25% and 2% were clearly separated by 4-ethylbenzaldehyde (C-23), 2,4-undecadienal (C-24), 1-3-ethyl-1-pentyn-3-ol (C-27) and 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (C-25) (Figure 59). Concentration 2% was characterised by 1-(1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3) (Table 21).

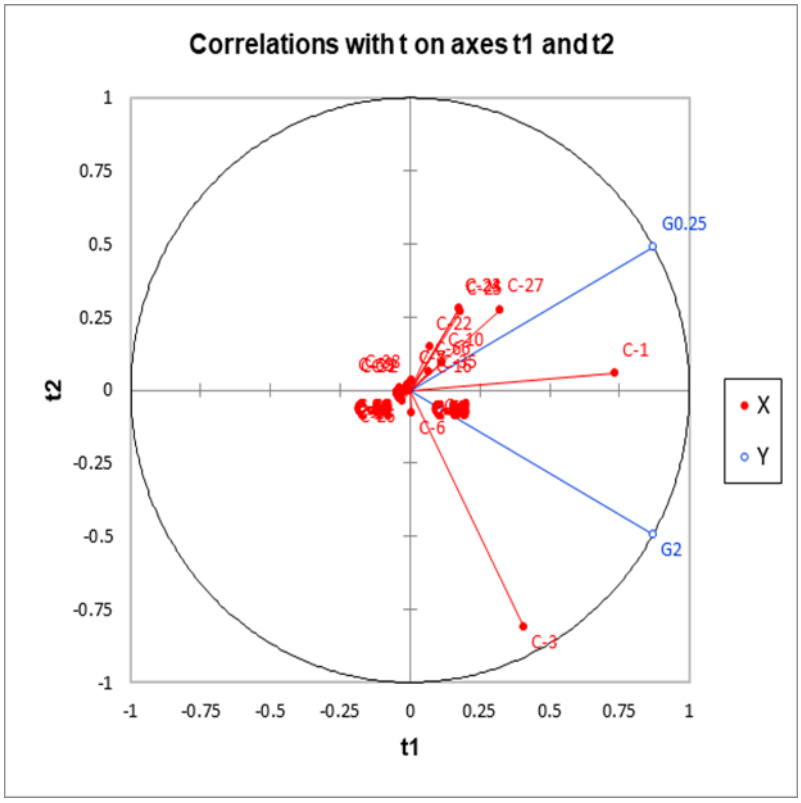

Figure 59 PLS correlation matrix comparing 0.25 and 2% glucose at Day 74, 4°C.

Table 21 Important identifiers of *Perna* at Day 74, 4°C storage, at two glucose concentrations in descending of relative dominance.

Compounds high for 0.25% glucose	Compounds high for 2% glucose
C-23 C-24 C-27 C-25	C-3

Concentration 0.25 versus concentration 2 at Day 74 (ambient storage)

At Day 74, ambient storage, concentrations 0.25% and 2% were clearly separated by dimethyl sulphide (C-1), 3-ethyl-1-pentyn-3-ol (C-27), 4-ethylbenzaldehyde (C-23), 2,4-undecadienal (C-24), 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (C-25) (Figure 60). Concentration 2% was again characterised by 1-(1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3) (Table 22).

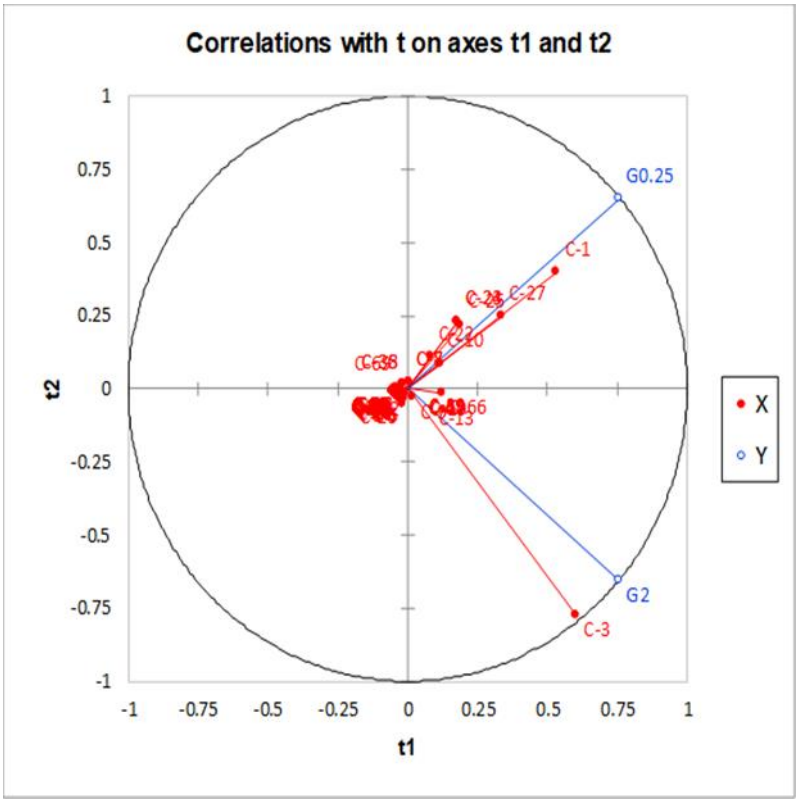

Figure 60 PLS correlation matrix comparing 0.25 and 2% glucose at Day 74, ambient storage.

Table 22 Important identifiers of *Perna* at Day 74, ambient temperature storage, at two glucose concentrations in descending of relative dominance.

Compounds high for 0.25% glucose	Compounds high for 2% glucose
C-1	C-3
C-27	
C-23	
C-24	
C-25	

Discussion

An overview of all the SPME results for *Perna* (Figure 45, 46, 56 and 57), fermented using different cultures and glucose showed the same general pattern of volatile compounds produced during fermentation and storage to Day 74. This means all *Perna* samples will have very roughly the same flavour as affected by these volatiles. Due to the high number of variables with wide variation of concentration within and between treatments, small differences due to treatments could not be easily seen from inspection alone. The use of PLS was the solution to see the detail. PLS can be applied when the number of observations is low and the number of explanatory variables is high.

The culture composition of cultures labelled as BC1, BC2, BC3, BC4 and BC5 and their contribution in flavour as described in Chr Hansen (2014) are as follows, (Table 23).

Table 23 The culture composition of cultures and their contribution in flavour formation as described in Chr Hansen (2014).

Culture	Culture type and role	Flavour contribution
BC1	*Pediococcus pentosaceus*	Lactic acid flavour
	Staphylococcus carnosus subsp. *utilis*	Flavour
BC2	*Pediococcus pentosaceus*	Lactic acid flavour
	Lactobacillus sakei	Lactic acid flavour
	Staphylococcus xylosus	Flavour
	Staphylococcus carnosus 1 undefined subsp.	Flavour
	Debaryomyces hansenii (a yeast)	Flavour
BC3	*Pediococcus acidilactici*	Lactic acid flavour
	Lactobacillus curvatus	Lactic acid flavour
	Staphylococcus xylosus	Flavour
BC4	*Lactobacillus sakei*	Lactic acid flavour
	Staphylococcus carnosus 2 subsp. undefined	Flavour
BC5	*Lactobacillus sakei*	Lactic acid flavour
	Staphylococcus carnosus 1 undefined subsp.	Flavour

Volatile compounds of particular interest in this study are shown in Table 24. They may or may not have high odor values, although sulphur compounds undoubtedly do (Dalton, 1996; Leonardos, Kendall, & Barnard, 1974). Thus, odor importance cannot be known from

these data, but the links between storage temperature, storage time, glucose concentration and culture composition might be inferred.

Table 24 Compounds of interest in *Perna* volatiles. They are grouped by likely chemical class.

Code	Compound name
C-1	Dimethyl sulphide
C-2	Methyl hydrogen disulphide
C-3	1-(1,3-Dithiolan-2-yl) pentane-1,2,3,4-tetrol
C-7	2-Ethylfuran
C-10	Lilac aldehyde
C-23	4-Ethylbenzaldehyde
C-24	2,4-Undecadienal
C-25	2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde
C-26	1-Pentanol
C-27	3-Ethyl-1-pentyn-3-ol
C-32	1-Octen-3-ol
C-36	Octane
C-39	1-Chloro-3-methylbutane
C-40	2,2,4,6,6-Pentamethylheptane
C-45	1,1-Dimethyl-2-(3-methyl-1,3-butadienyl)-cyclopropane
C-49	Limonene
C-50	2-Formyl-4,6-dichlorophenyl-6-fluoro-2-trifluoromethylbenzoate

All LABs in starter cultures used in this study were responsible for lactic acid flavour and acidification, but the *Staphylococci* and yeast are likely to have been contributed to differences in flavour type and intensities (Figure 45 and Table 23). That is certainly the claim in Chr. Hansen (2014).

When the mean of three replicates of 69 compounds tentatively identified at Day 0 and Day 4 for five cultures were analysed using PLS correlation, it showed the biggest change

from Day 0 to Day 4, was for 2-ethylfuran (C-7) and the complex alkene/ane 1,1-dimethyl-2-(3-methyl-1,3-butadienyl)-cyclopropane (C-45), which became more dominant after fermentation (Table 13). *Staphylococcus xylosus* and *Staphylococcus carnosus* are able to transaminase and decarboxylate the branched chain amino acids valine, leucine, and isoleucine into the corresponding branched chain aldehydes, carboxylic acids, and alcohols (Olivares, Navarro, & Flores, 2015; Ravyts et al., 2010), but C-7 and C-45 do not fit this description. In respect of compounds lost, limonene (C-49), a characteristic of fresh seafoods (Nollet & Toldrá, 2009) was in Cultures 3, 4 and 5 (Figure 46), but not in Cultures 1 and 2.

Comparing cultures at Day 4, Cultures 1 and 2 were clearly differentiated from Cultures 3, 4 and 5 by C-7, 2-ethylfuran, essentially absent in Cultures 1 and 2, similarly C-1, dimethylsulfide (Table 13). Knowing this, the *Staphylococcus* species in Cultures 1 and 2 were either incapable of producing these two compounds or, as will be shown next, were slow to do.

Comparing five cultures, stored at 4°C to Day 74, 2-ethylfuran (C-7) in Cultures 1 and 2 increased to be equivalent to the profile of Culture 5, indicating they were slow to produce 2-ethylfuran and dimethylsulfide. Inspection of Figure 45 shows this to be the case. Dimethylsulfide was important in all cultures but more so in Cultures 3 and 4. The alkane 2,2,4,6,6-pentamethylheptane (C-40) was observed to be dominant for Cultures 3 and 4. Hydrocarbons have low odor values, due to their high odor thresholds (Le Guen, Prost, & Demaimay, 2000), so 2,2,4,6,6-pentamethylheptane is unlikely to be important in *Perna* flavour.

Storage at ambient for 74 days showed a marked differentiation between only Culture 1 (C-1, dimethylsulfide) and Culture 5 (C-7, ethylfuran). Other cultures lay between these extremes. Day 4 and Day 74 are extremes of the fermented state and it was useful to pool all data between these days (Days 4, 11, 18, 46 and 74), because consumer could eat *Perna* at any time between the extremes (Figure 51 and 52, Table 16 and Table 17). Cultures stored in 4°C showed small difference but were still divided into two groups. Cultures 1 and 2 were similar, characterised by methyl hydrogen disulphide (C-2), 1-chloro-3-methylbutane (C-39), octane (C-36), 3-ethyl 1-pentyn-3-ol (C-27) and lilac aldehyde (C-10) which increased for these cultures. 1-(1,3-Dithiolan-2-yl) pentane-1,2,3,4-tetrol (C-3) increased in Cultures 3, 4 and 5.

When the equivalent storage day data were pooled for ambient temperature, the differences between two groups – Cultures 1 and 2 versus Cultures 3, 4 and 5 – slightly

increased. Compounds dimethyl sulphide (C-1), butane, 1-chloro-3-methyl (C-39), octane (C-36), 1-pentyn-3-ol, 3-ethyl (C-10) characterised Cultures 1 and 2, and Cultures 3, 4 and 5 were characterised by 1-(1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3) and 2-ethyl furan (C-7). Temperature was the obvious driver of these greater between-culture differences.

Comparing glucose concentration at Day 4, concentrations 0.25, 0.5 and 0.75 % were clearly differentiated from concentrations 1 and 2 by dimethyl sulphide (C-1), for the earlier group and 1-(1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3) for the latter group (Table 20). Comparing 0.25 and 2% glucose concentrations stored at 4°C on Day 74, 4-ethylbenzaldehyde (C-23), 2,4-undecadienal (C-24), 3-ethyl-1-pentyn-3-ol (C-27), 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, (C-25) separated 0.25% from 2%, which appeared to be characterised by 1-(1,3-dithiolan-2-yl)pentane-1,2,3,4-tetrol (C-3). The equivalent comparison at ambient temperature on Day 74 showed the same characterizing compounds, but more strongly, and dimethylsulfide (C-1) was now in the list.

The cultures supplied by Chr. Hansen are used commercially with mammalian meats to produce different styles of salami, and it is likely that equivalent SPME experiments with these cultures and say, unspiced beef, would yield analogous differences, but complicated by the fact that salami is often dried to low water activities. This is not done with *Perna*. Thus, is likely that the differences demonstrated here would yield different *Perna* flavors, but their perception is beyond the scope of this thesis.

However, considering the fact that at 0.25% glucose was limiting to the point that pH did not fall below 5, and that deamination was likely to be occurring at ambient temperature (Figure 55), it is likely that these compounds would be a cause of poor flavour on consumption: dimethylsulfide (C-1), 4-ethylbenzaldehyde (C-23), 2,4-undecadienal (C-24), 3-ethyl-1-pentyn-3-ol (C-27) and 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (C-25). Recall that lower glucose treatments smelt strongly when opened for pH measurement and SPME sampling. The smell was not attractive. It is well known that sulphur compounds have low odor threshold (Leonardos et al., 1974), so dimethylsulfide is likely to be involved. Dimethyl sulphide has a characteristic smell commonly described as cabbage-like. It becomes highly disagreeable at even quite low concentrations. According to PubChem (2018) 4-ethylbenzaldehyde is described as “present in roasted chicken, cider, tea and roasted peanuts. 4-Ethylbenzaldehyde is a flavoring ingredient” and described elsewhere (Thegoodscentscompany,(2019)) as “bitter almonds”. 2,4-Undecadienal is a typical fat oxidation product, and as such is likely to have a rancid odor. Odor descriptions of 3-ethyl-1-

pentyn-3-ol and 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde have not been found.

From a commercial point of view, *Perna* should be prepared with glucose at or above 1.0% and should be stored chilled although it still has remarkable volatiles stability at higher temperatures. However, the darkening Maillard reaction with free glucose proceeds more rapidly at higher temperatures, so *Perna* storage should be under chilled conditions.

Some other points should not be overlooked: the contribution of cooking, residual microorganisms, and other contributions to fermentation and volatiles generation. The obvious contribution from cooking is through the Maillard reaction, but there will be other effects arising from fat oxidation and protein denaturation. In respect of residual microorganisms, *Perna* is based on the novel cook-then-ferment preservation technology developed at AUT University, but this does not mean the native mussel microflora would be totally destroyed. However, the added starter culture should be able to be compete if the matrix environment is conducive to growth. It is not uncommon for inoculated starter cultures species to have difficulties in adapting to specific fermented food matrices (Arana-Sánchez et al., 2015; Fonseca, Cachaldora, Gómez, Franco, & Carballo, 2013). Cooked mussel is not the intended matrix for meat salami cultures; cooked mussel is new territory and microbial responses are unknown beyond the fact that pH usually fell to preservation levels, provided enough glucose was present. It has been shown in the glucose concentration experiment (0.25 to 2%) that with insufficient glucose it is likely that the surviving microflora can successfully compete with the starter culture microflora. To make implementation even more complex, it has been shown that even if promising *Staphylococcus* strains are able to survive in a fermented food matrix, in situ expression of their desirable characteristics does not always occur (Villani et al., 2007). *Staphylococci* can be metabolically inactive due to the processing conditions, for instance displaying a reduced proteolytic activity at too low pH values (Montel et al., 1998). Chapter 7 will discuss microbiological characteristic of *Perna* in detail. During cooking there is a possibility of Maillard reaction for production of some flavour compounds.

Chapter 7

Basic microbiological characteristics of *Perna*

Introduction

Perna is a novel cook-then-ferment product made from the New Zealand greenshell mussel, *Perna canaliculus*, a filter feeder shellfish. Accumulation of microorganisms by filter feeding is by far the most important root cause of shellfish product problems of safety and eating quality. Although *Perna* is cooked, residual microorganisms may still reduce safety and quality. Moreover, although the boiling water is somewhere near 100°C, the temperature in the thermal center of gravity of each mussel would almost certainly be less than that in a 7 minutes cook from cold mussels into boiling water.

Common pathogenic bacteria in seafood are bacteria that belong to the natural microflora of fish such as *Clostridium botulinum*, pathogenic *Vibrio* spp., *Aeromonas hydrophila*; bacteria from faecal and/or environmental contamination, such as *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*; and bacterial contaminants acquired during processing, storage, or preparation for consumption, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium botulinum*, *Salmonella* spp. (Calo-Mata et al., 2008; Mejlholm et al., 2008).

This chapter is in two parts. The first describes the microbiology of *Perna* prepared with one culture fermented and stored at different temperatures. The second focuses on the comparison of five different starter cultures from Chr. Hansen to produce *Perna*, and where work extended to *Listeria monocytogenes*.

Summary of methods

These are described in detail in Chapter 2 but summarised here for convenience. A peptone water extract of *Perna* was serially diluted and used for the determination of aerobic plate count (APC), faecal coliforms, also *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and the coagulase negative staphylococci and LAB bacteria. These determinations were applied to *Perna* preparations using one starter culture (BFL-F02) with 2% glucose at different time of mussel harvest (May, June and July 2016) and storage temperatures (4°C, ambient and 35°C).

APC was done by pour plating on APC agar. Faecal coliforms and *Escherichia coli* were determined by the most probable number (MPN) method in three stages: presumptive test for faecal coliforms and *E. coil*; confirmed MPN test for faecal coliforms and *E. coli*; completed MPN test for *E. coli.* The MPN method was also applied to *S. aureus* and coagulase negative staphylococci. Determination of *V. parahaemolyticus* required a specific agar plated with serial dilutions. Determination of LAB required serial dilutions, then pour plating on MRS agar. *Salmonella* in *Perna* was detected as presence/absence using specialized broth, lactose and RVS broth and plating techniques on XLD agar.

Comparison of five different starter cultures from Chr.-Hansen to produce *Perna,* preparations were done as detailed in Chapter 2, using 1.5% glucose. The outputs of this work were APC, pH, and *L. monocytogenes* count, the last being only presumptive. *L. monocytogenes* is of concern in shellfish and fermented foods. The questions were: was it present and does it survive storage? *L. monocytogenes* in *Perna* was detected with a presence/absence test on a combined selective and differential agar, as detailed in Chapter 2. Preparations were made in November and December 2016, and January 2017. APC was determined only in November because of time resource constraints.

Results

Effect of storage temperature using one culture

Changes in the microbial flora in Perna for three trials stored at 4°C

Changes in the microbial flora in *Perna* before and after fermentation and during storage are shown in Figure 61 and Table 25. The APC cell population of *Perna* prepared with one culture for three trials was counted at 7.25, 7.58 and 7.20, $\log_{10}$ cfu^{-1} before fermentation, increasing to 8.47, 8.52 and 8.78 $\log_{10}$ cfu^{-1} after fermentation. For the three different months of sampling, May, June and July, the difference in microbial counts were not significantly different. APC showed negligible changes during storage.

The LAB (presumably *Pediococcus pentosaceus* from BFL-F02*)* increased in numbers (Figure 61). The counts were almost the same as for APC, indicating that LAB were dominant. *Staphylococcus carnosus* count as determined in the selective broth dictated by the standard method was stable throughout fermentation and storage (Table 25). Reductions of pathogenic microorganisms were observed (Table 25). After fermentation, *S. aureus* was not observed in any product. *V. parahaemolyticus*, which is common in shellfish, was either

totally absent or was sensitive to heat in this matrix because it was not observed from Day 0 after mussels were cooked for 7 minutes. The safety indicator microorganisms (Table 25) indicated the safety of *Perna* as defined by New Zealand Microbiological Criteria (1995) in respect of enteric bacteria. However, the pathogenic *L. monocytogenes* is not included in these criteria.

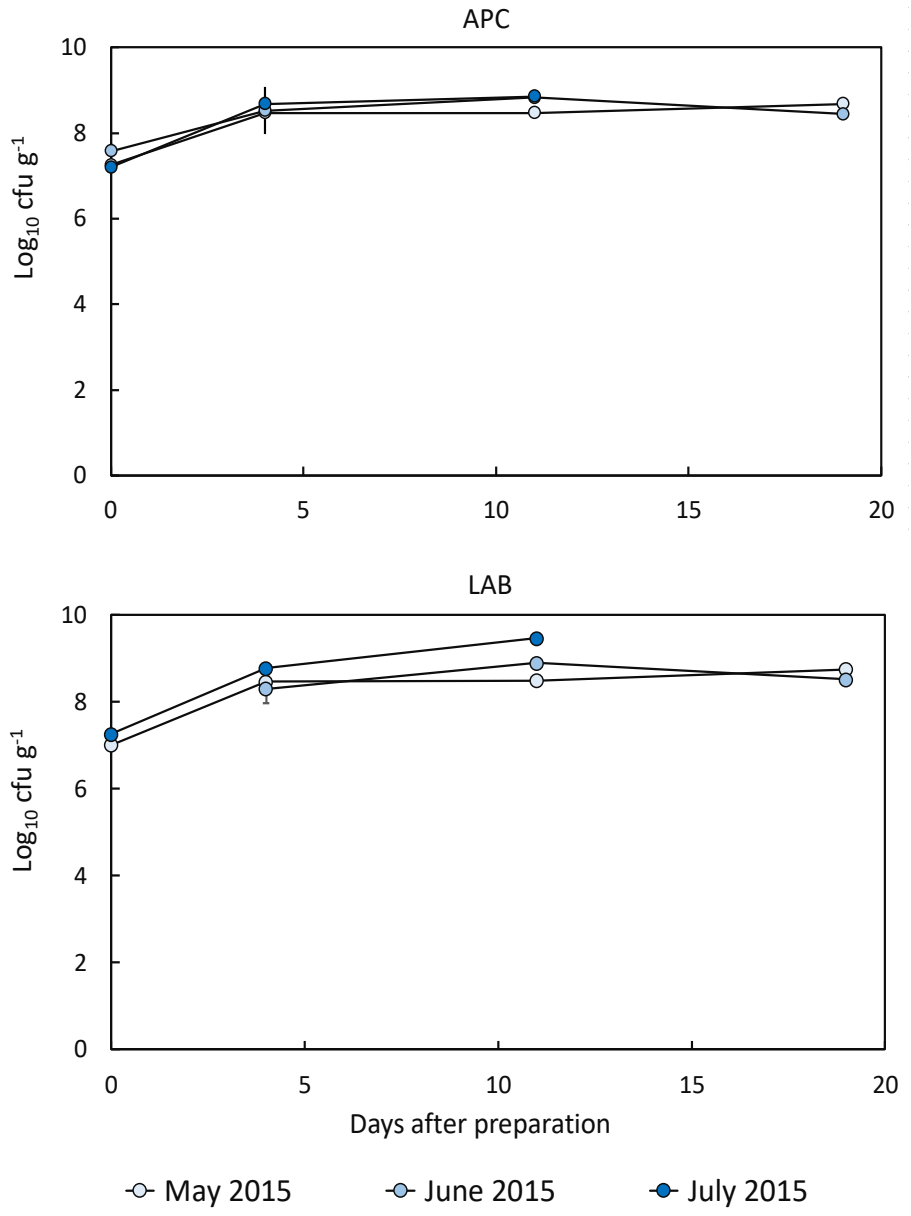

Figure 61 Effect of storage on APC and LAB in three preparations. Data are means of triplicate barrier bags with standard deviations on each preparation date, LAB on Day 18 on the July 2015 was not analysed. Standard deviations were low and are difficult to see with this scaling.

Table 25 Storage effect on *Staphylococcus carnosus* and *aureus*, faecal coliforms, *Escherichia coli* and *Vibrio parahaemolyticus* on three preparation dates. Data as $\log_{10}$ cfu g^{-1}were averaged for three replicate plates, then averaged over the three trials, all stored at 4°C, 0 indicate below the threshold of the used method for enumeration.

		Days after preparation			
Date 2015	Bacteria	0	4	11	19
May	*S. carnosus*	7.0	8.0	8.0	8.0
June		8.0	8.0	8.0	8.0
July		8.0	9.0	9.0	No data
May	*S. aureus*	2.6	0.0	0.0	0.0
June		0.0	0.0	0.0	0.0
July		0.0	0.0	0.0	No data
May	Faecal coliforms	3.2	3.1	0.0	0.0
June		0.0	0.0	0.0	0.0
July		5.8	2.8	0.0	No data
May	*E. coli*	1.6	0.0	0.0	0.0
June		0.0	0.0	0.0	0.0
July		0.0	0.0	0.0	No data
May	*V. parahaemolyticus*	0.0	0.0	0.0	0.0
June		0.0	0.0	0.0	0.0
July		0.0	0.0	0.0	No data

Comparison of microflora of Perna at three storage temperatures

When *Perna* storage temperature was explored using three batches (with replicated barrier bags) prepared on the same day, APC was increased by almost 1 log to Day 4. It then remained constant to Day 74 at 4°C (Figure 62, APC) and was dominated by LAB (Figure 62, LAB). The numbers of microorganisms decreased with storage at higher temperatures from Day 18 (ambient and 35°C), especially at 35°C. LAB was always dominant for all temperatures. *Staphylococcus carnosus* (Figure 62) was constant at 4°C to Day 11, after which it increased to Day 18. Numbers for ambient storage increased from Day 4 to 11, were constant to Day 18, then declined slightly. At 35 °C, *S. carnosus* was constant to Day 18, then declined to zero by Day 74. *Staphylococcus carnosus* clearly did not tolerate higher temperature storage.

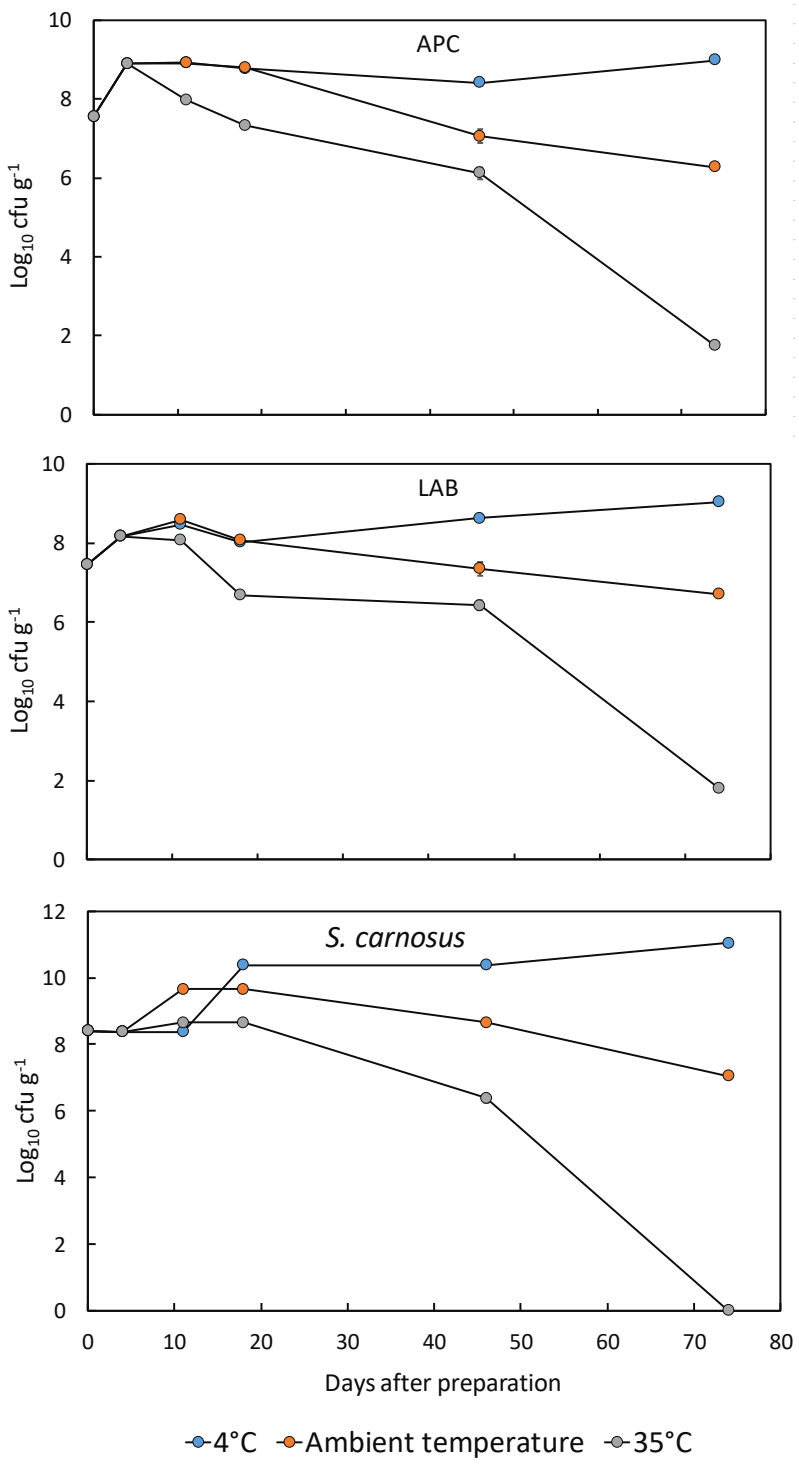

Figure 62 Effect of storage temperature on APC, LAB and *Staphylococcus carnosus*. Data are means of three batches made on one day with standard deviations, where batch values were means of triplicate barrier bags. Standard deviations were low and seldom visible with this scaling.

For the three batches (Figure 63), pH declined within the 96 hours of incubation at 30°C (Figure 16) then appeared to remain rather stable during 74 days of chill storage but subtlety decreased to Day 18 at the higher temperatures, and slightly beyond to Day 74. At 4°C, pH was more stable.

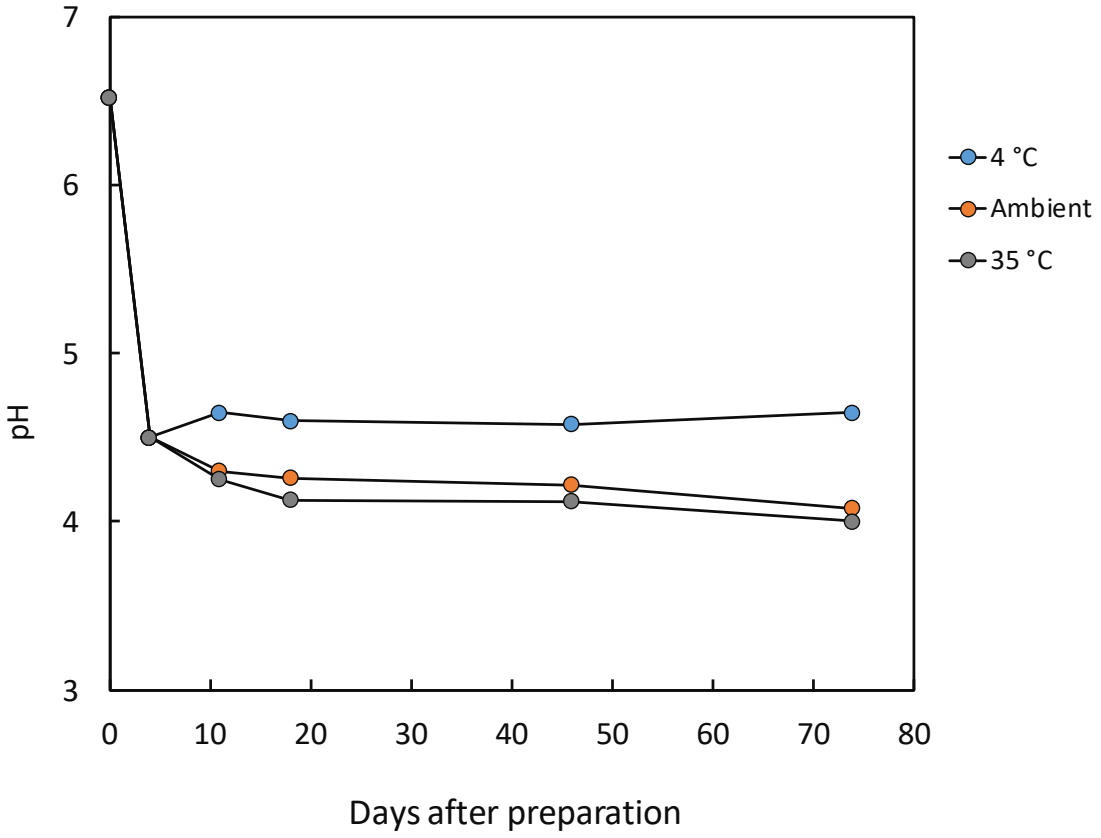

Figure 63 Effect of storage temperature on pH. Data are means of three batches with standard deviations, where batch values were means of triplicate barrier bags. Standard deviations were low and impossible to see with this scaling.

Comparison of five different starter cultures from Chr. Hansen to produce *Perna*

The study included monitoring of pH, APC and presumptive *L. monocytogenes.* In all cases the glucose concentration was 1.5%. There were two storage temperatures, 4°C and ambient temperatures.

APC and pH

Three preparations were made three weeks apart, in November and December 2016, and January 2017. The microbial flora in *Perna* prepared using five starter cultures for three batches prepared on November 2016, are shown in Figure 64. The APC cell population of *Perna* prepared with five cultures showed the same trend as for the single culture trials with BFL-F02 (Culture1) shown elsewhere. APC increased by Day 4 by about one log for Cultures 1, 3 and 4, and half a log for 2 and 5. Thereafter the counts peaked by Day 14 at ambient but were slower to peak at 4°C. Interestingly, the pattern for each culture established by Day 4 was maintained to at least Day 18. After Day 46, counts decreased at both temperatures, but more so at ambient.

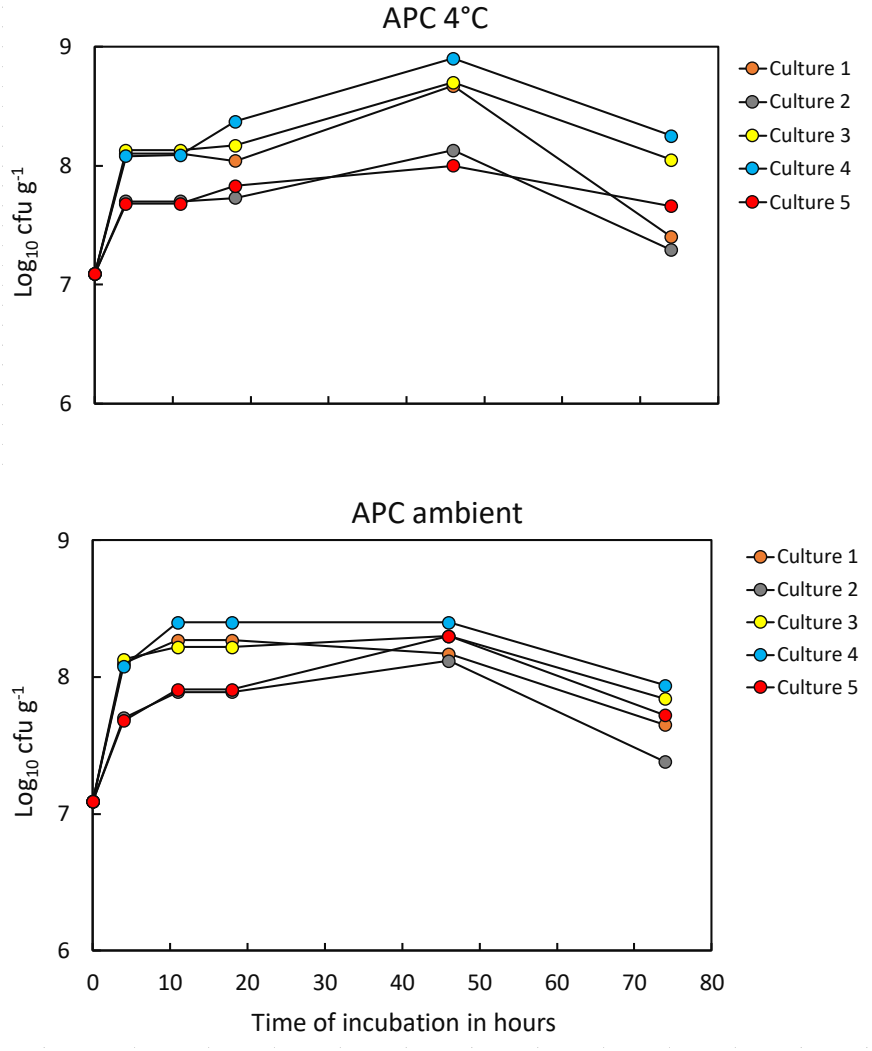

Figure 64 APC in *Perna* prepared using five starter cultures, before and after fermentation and during storage to 74 days in 4°C and ambient temperature. Data are batch means of the November 2016 preparation.

The pH data that matches Figure 64 is shown in Figure 65. Strikingly, there was a pH increase between Days 11 and 18, and that was true for both storage temperatures. Prior work reported in Chapter 3 (Figure 16) showed that storage from single culture experiments did not show a pH increase on storage. On the contrary, pH subtlety declined. This begs the question: what was different about these contrasting experiments? The answer is likely to be the glucose concentration used, 2% in Figure 16 and 1.5% here. How this could affect pH is

addressed in the Discussion.

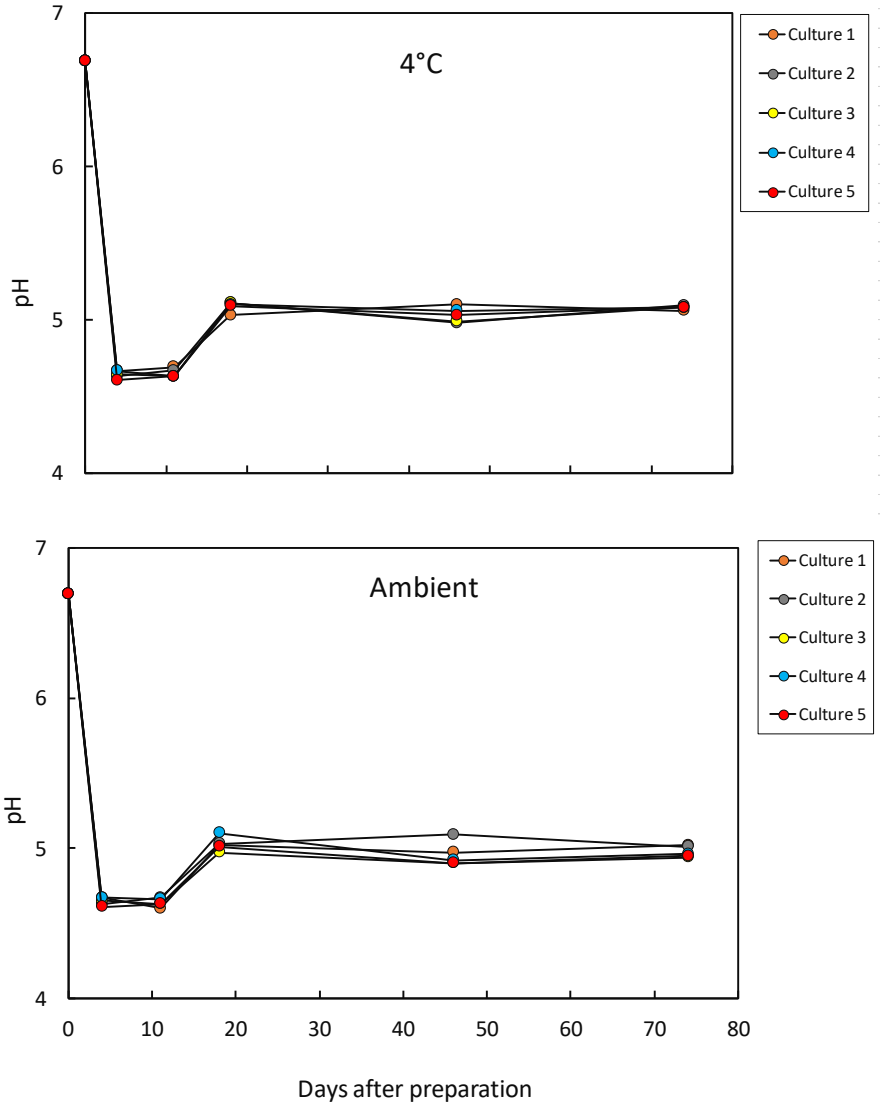

Figure 65 pH in *Perna* prepared using five starter cultures, before and after fermentation and during storage to 74 days in 4°C and ambient temperature. Data are batch means of the November 2016 preparation.

Presence of Listeria monocytogenes in Perna during fermentation and storage at 4°C and ambient temperature

The presence/absence of *L. monocytogenes* was determined in 25 g samples for the three preparation dates (Table 26, Table 27 and Table 28). In these tables a +, and –indicate positive and negative for *L. monocytogenes*, a judged by black colonies. Table 27 and Table

28 showed that although *L. monocytogenes* was present at Day 0, the bacterium was subsequently inactive. Moreover, the density of colonies was lower than for Trial 1 (Table 26) (data not shown), meaning that the mussels sourced for Trial 1 were most strongly contaminated with this bacterium. The cause of this will be somewhere up the supply chain but unknown. Table 26 was the most interesting because the bacterium persisted in Cultures 1, 2, 3 and 4, particularly in 4°C storage. Culture 5 was the standout; by Day 4 *L. monocytogenes* was absent after fermentation.

Table 26 *Listeria monocytogenes* presence/absence in *Perna* made with five starter cultures to Day 74 – Preparation in November 2016. Symbols + and – indicate detected and not detected respectively for *L. monocytogenes* in 25 grams of sample.

	Days after preparation									
	0	4	11	11	18	18	46	46	74	74
Culture			4°C	Amb.	4°C	Amb	4°C	Amb	4°C	Amb.
1	+	+	–	+	–	–	+	–	+	–
2	+	–	+	–	–	+	+	+	+	+
3	+	+	+	+	+	–	+	–	+	–
4	+	+	+	+	+	–	+	–	+	–
5	+	–	–	–	–	–	–	–	–	–

Table 27 *Listeria monocytogenes* presence/absence in *Perna* made with five starter cultures to Day 74 – Preparation in December 2016. Symbols + and – indicate detected and not detected respectively for *L. monocytogenes* in 25 grams of sample.

	Days after preparation					
	0	4	46	46	74	74
Culture			4°C	Amb.	4°C	Amb.
1	+	–	–	–	–	–
2	+	–	–	–	–	–
3	+	–	–	–	–	–
4	+	–	–	–	–	–
5	+	–	–	–	–	–

Table 28 *Listeria monocytogenes* presence/absence in *Perna* made with five starter cultures to Day 74 – Preparation in January 2017. Symbols + and – indicate detected and not detected respectively for *L. monocytogenes* in 25 grams of sample.

	Days after preparation					
	0	4	46	46	74	74
Culture			4°C	Amb.	4°C	Amb.
1	+	–	–	–	–	–
2	+	–	–	–	–	–
3	+	–	–	–	–	–
4	+	–	–	–	–	–
5	+	–	–	–	–	–

Discussion

Effect of storage temperature using one culture

The aerobic plate count, APC, is used to indicate the crude level of bacteria in a food product or other material. It returns the number of bacteria that grow in the presence of oxygen (aerobic). APC results for fermented foods including fermented and cured meats, fermented vegetables, ripened cheeses, yoghurts, cultured butter, etc. do not apply to safety regulations(Agency, 2009) (Health Protection Agency, (2009) because of the microflora's nature. *Perna* as a fermented food has high APC counts, mainly comprising the starter cultures used. The counts in *Perna* indicated that LAB dominates the microflora of product (Figure 61) LAB counts increase rapidly in the first four days of fermentation. Fast LAB growth was usually accompanied with high acidification rates (Figure 14). Rapid pH falls are likely to prevent pathogenic bacteria growth (Table 25). High counts of *S. carnosus* (Table 25) in acidic conditions indicate that this bacterium is resistant to acidic conditions and is chosen for inclusion in culture presumably because it will remain active and contribute to flavour. Several *Staphylococcus* species have an important role in aroma development through their protease and lipase activities (Montel et al., 1998; Stahnke, 1994).

The effect of storage temperature at 4°C, ambient and 35°C reported in Figure 62 showed an increase in APC and LAB numbers until Day 4, inversely correlated with pH (Figure 63). The LAB was always dominant for all temperatures, and their negative correlation with pH indicates that the principal role of LAB is to acidify the *Perna*. Between Day 4 and Day 11 the pH decreased more at the higher temperatures. This may be in response to higher LAB numbers at some point between Days 4 and 11, and/or the bacteria were simply more metabolically active at the higher temperatures. Rapid lowering of pH is important. Several authors have also reported that growth of most spoilage bacteria and pathogens present in minced fish was inhibited more effectively by rapid attainment of low pH (Hu, Xia, & Ge, 2007; Yin, Pan, & Jiang, 2002).

Although the numbers of useful bacteria decreased with storage at higher temperatures, pH values remained largely constant beyond Day 18 (Figure 63). Fermented foods can contain low counts of live or viable organisms simply due to inhospitable environmental conditions that reduce microbial populations over time. However, the absence of live microbes in the final product does not preclude a positive functional role. Broadly, food fermentation microbes may produce vitamins or other bioactive molecules in situ or

inactivate anti-nutritional factors, and yet be absent at the time of consumption (Rezac, Kok, Heermann, & Hutkins, 2018). Higher storage temperature can reduce pH to very safe levels, but consumers may not like very acidic *Perna* products. Thus, storage at 4°C is the best storage temperature for *Perna*.

Basic microbiology of *Perna* prepared using five starter cultures

By Day 4 there was increase of about one log APC for Cultures 1, 3 and 4, and half a log for 2 and 5 (Figure 64). There are several possible reasons for this. The initial inoculum may be have been lower for Cultures 2 and 5 (but unknown) and/or they were slower to multiply. These possibilities are explored in Chapter 8 where detailed monitoring was done between Day 0 and Day 4, so discussion is deferred.

Interestingly, the APC pattern for each culture established by Day 4 was maintained to at least Day 18. Possibly the glucose concentration was the same in all cultures by Day 18. After Day 46, counts decrease at both temperatures, but more so at ambient. What is the reason for this? One possibility is that the bacteria are undergoing the normal phases of lag, exponential, stationary, and death depending on conditions. The important condition may be that glucose becomes limiting, realizing that the concentration of glucose was only 1.5%. The pH data (Figure 65) that matches Figure 65 shows that pH increased between Days 11 and 18, and that was true for both storage temperatures. Such an increase has not been shown elsewhere in this thesis, where 2% glucose was the chosen level. With insufficient glucose, the microflora metabolizes other substrates instead, the most likely being amino acids. Deamination of these liberates ammonia, which is alkaline and is likely to be the cause of the pH increase. More generally, amino acids can be broken down into amines, ammonia or aromatic components(Montel et al., 1998). The degradation of amino acids into volatile molecules have been reported to play an important role in the characteristic flavour of dry sausage. *Staphylococcus* has been reported to play a more important role than the LAB (*Lactobacillus sakei, P. acidilactici* or *P. pentosaceus*) in the production of these volatile compounds (Berdagué, Monteil, Montel, & Talon, 1993). Viewed this way, a lower glucose concentration may be desirable for flavour.

Presence of *Listeria monocytogenes* in *Perna* during fermentation and storage in 4°C and ambient temperature

The presence/absence of *L. monocytogenes* was determined in 25 g samples in three trials. The density of colonies for Trials 2 and 3 (Table 27 and Table 28) were lower than for Trial 1 (Table 26) (data not shown), meaning that the mussels sourced for Trial 1 were most strongly contaminated with *L. monocytogenes*. Contamination at this level means that somewhere in the supply chain conditions were favourable for *L. monocytogenes*. This chain includes possible contamination in the mussel farm water, perhaps from land runoff in rainstorms. It may also be due to poor handling. All starter cultures were able to inhibit the growth of *L. monocytogenes* in less contaminated Trials 2 and 3. Toroi, a traditional Maori food, is a fermented mixture of boiled green vegetable and uncooked mussel meat. It traditionally relies on endogenous lactobacilli and is typically stored chilled for two weeks to two months before being eaten. Using a culture to prepare toroi spiked with 2.4×10^3 cfu g^{-1} *L. monocytogenes*, Dixon, Donnison, Ross, and McDonald (2008) found that *Lactobacillus sakei* Lb706, caused a rapid decrease in *L. monocytogenes* to a very low level (< 10 cfu g^{-1}) with no increase even over two months. However, was not effective for toroi that contained a 100 fold higher 2×10^5 cfu g^{-1} *L. monocytogenes*.

Although Trial 1 was highly contaminated with *L. monocytogenes*, Culture 5 was the standout; by Day 4, *L. monocytogenes* was absent after fermentation. The *Lactobacillus sakei* strain in Culture 5, is able to produce the bacteriocin sakacin P, an inhibitor originally isolated from naturally fermented foods (da Costa, Voloski, Mondadori, Duval, & Fiorentini, 2019). It has been previously shown that sakacin P inhibits *L. monocytogenes* without development of resistant strains in food model experiments (Katla, Naterstad, Vancanneyt, Swings, & Axelsson, 2003). In Cultures 1, 2, 3 and 4 for Trial 1, *L. monocytogenes* persisted more at 4°C. This could be because *L. monocytogenes* is a psychrotrophic bacterium and can multiply at low temperatures, both under aerobic and anaerobic conditions (Arevalos-Sánchez, Regalado, Martin, Domínguez-Domínguez, & García-Almendárez, 2012). It is likely that it will be able to dominate at 4°C while other microorganisms are growing slowly.

In this work I found that only one Chr Hansen starter cultures, Culture 5, was fully active against *L. monocytogenes* in *Perna*, and that its use as starter culture might contribute to improved safety of traditional fermented sausages. The results suggest the need for further studies of *Perna* challenged with the pathogen and the bacteriocins-producing strains. The next and final experimental chapter aims to evaluate the effectiveness of the bacteriocins-

producing cultures at inhibiting *L. monocytogenes* in vitro and in situ. The experiments also focus on the kinetics of pH change relating to bacterial growth during fermentation and the maturation of *Perna.*

Chapter 8

The ability of starter cultures to inhibit growth of *Listeria monocytogenes* and *Clostridium botulinum*

Introduction

The previous chapter established that several pathogens, *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Vibrio parahaemolyticus* were not detectable in three preparation trials in 2015. This strongly suggested that cooking, reduction of pH and other unidentified factors were responsible for generating a product safe from these pathogens. However, another three trials, performed on different dates in 2016/2017, showed that *Listeria monocytogenes* could still pose a serious food safety threat for *Perna*. However as seen in Chapter 7, one Chr Hansen culture was able to suppress the growth of this pathogen. In fermented foods, *L. monocytogenes* and the spore forming anaerobe *Clostridium* spp. in particular, are pathogens of concern (O'Mahony & Mitchell, 1990; Gahan et al., 1996; Chiou et al., 2002).

Listeria monocytogenes strains, its presence in raw mussels and processed mussel products, and the importance of cross-contamination during processing has been confirmed in New Zealand (Cruz & Fletcher, 2011). *Listeria monocytogenes* is a psychrotrophic bacterium and can multiply at low temperatures, both under aerobic and anaerobic conditions (Arevalos-Sánchez et al., 2012). This chapter has a focus on inhibition of *L. monocytogenes.* For *L. monocytogenes* the authentic live microbes were used as a challenge to different Chr Hansen cultures (Chapter 2, Table 4).

Clostridium botulinum is another dangerous bacterium in foods. It is practically everywhere, but most of the time this bacterium is dormant. It becomes a problem when conditions that are favorable for its growth and toxinogenesis are met. *Clostridium botulinum* prefers an anaerobic environment that is not too acidic, not too salty, not too crowded with other bacteria, and it does especially well at warm temperatures. Spores can survive high temperatures for relatively long periods of time.

Although *Perna* received heat treatment, the heat applied is insufficient to inactivate spores, so spores may survive and persist in *Perna*. Further, the cooking of mussels for 7 minutes would destroy most potentially competing microorganisms. Thus the surviving *C. botulinum* spores could germinate and grow without significant microbial competition. And

because common spoilage microflora that would indicate that a product was no longer fit for consumption would be destroyed, the *Perna* may become toxic while still organoleptically acceptable. Finally, vacuum packaging of *Perna* creates anaerobic environment favourable to growth of *C. botulinum*. For theoretical reduction of *Clostridium*, the criterion of safety was the kinetics of pH fall.

Summary of methods detailed in Chapter 2

In situ and in vitro studies

The five starter cultures from Chr. Hansen were used in one in situ method and in four in vitro methods, to evaluate their inhibitory performance on *L. monocytogenes* in *Perna*. The inputs and outputs of these methods are in Figure 66 and Figure 67, which are summaries of the methods detailed in Chapter 2.

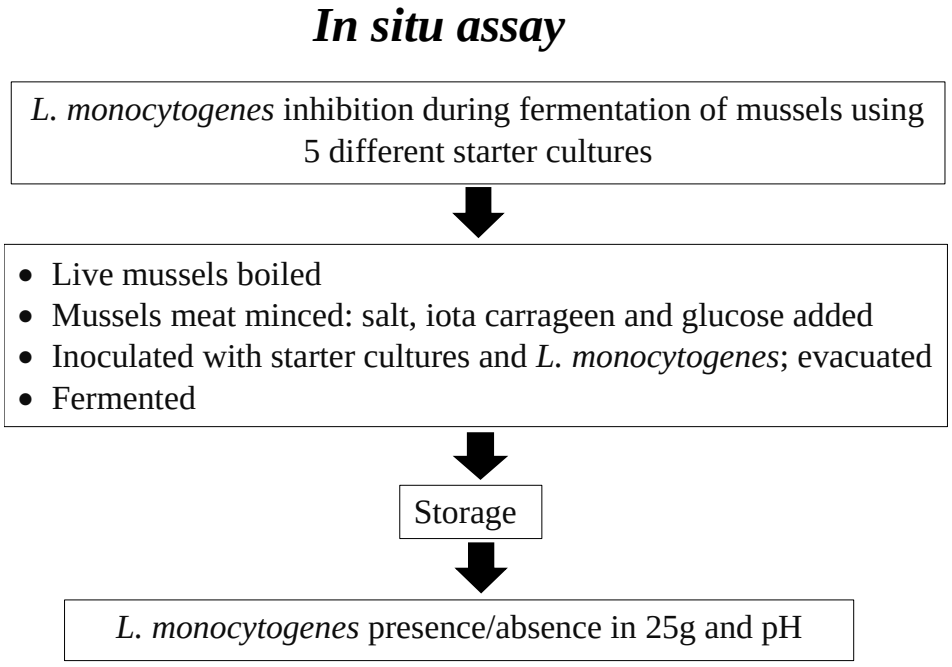

Figure 66 In situ assay for the inhibition capability of five starter cultures from Chr Hansen on *L. monocytogenes* in *Perna*.

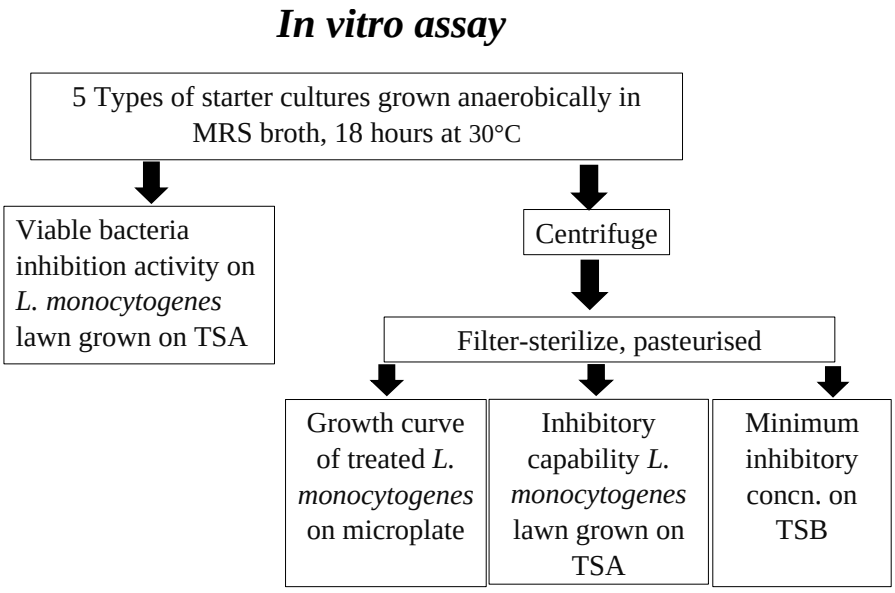

Figure 67 Four in vitro assays for the inhibition capability of five starter cultures from Chr Hansen on *L. monocytogenes* in *Perna*.

Identification of colonies

The identity of *Listeria monocytogenes* was confirmed by sequencing 16s ribosomal DNA. The full length 16s primers was used and the primer was set at 1492r primer 5′-TACCTTGTTACGACTT and 27f-CM 5′-AGAGTTTGATCCTGGCTCAG. The bioinformatics software tool Geneious-Primer 2019.1.1 was used for interpreting the sequences. Sequences obtained were compared to sequences stored on the NCBI BLAST 16s ribosomal database in order to identify bacteria. More detail of the method is in Chapter 2

Kinetics of acidification activity during fermentation of *Perna*

Perna was prepared using five starter cultures from Chr Hansen and growth rate of each culture in *Perna* were evaluated by pH. Unlike in Chapter 7, where the basic microbiological safety of *Perna* was described, the glucose concentration was restored to 2%, because of the unwanted pH increases arising from 1.5%, as described in Chapter 7. The results are means of two trials done one week apart, where the day means of replicate barrier bags were averaged and a standard deviation calculated from data from these two trials.

Results

In situ assay using five starter cultures

One preparation was made, spiked with *L. monocytogenes* and inoculated with the five cultures (Table 29), and where the glucose concentration was 2%. Although cream-coloured colonies on selective and differential medium for *Listeria,* Modified Oxford Medium were detected in Chapter 7, they were only observed, but otherwise ignored. Here however, where media was spiked with *L. monocytogenes,* it was of interest to identify the cream and black colonies observed (Figure 68).

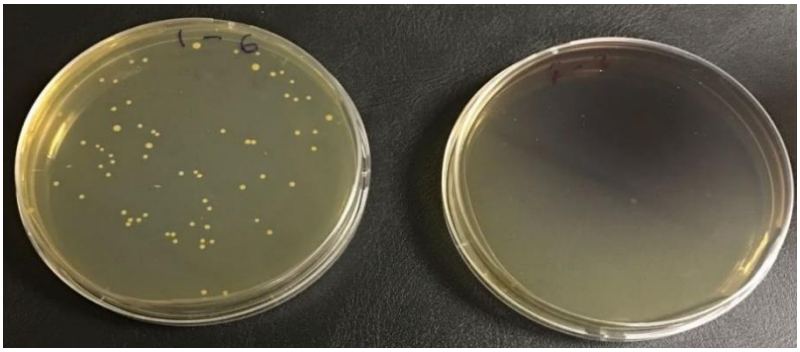

Figure 68 Cream and black colonies observed in Modified Oxford Medium. This medium was unable to definitively identify the colonies, so sequencing was used instead.

Table 29 *Listeria monocytogenes* inhibition capability of five starter cultures in fermented *Perna* at Days 0, 4 and 46 – Preparation 1. Colour indicates colony colour; black indicates positive for *L. monocytogenes* based on media selectivity; no information indicates no colonies observed.

	Days after preparation			
	0	4	46	46
			4°C	Ambient temperature
Culture 1	Black	Cream/black	Cream/black	Cream/black
Culture 2	Black	Cream/black	Cream	Cream/black
Culture 3	Black	Cream	Cream	Cream
Culture 4	Black	Cream/black	Cream/black	Cream
Culture 5	Black	Black		

As judged by the black colonies, all cultures were positive for *L. monocytogenes* at Day 0 as expected, but black colonies persisted in Cultures 1, 2, 4 and 5 at Day 4 and in Cultures 1, 2 and 4 at various subsequent days. These latter black colonies may or may not be *L. monocytogenes*, but this identity was assumed for Cultures 1 and 2 because black colonies were clearly persistent.

Attention was therefore directed to Cultures 3, 4 and 5, which were more promising as being inhibitory for *L. monocytogenes*.

The cream colonies in Culture 3 were most likely *Staphylococcus* spp. (Table 30), but the genus was not resolved to species level. Evidently this species cannot hydrolyse the esculin included in the Modified Oxford Media (see Discussion).

The cream colonies in Culture 4 were also most likely *Staphylococcus* spp. (Appendix XI). However, black colonies persisted at Day 46, and were the spiked *L. monocytogenes* (Table 31).

The black colonies in Culture 5 (no cream colonies at all) were *L. monocytogenes* (Table 32), still present at Day 4 but not at Day 46; it is not known exactly when *L. monocytogenes* was no longer present because there were no time points between 4 and 46 days. Replicated results are showed in Appendix XII, Appendix XIII, Appendix XIV, and

Appendix XV.

Table 30 Identification of the cream colony grown in Oxford modified medium from *Perna* fermented by Culture 3, based on BLAST bioformatics.

Description	Max Score	Total Score	Query Cover (%)	Expectation value	Percent Confidence
Staphylococcus sp. IGCAR-2/07 16S, partial sequence	780	1619	100	0	94
Staphylococcus sp. SDB 2975 16S, partial sequence	472	942	100	3.00E-129	100
Staphylococcus sp. SDB 2975 chromosome, complete genome	472	5653	100	3.00E-129	100

Table 31 Identification of the black colony grown in Oxford modified medium inoculated with authentic *Listeria* monocytogenes, based on BLAST bioformatics.

Description	Max Score	Total Score	Query Cover (%)	Expectation value	Percent confidence
Listeria monocytogenes strain DPMP-TSN11 16S, partial sequence	1371	1371	100	0	100
Listeria monocytogenes strain DPMP-TSN6 16S, partial sequence	1371	1371	100	0	100
Listeria monocytogenes strain DPMP-TSN4 16S, partial sequence	1371	1371	100	0	100

Table 32 Identification of the black colony grown in Oxford modified medium from *Perna* fermented by Culture 5, based on BLAST bioformatics.

Description	Max Score	Total Score	Query Cover (%)	Expectation value	Percent confidence
Listeria monocytogenes strain FDAARGOS_555 chromosome, complete genome	518	6173	100	4.00E-143	100
Listeria monocytogenes strain FDAARGOS_554 chromosome, complete genome	518	6173	100	4.00E-143	100
Listeria monocytogenes strain 3 16S, partial sequence	518	1028	100	4.00E-143	100

In vitro assay using five starter cultures

Results for the in vitro work (Figure 66) were as follows, divided in four parts that were bolded in Chapter 2, page 49.

Determination of the inhibition activity of starter culture spots on a *Listeria monocytogenes* lawn

The five viable cultures spotted on TSA agar (Figure 69) were used to determine the inhibition zone of each starter culture. Cultures 3, 4 and 5 inhibited growth at all three L. monocytogenes concentrations (104 to 106 cfu mL-1), but Cultures 1 and 2 did not (Figure 69). However, although not clear from the photographs (Figure 69), the inhibition zone decreased (Table 33) with higher L. monocytogenes concentrations. Culture 5 was the most inhibitory, followed by Culture 3 then 4. Cultures 1 and 2 showed no activity at any concentration. In summary, Culture 5 had the highest inhibitory ability, followed by Culture 3, then 4. Cultures 1 and 2 had no activity against the lawn of L. monocytogenes.

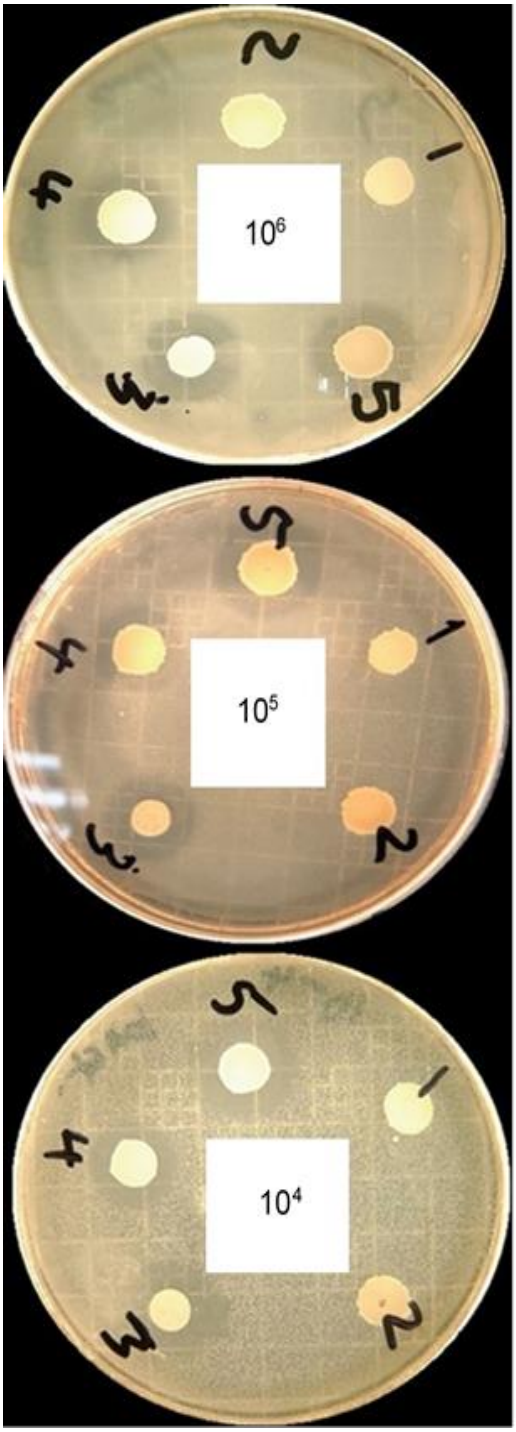

Figure 69 LAB inhibition activity on *L. monocytogenes* grown as a lawn on tryptic soy agar, where values are cfu mL^{-1}. Two of the five cultures had negligible activity.

Table 33 LAB inhibition zones on *L. monocytogenes* grown as a lawn on tryptic soy agar, where values are mm.

	Dilutions of *L. monocytogenes* grown as a lawn (cfu mL^{-1})		
	10^6	10^5	10^4
Culture 1	0	0	0
Culture 2	0	0	0
Culture 3	16 ± 1	18 ± 0	19 ± 0
Culture 4	16 ± 1	18 ± 0	19 ± 0
Culture 5	17 ± 1	18 ± 1	19 ± 0

The inhibition activity of starter culture supernatants on a L. monocytogenes lawn

Lawns as prepared above were spotted with 10 µL of filter-sterilized and pasteurized *Perna* supernatant incubated for 18 h at 35 °C and monitored for inhibition zones. Inhibition results (Figure 70, Table 34 showed the same trends as for using live culture. Peculiar growth within the zones of inhibition (Figure 70) were observed for the supernatant inhibition zones for Cultures 3 and 4 for all concentration of *L. monocytogenes* and for some extent for Cultures 1 and 2 at the high concentration of *L. monocytogenes*. The growth appeared to consist of larger colonies comparing to lawn colonies of *L. monocytogenes*, and creamy in the site where supernatant was applied. None were expected on account of filter sterilization and pasteurization that is supposed to remove all bacteria. Culture 5 did not show this phenomenon; the inhibition zone was clear.

Another feature of Figure 70 – one that is not immediately obvious – is that creamy colonies grown on the site where supernatant was applied were greatest in number where *L. monocytogenes* was most concentrated. If the creamy colony derived from the supernatant, one would expect to see fewer at the highest *L. monocytogenes* lawn concentration, 10^7 cfu mL^{-1}. Moreover, such a predicted effect would be most likely for Culture 1, which had the lowest inhibitory capacity. However, the reverse was seen, true for all culture except Culture 5, and what this means is examined in Discussion.

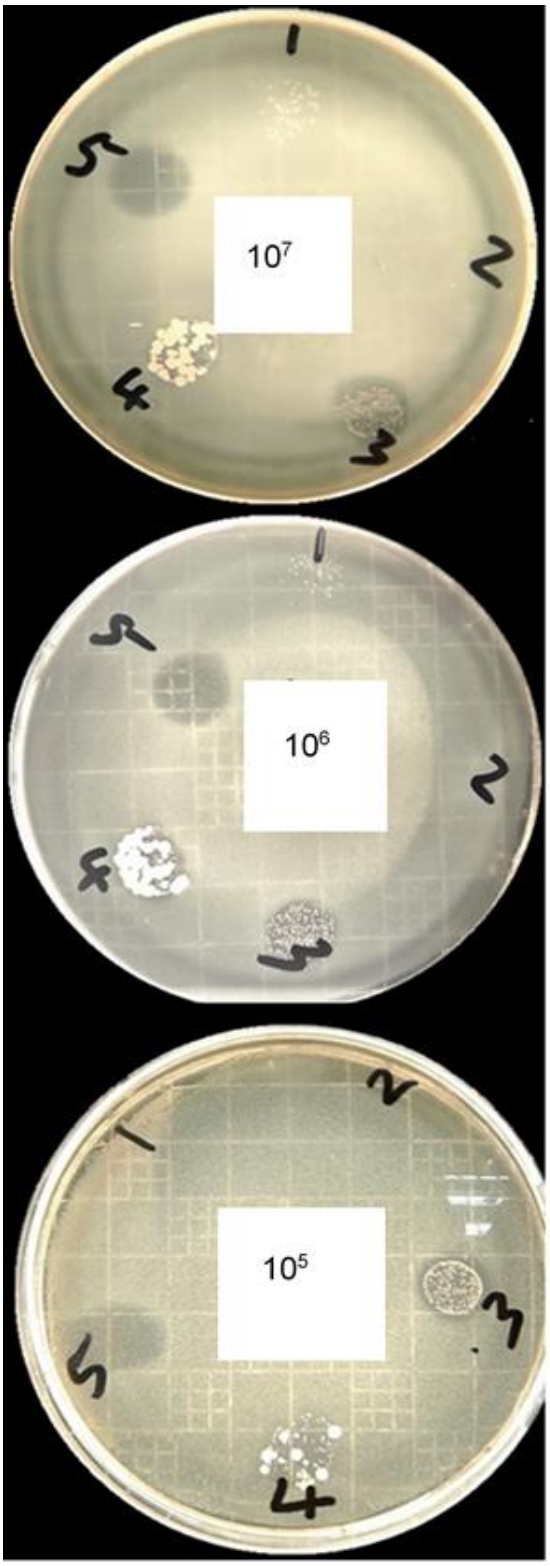

Figure 70 Supernatant inhibition activity on *L. monocytogenes* grown as a lawn on TSA, where values are cfu mL^{-1}.

Table 34 Supernatant inhibition activity on *L. monocytogenes* grown as a lawn on TSA, where values are mm.

	Dilutions of *L. monocytogenes* grown as a lawn (cfu mL^{-1})		
	10^7	10^6	10^5
Culture 1	0	0	0
Culture 2	0	0	0
Culture 3	10 ± 0	12 ± 0	12 ± 0
Culture 4	9 ± 0	10 ± 0	10 ± 1
Culture 5	10 ± 0	12 ± 0	12 ± 0

The inhibition activity of starter culture supernatants by microplate assay

To determine if the inhibition of *L. monocytogenes* by starter cultures was caused by secretion of antimicrobial compounds, a microtitre well assay was performed using cell free supernatant of starter cultures. Aliquots (100 μL) of filter-sterilized supernatant of each culture applied to a clear 96-well microplate and inoculated with *L. monocytogenes* at 10^4 cfu mL^{-1}. As judged by absorbance due to turbidity at 595 nm, all culture supernatants were able to increase lag phase and reduce the growth of *L. monocytogenes*. Culture 5 showed the greatest inhibition of growth of *Listeria* while Culture 1 showed the lowest inhibition (Figure 71). After the lag phase the turbidity became more complex, but beyond the scope of this study. However, two points are important: all supernatants had some inhibitory activity, but that from Culture 5 was clearly the best.

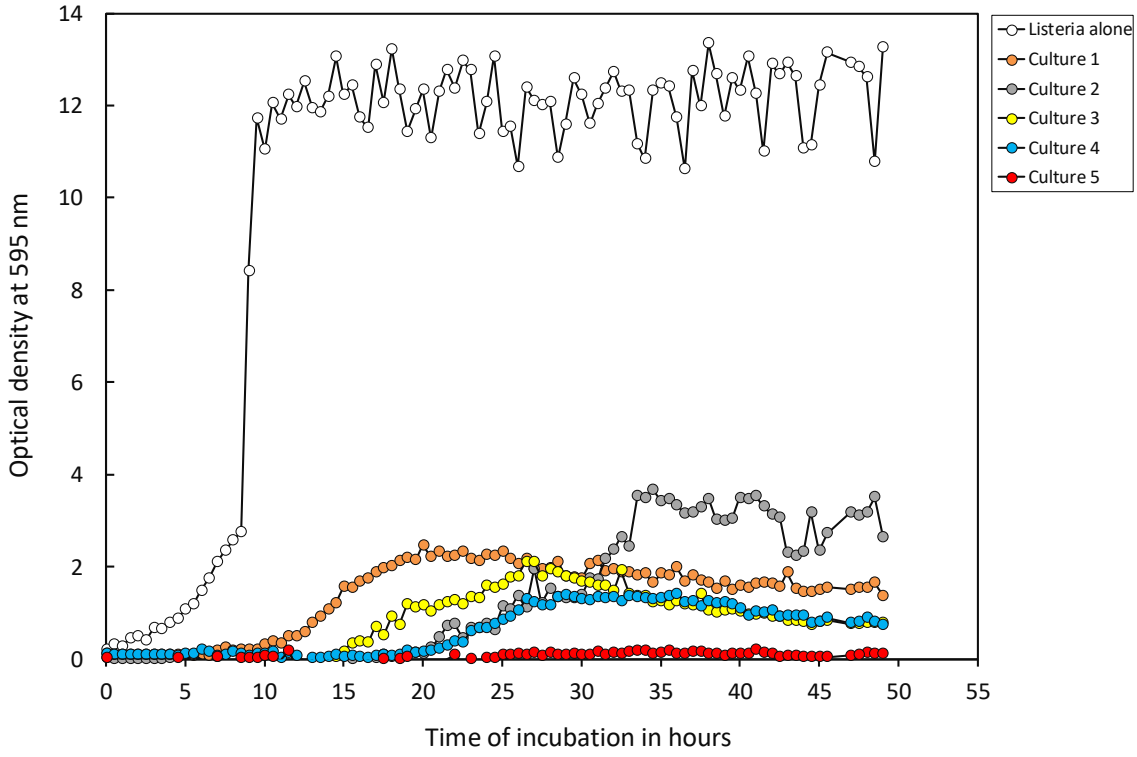

Figure 71 Growth curve of *L. monocytogenes* treated with starter culture supernatants, as determined by turbidity.

Determination of minimum inhibition concentration of starter culture supernatants on L. monocytogenes

The supernatants of all cultures showed the lowest concentration for complete inhibition was for undiluted samples (Figure 72). At all concentrations, Culture 5 was more effective than Cultures 1, 2, 3 and 4 at all dilutions, many differences of which would be statistically significant, particularly at more concentrated concentrations.

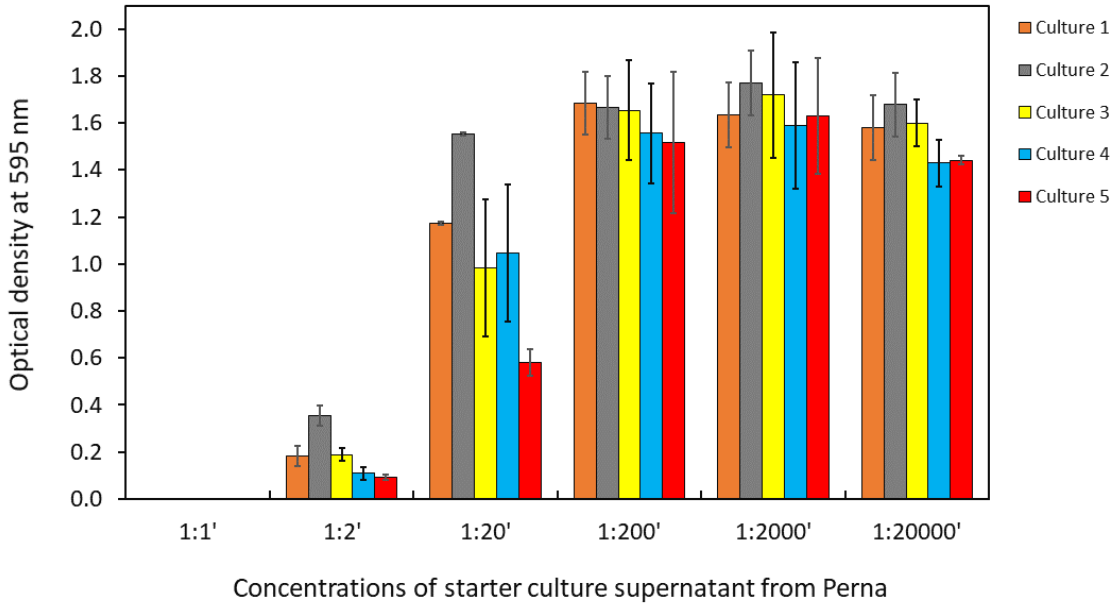

Figure 72 Minimum inhibition concentration of starter culture supernatants against *L. monocytogenes* at 10^4 cfu mL^{-1}. Data are means of optical density of three replicate incubations, as measured in a spectrophotometer with 10 mm light path. Bars are standard deviations.

Growth rate of five cultures with respect to pH change in Perna

All bacteria were able to reduce pH to 5.2 within 48 h (Figure 73) as required by the New Zealand standard for fermented food safety (MPI, 2017). Culture 5 was able to reduce the pH faster in the first 6 h when compared to the other cultures (Figure 73). Standard deviations, which were small are not shown for clarity, but by inspection of annotated points, many differences were obviously significant.

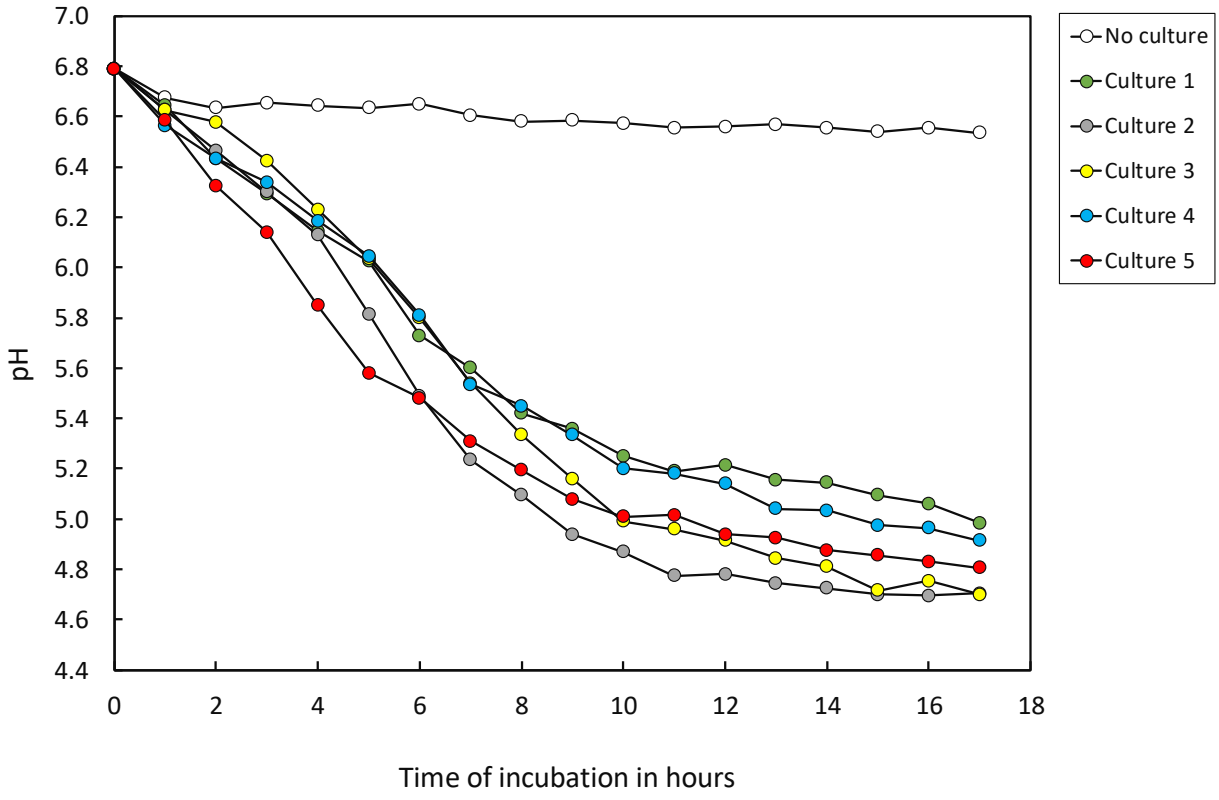

Figure 73 The pH of *Perna*, prepared using five starter cultures, with measurements taken hourly from preparation time to 17 hours. Standard deviations are omitted for clarity.

Discussion

The Table 35 shows the composition of microorganism in each culture and its acidification characteristics according to manufacturer, Chr Hansen. For the in situ work, the inhibitory capability of five cultures against *L. monocytogenes* were compared by investigating persistent growth of *L. monocytogenes* and other microorganisms on *Perna.*

Culture 5 with a combination of *Lactobacillus sakei* and *Staphylococcus carnosus* appeared to be effective in inhibition of *L. monocytogenes* (Table 29 and Table 35). Culture 5 appeared to produce a safe product, although there are no data for the first 42 days. Elimination of *L. monocytogenes* was observed by Day 46.

Table 35 The culture composition of cultures and their contribution in acidification as described in Chr Hansen (2014).

Culture	Culture species	Acidification as described by manufacturer
1	*Pediococcus pentosaceus*, *Staphylococcus carnosus* subsp. *utilis*	Fast pH fall
2	*Pediococcus pentosaceus*, *Lactobacillus sakei*, *Staphylococcus xylosus*, *Staphylococcus carnosus* 1 undefined subsp., *Debaryomyces hansenii* (a yeast)	Fast pH fall at high temperature
3	*Pediococcus acidilactici*, *Lactobacillus curvatus*, *Staphylococcus xylosus*	Moderate pH fall
4	*Lactobacillus sakei*, *Staphylococcus carnosus* 2 subsp. undefined	Fast pH fall
5	*Lactobacillus sakei*, *Staphylococcus carnosus* 1 undefined subsp.	Fast pH fall due to fast growth

For Cultures 3 and 4 the cream colonies on Modified Oxford Medium were *Staphylococcus* spp. (Table 30, Appendix XI). The esculin in the Modified Oxford Medium is normally hydrolysed by *Listeria* species. and the resulting compound reacts with ferric ions (from the ferric ammonium citrate) to produce 6,7-dihydroxycoumarin and thus blackening of the media surrounding the colonies (APHA (2001). This suggests that the *Staphylococcus* spp. identified lacks the ability to hydrolyse esculin. Of more general microbiological interest was the occurrence of *Staphylococcus* spp in Culture 3 and 4 (Table 30, Appendix XI). The Modified Oxford Medium contains moxalactam to inhibit staphylococci, bacilli and *Proteus* species (APHA (2001). However,Curtis, Mitchell, King, and Griffin (1989) reported *Staphylococcus* spp. that may occasionally grow on Modified Oxford Medium as yellow/cream colonies. Moreover, it has been reported by Thermo Scientific (2009) that some staphylococci may grow as esculin-negative colonies in Modified Oxford Medium; these colonies will not be black. The origin of *Staphylococcus* spp. could be from the cultures, because Culture 3 contains *Staphylococcus xylosus* and Culture 4 contains *Staphylococcus carnosus* (Table 35). Further research work needs to be done to confirm the origin of these bacteria species.

Bacterial features exploited for functional and safe foods design have been demonstrated to be strain-dependent rather than species dependent (Capozzi et al., 2011). This could be main reason for the inhibitory capability of Cultures 2, 4 and 5 to differ, although all contains species of *L. sakei* and *S. carnosus* (Table 35). In this respect, development of new starter cultures have been stated to involve a combination of screening for naturally occurring strains with the desired properties, and improvement of existing strains by means of modern microbiological methods to get the desired characteristics (Derkx et al., 2014; Johansen, Øregaard, Sørensen, & Derkx, 2015; Kuipers, 2015).

The in vitro experiment was divided into four sections. The first section, on the inhibition activity of starter culture spots on a *Listeria* lawn (Figure 69), was on growth media. Culture 5 was most inhibitory followed by 3 then 4 while Cultures 1 and 2 were not.

Previous studies have focused on bacteriocin producing LAB such as the pediocin[14]-producing bacteria like *Pediococcus pentosaceus* and *Pediococcus acidilactici* used in cultures and reported to be active against *L. monocytogenes* in fermented meat and milk products (Banwo, Sanni, & Tan, 2013; Bhunia, Johnson, & Ray, 1988; Lewus, Kaiser, & Montville, 1991). *Pediococcus acidilactici* in this study was present only in Culture 3 (Table 35). Chr

[14] Pediocin is a bacteriocin specific to *Pediococcus.*

Hansen (2014) have developed Culture 3 for fermented sausages as a patented culture blend capable of preventing growth of *Listeria.* The cultures containing *P. pentosaceus,* Cultures 1 and 2 (Table 35), were less inhibitory (Figure 69) than cultures containing *Lactobacillus sakei.* This bacterium has been reported as producing sakacin bacteriocins at the early logarithmic growth phase (Avaiyarasi et al., 2016) and appears to be a candidate for inhibition of *L. monocytogenes* in food.

Katla et al. (2003), analysed 200 strains of *L. monocytogenes* collected from food and the food industry for susceptibility test to bacteriocins sakacin P, sakacin A, pediocin and nisin. The individual 50% inhibitory concentrations variously ranged between 0.01 and 781 ng mL^{-1}, a very wide range. Sakacin P was the most effective to inhibit *L. monocytogenes* even at low concentrations (50% between 0.01 to 0.61 ng mL^{-1}) than sakicin A, although both are produced by strains of *Staphylococcus sakei*. It seems likely that species is not a perfect guide to *L. monocytogenes* inhibition. Strain selection is very important too (Avaiyarasi et al., 2016; Katla et al., 2003).

In the second part of in vitro experiment was determination of the inhibition activity of starter culture supernatants on a *L. monocytogenes* lawn. The supernatant from Cultures 1, 3 and 4 for all concentration of *L. monocytogenes* – and for Culture 2 at the highest concentration of *L. monocytogenes* – showed growth of large cream colonies compared to tiny lawn bacterial colonies in the inhibition zones.

It was expected that at higher concentration of *L. monocytogenes* (10^7 cfu mL^{-1}), any contaminating bacteria in the supernatant would be suppressed. Conversely, at low *L. monocytogenes* concentrations (10^5 cfu mL^{-1}) any contaminating bacteria in the supernatant would be encouraged to grow, but the vice versa was true, and most obvious for Culture 1.

The filtration/pasteurization system was thorough, indicated by for absence of any growth in the inhibition zone of Culture 5, as this was consistent over three experimental replicates. Possibly the supernatant induced a spontaneous mutation in *L. monocytogenes*. Again Culture 5 was the best, the inhibition zone was clear without any mutation effect to *L. monocytogenes*.

Sakacin P from certain *Lactobacillus sakei* strains can inhibit *L. monocytogenes* without development of resistant *L. monocytogenes* strains. However, sakacin A produced by other strains of *L. sakei* (and pediocin and nisin), permitted development of resistant *L. monocytogenes* strains (Katla et al., 2003). Vijayakumar and Muriana (2015), identified growth colonies on the inhibition zone and were reported to be *L. monocytogenes* having developed resistance to supernatants highly likely to contain LAB bacteriocins. Vignolo et al. (2000), described a similar concept. Thus, it is concluded that the cream colonies of interest are spontaneous mutants of *L. monocytogenes*.

The growing problem of bacterial resistance to antibiotics in food could be faced by using cultures with strains like those in Culture 5.

The third part of in vitro experiment was the growth curve of *L. monocytogenes* treated with starter culture supernatants in a microplate assay (Figure 71). The maximal growth rate and lag phase of *L. monocytogenes* treated with supernatant of Cultures 1, 2, 3, 4 and 5 was compared to that of original an untreated culture control. All culture supernatants increased the lag phase and reduced the growth rate of *L. monocytogenes* compared to the control, but the effectiveness depended on the culture. In the microplate assay (Figure 71), the supernatants of all cultures were more inhibitory that when the same supernatants were applied to *L. monocytogenes* lawn. This difference was attributed to higher supernatant concentrations in microplate assay. In both assays Culture 5, which contained *Lactobacillus sakei* and *Staphylococcus carnosus* (one undefined subspecies) was the most inhibitory.

Several bacteriocinogenic lactic acid bacteria have been used in food manufacturing processes in attempts to inhibit growth of *L. monocytogenes* (De Martinis, Alves, & Franco, 2002; Kouakou et al., 2009; Mataragas, Drosinos, & Metaxopoulos, 2003). It has been reported that, at pH 5 or lower, bacteriocin is more available for inhibitory functionality compared to higher pH (Bailey & Hurst, 1971; Bhunia et al., 1988; Jack & Tagg, 1992; Vijayakumar & Muriana, 2015). The use of *L. sakei* and *S. carnosus* in suitable combination as a starter culture has been suggested by Bingol et al. (2014). Many studies have reported bacteriocin production by different *L. sakei* strains (Avaiyarasi et al., 2016; Castellano et al., 2008; Møretrø et al., 2005). In red meat storage trials under vacuum, five *L. sakei* isolates exhibited inhibitory properties against *Bacillus thermosphacta*, *L. monocytogenes*, and *Clostridium estherteticum*, as shown by Jones, Zagorec, Brightwell, and Tagg (2009). Inhibition of *L. monocytogenes* by the combination of bacteria in Culture 5 showed the combination that worked in *Perna*.

The fourth part of in vitro experiment was to determine the minimum inhibition concentration of starter culture supernatants on *L. monocytogenes*. The undiluted supernatant showed lowest complete inhibition of *L. monocytogenes* at 10^4 cfu mL^{-1} (Figure 72). Culture 5 was again the most effective at all concentrations.

Antimicrobial activity can be highest at lower pH (Avaiyarasi et al., 2016). This could be one of reason of decrease of inhibitory ability with dilution. Dilution would decrease the concentration of H+ in the supernatant solutions and therefore increase the pH. Inhibitory action of LAB has been reported to be due to complex antagonistic systems produced by the starter cultures (Daeschel, 1989; Klaenhammer, 1988). (Ray, 1995), reported that certain conditions are required for the secretion of active pediocin molecules, particularly a low pH. The acidic status of *Perna* provides favorable conditions for bacteriocin production.

Now consider *Clostridium botulinum*. Among foodborne pathogens in fermented products, *C. botulinum* is a major concern (Akhtar, Paredes-Sabja, Torres, & Sarker, 2009; Linton, Connolly, Houston, & Patterson, 2014). *C. botulinum* requires anaerobic conditions, so it can only grow in the absence of oxygen. Under the right conditions, it creates heat-stable spores that can survive in foods that are incorrectly or minimally processed. *C. botulinum* produces a potent neurotoxin formed during vegetative cell growth, and illness is caused by the ingestion of foods containing this neurotoxin. The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating at 80°C for 10 min (Mataragas et al., 2003). This condition is probably met during mussel cooking, but spores could still represent a danger.

Lalitha and Gopakumar (2005), reported that with 30°C incubation the earliest time for *C. botulinum* toxicity in a fermenting anaerobic meat medium was one day. However, heat treatment at 80°C for 20 s reduced the number of spores able to germinate by 93% and extended the lag time and increased the lag variability of the surviving spores. This shows the value of heat treatment, which is notably fundamental to the *Perna* process. Cooking *Perna* has advantage of widening of lag phase of *Clostridium* spores remaining after mussel cooking. This allows enough time for LAB to the drop the pH and at same time be able to start production of bacteriocin.

At the lowest pH at which germination of *C. botulinum* spores was detected in fermented products, pH 4.8, a lag time of 20 to 53 days was required depending on strain (Graham & Lund, 1987; Lalitha & Gopakumar, 2005) As for storage temperature, the minimum pH for growth of most strains at 30°C was between 4.8 to 5.0, but at chill temperatures the minimum pH for growth was raised to 5.5 (Graham & Lund, 1987; Lalitha & Gopakumar, 2005). These experiments show the value of a low pH.

Turn now to the rate of acidification experiment during *Perna* fermentation with the five starter cultures. Previous pH determinations in other chapters have simply recorded pH at Day 0 and then at Day 4. From this study, all cultures were able to drop pH below 5.2 within 17 hours of fermentation but Culture 5 was the fastest (Figure 73).

A guidance document from Ministry of Primary Industry on production of uncooked fermented salami (MPI, 2017) indicate that, “If it takes longer than 48 hours for the pH to drop below 5.2, or if the pH does not drop far enough, then there is a likelihood that pathogenic bacteria or toxins will still be present”. In this experiment Culture 5 was able to reduce pH faster than other cultures (Figure 73) but all cultures were able to drop pH below 5.2 within 17 h of fermentation, greatly faster than the requirement of 48 h (MPI, 2017). Long term inhibitory against *C. botulinum* can best be achieved in *Perna* for refrigerated storage along with proper starter culture for fermentation process for suitable pH fall during fermentation to ensure the

public health safety.

Chapter 9

Concluding discussion

This study has investigated detailed physicochemical and microbiological of a completely new food product, *Perna*. Completely new knowledge regarding lactic-fermented molluscs has been generated. Variations in physicochemical and consequently nutritional components of *Perna* and understanding the magnitude of these variations has defined a base commercial product produced with commercial starter cultures.

The most important activities of these starter cultures are conversion of lactose into lactic acid by glycolysis (pH change), hydrolysis of protein chains into peptides and amino acids (proteolysis), hydrolysis of fatty acids (lipolysis) and subsequent metabolism into keto-acids, ketones and various esters, many of which are responsible for odor and flavour. Another activity is suppression of pathogens, by pH alone and by bacteriocins. Related to these collective activities is the effect of longer-term storage after fermentation.

The microbiological status of *Perna* throughout the production and storage events has been done according to New Zealand microbiological criteria set by the New Zealand Food Safety Authority to confirm or otherwise that *Perna* meets safety standards. The study has identified the best cultures for use in production of *Perna* at a commercial level through observing physicochemical and microbiological terms of *Perna* prepared using different five cultures. Inhibition capability of LABs to stubborn pathogen in seafoods, the *Listeria monocytogenes* have been investigated, fast acidifying culture in order to be capable to inhibit dangerous pathogens in fermented sea foods has been identified such as *Clostridium botulinum*. The major findings in this study are the following:

Seasonal changes

The pH of *Perna* at the day of preparation showed no obvious pattern with season. Had the mussels been harvested in a controlled way useful information might have been obtained. However, the source was not known, sea conditions were unknown, and the time between harvest and sale was also unknown. pH changes due to lactic fermentation between Day 0 and Day 4 in 15 trials between August 2014 and July 2015 appeared to be random. A possible reason for this randomness could be first, there was also no seasonal effect and second, the viability status of added culture may have varied. Subsampling over cultures is not ideal for maintenance of high viability and thus consistent fermentation. Another unknown was the activity of competing biota surviving the cooking treatment. Chapter 7 showed that *Listeria monocytogenes* contamination varied with date of preparation. Survival of other unmeasured

bacteria could also vary.

After fermentation, the *Perna* pH continued to decline slowly with storage at 4°C. This property assures that *Perna* product will continue to be safe, because most pathogens do not grow in acidic conditions. The spores of anaerobic bacteria such as *Clostridium* which can germinate and produce toxin at as low as pH 5.5 at chill temperature will continue to be suppressed (Graham & Lund, 1987; Lalitha & Gopakumar, 2005).

The gonad colour is orange/apricot for female and creamy white for males, but mincing caused gonad colour to be largely swamped by the colour of the viscera. *Perna* became markedly brown. As is discussed in the final section of this chapter, this is an important for marketing. Long storage at ambient temperature markedly darkened the colour of *Perna*, but colour was stable at chill temperature storage. The darkening in ambient temperature was probably due to Maillard reaction (Davies et al., 1997; Leong & Wedzicha, 2000). Storage at 4°C is best option for *Perna* product stability, but the temperature storage requirements are not particularly rigorous.

During the early phase of fermentation, contaminating microorganisms on raw materials, utensils and from the environment compete for nutrients with LAB from starter cultures. Effectiveness of the starter culture depends on the hygienic quality of the raw material used, as is spectacularly confirmed in Figure 8 (Chapter 1) where fermentation of uncooked mussel was attempted. Mussels are filter feeders and are known to be highly contaminated with marine microorganisms. Therefore, the cooking step to reduce the native biota is very important. Higher storage temperature can reduce pH to very safe levels, but consumer response to a very acidic and dark *Perna* would likely be jeopardized.

Proteins, peptides and amino acids

Significant increases in concentrations of protein hydrolysis products was anticipated in Perna, arising from enzymes secreted by the surviving endogenous microflora and culture bacteria. These increases of more generally changes in concentration were measured in four ways, amino nitrogen, UV absorbance, soluble protein, and free amino acid analysis by liquid chromatography.

Amino nitrogen as measure of amino groups that were chemically available. Storage at higher temperature, 35°C, resulted in big increases in amino nitrogen suggesting enzymatic hydrolysis to the point of spoilage. Long term storage at lower temperature resulted in slightly reduced amino nitrogen perhaps arising from the Maillard reaction or loss due to microorganisms converting free amino groups to stable products.

The similarity trends of UV absorbance data between 275 to 292 nm and 200 to 400 nm

indicated that proteinaceous matter was dominant the in water-soluble portion of *Perna* rather than nucleic acids.

A loss in soluble protein with storage time was clear, but could not be easily reconciled with the amino nitrogen results at 4°C and ambient. One reason could be that peptides below 2,000 Da, which accumulated with time were not measurable by the BCA assay, but measurable with amino nitrogen and UV absorbance. Hydrolysis would minimally affect UV absorbance because absorbance due to aromatic amino acids would occur whether peptide-polymerized or not. Indeed, changes in UV absorbance were minor compared to the apparent loss of soluble protein.

Turning now to amino acid analysis, all peaks in chromatograms were assigned to an amino acid(s), with no unknown peaks or small peptides. However, the individual amino acids showed clear seasonal changes, with some amino acids showing an inverse correlation to each other. The data for this study broadly matched data from with other researchers, Fuentes et al. (2009) and (Babarro et al., 2006) working with different mussel species. The proportions of arginine were particularly striking, leading to three patterns amino acid patterns at different times of the year. It was argued that in summer arginine accumulated as an energy reserve because food supply was not limiting. At other times, particularly late winter, arginine was clearly limiting. The free amino acids alanine, glutamic acid and glycine which are greatly responsible for flavour were present in high percentage in *Perna* extract. Data sets presented in for this section collectively suggest that these amino acids are important definers of green shell mussel flavour. In respect of the individual cultures, the Culture 4 and 5 were the fastest metabolizers to create an acidic environment in *Perna*. Culture 5 has been shown to be a standout in terms of changing the amino acid profile quickly, as is clear in Figure 39 for example.

LAB and staphylococci contributed to biogenic amine formation in *Perna* when the fermentation process was delayed, but not with advanced storage where biogenic amines were completely absent. Even at early time, biogenic amine concentrations were below regulated food safety limits.

Fatty acids in *Perna*

The fatty acid profile of *Perna* was dominated by palmitic acid, followed by DHA, then EPA, and subsequently a host of more minor fatty acids. As filter feeders, the fatty acid profile will reflect the mussel's diet. All unsaturated fatty acids are prone to oxidation, which at the very least generates off-flavors from oxidation products (aldehydes, ketones etc.) and in chronic consumption are also major risk factors for cardiovascular and inflammatory diseases. The fatty acid profile was tracked throughout *Perna* preparation and subsequent long-term storage. Any

significant and thus deleterious oxidation would be a fall in the proportion of EPA and DHA, the major unsaturated – and omega-3 – fatty acids in *Perna*. They were shown to be extraordinarily stable. Thus, the cooking, an anaerobic fermentation step and subsequent storage under vacuum has proved to be a very good way of maintaining fatty acid stability.

Volatile compounds

All LABs in starter cultures used in this study were responsible for lactic acid flavour and acidification, but the staphylococci and yeast were likely to have been contributed to differences in flavour type and intensities. Staphylococci has been reported to play a more important role than the LAB (*Lactobacillus sakei*, *Pediococcus acidilactici* or *P. pentosaceus*) in the production of these volatile compounds. From a commercial point of view, *Perna* should be prepared with glucose at or above 1.0% and should be stored chilled although it still has remarkable volatiles stability at higher temperatures. Below 1.0% flavour problems will develop.

Cooked mussel is not the intended matrix for meat salami cultures; cooked mussel is new territory and microbial responses are unknown beyond the fact that pH usually fell to preservation levels, provided enough glucose was present. It has been shown in the glucose concentration experiment (0.25 to 2%) that with insufficient glucose it is likely that the surviving microflora can successfully compete with the starter culture microflora. To make implementation even more complex, it has been shown that even if promising *Staphylococcus* strains are able to survive in a fermented food matrix, they may sometimes be metabolically inactive due to the processing conditions, for instance at too low pH values.

Microbiological characteristics of *Perna*

Although the green shell mussel is grown in clean coastal waters as required by food safety standards (New Zealand Food Authority, (2006), the potential for health risk remains, along with the associated loss of reputation and costs of a product recall due to pathogenic bacterial contamination. Contamination of green shell mussel has been linked to land use and flooding, sewage contamination of ocean water and microbial cross-contamination during processing (Aquaculture, 2012).

A lactic culture suited to raw meat fermentation (BFL-F02) was chosen for *Perna* work simply because its use was suggested by Chr. Hansen, the world's leading culture supplier, and although the results are largely acceptable, the effects of other cultures were completely unknown, and some have shown to be superior, to BFL-F02 in quality and safety of *Perna*. Culture 5 was the standout in this study and was fully active against *L. monocytogenes* in *Perna*. Its use as starter culture for *Perna* seems obvious, particularly as other quality indicators (pH, free amino acids, fatty acids, and volatiles) did not deviate to any extent from averages.

Inhibition of pathogens in *Perna*

In vitro experiment shows certain strain of *L sakei* have inhibitory ability to *L. monocytogenes*. Strain selection is very important selection of inhibitory starter cultures *to L. monocytogenes* during fermentation of mussel to produce *Perna*. Some of LAB have shown to cause spontaneous mutation on *L. monocytogenes* during fermentation but Culture five did not produce spontaneous mutation. The growing problem of bacterial resistance to antibiotics could be faced by using antibiotic can be substituted with use of these cultures with strains like Culture 5. Inhibition of *L. monocytogenes* by the combination of bacteria in Culture 5 showed the particular combination that worked in *Perna*. Undiluted acidic supernatant of *Perna* showed to provide favorable condition for *L. monocytogenes* inhibition as it has been reported enzyme responsible for production of bacteriocin are active in acidic condition (Ray, 1995). This indicates the safety status for fermented mussels, *Perna,* because rapid pH falls are likely to prevent pathogenic bacteria growth the same time produce favorable condition for enzyme responsible for production of bacteriocin.

In this experiment Culture 5 was able to reduce pH faster than other cultures (Figure 73) but all cultures were able to drop pH below 5.2 within 17 h of fermentation, greatly faster than the requirement of 48 h (MPI, 2017). Long term inhibitory against *C. botulinum* can best be achieved in *Perna* for refrigerated storage along with proper starter culture for fermentation process for suitable pH fall during fermentation to ensure the public health safety.

Market prospects and where from here

Prior to the start of this study and to the present time, attempts have been made to introduce the concept of cook-then lactic-ferment green shell mussels to commercial mussel processors. These attempts have been unsuccessful to date, and according to the inventor of the process there are several reasons for this. First, *Perna* is completely new territory for an industry with a commodity focus; second, although the capital equipment outlay would be minimal, margins in the mussel industry are low and a completely new product would be risky. Other reasons are that the brown colour is nominally unattractive, who would a supermarket target for sales, and would a consumer buy and try a completely novel product? How would they use it? As a spread on crackers? What would be an ideal pack size? These are unanswered marketing questions. However, there would be some merchandising advantages for *Perna*. Provided it is stored cool, with some temperature flexibility, the shelf life would be long with a 'best by' date months after preparation.

The discussion above focuses on the New Zealand or Australasian market, but another major market could be northeast Asia, where fermented foods are well accepted, unlikely Australasia where fermented milk dominates the market. However, it has been pointed out that a relatively homogeneous product like *Perna* might be greeted with suspicion particularly in China with its history of food fraud. *Perna* may be perceived as masking contaminating ingredients. As a counter to this, work at AUT has shown that whole cooked mussels can also be fermented, but to date that work has not been pursued.

Future work is likely to center on recipe development of several *Perna* formats (very finely comminuted, *Perna* as described here, whole mussel format), which is beyond the scope of this thesis. That is work for creative chefs.

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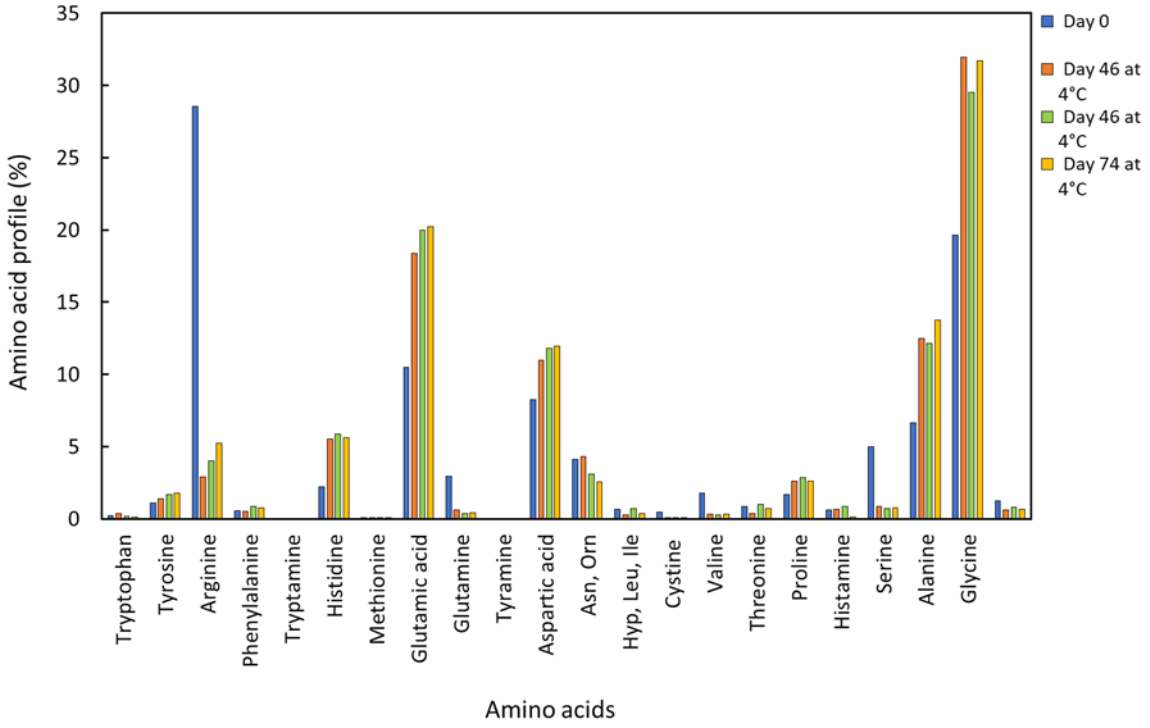
Appendices

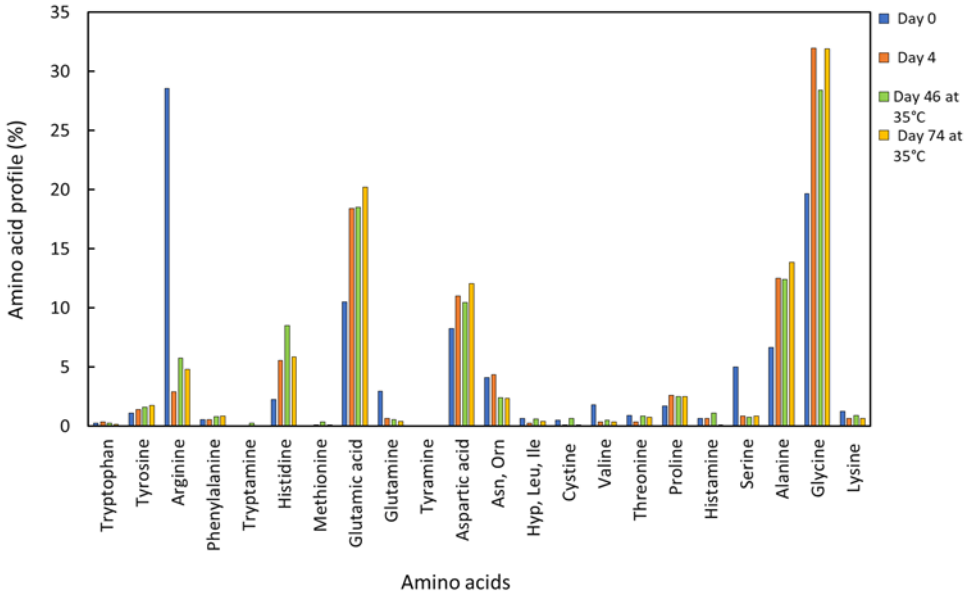
Appendix I	Appendix Standards injection preparation				
	Volume neutralising solution			450	uL
	Volume amino cpds			50	uL
	Total volume			500	uL
Maximum	Concn amino cpds	1.111111	uM		
Maximum	Concn d4-alanine	1.103737	uM		
Dilution	Concn amino cpds	0.555556	uM		
Dilution	Concn d4-alanine	0.551868	uM		
Dilution	Concn amino cpds	0.277778	uM		
Dilution	Concn d4-alanine	0.275934	uM		
Dilution	Concn amino cpds	0.138889	uM		
Dilution	Concn d4-alanine	0.137967	uM		
Dilution	Concn amino cpds	0.069444	uM		
Dilution	Concn d4-alanine	0.068984	uM		

Appendix II Sample and blank injection preparation		
Volume neutralising solution	450 uL	
Volume of sample and blank	50 uL	1
Total volume	500 uL	10
Concn sample	0 uM	
Concn d4-alanine	0.41763 uM	
Deproteinisation dilution factor		2
Total dilution of original 20 mL		180
Total volume with 2 g added		20 mL
Actual volume injected		0.01 mL
Dilution-adjusted volume injected		5.55556E-05 mL
Mass of Perna injected		0.005555556 mg
Mass of Perna injected		5.55556E-06 g

Appendix III Amino acids before fermentation at Day 0 and after fermentation at Day 4, Day 46, and Day 74 stored in 4°C after fermentation, three replicates averaged over trials of 3 batches

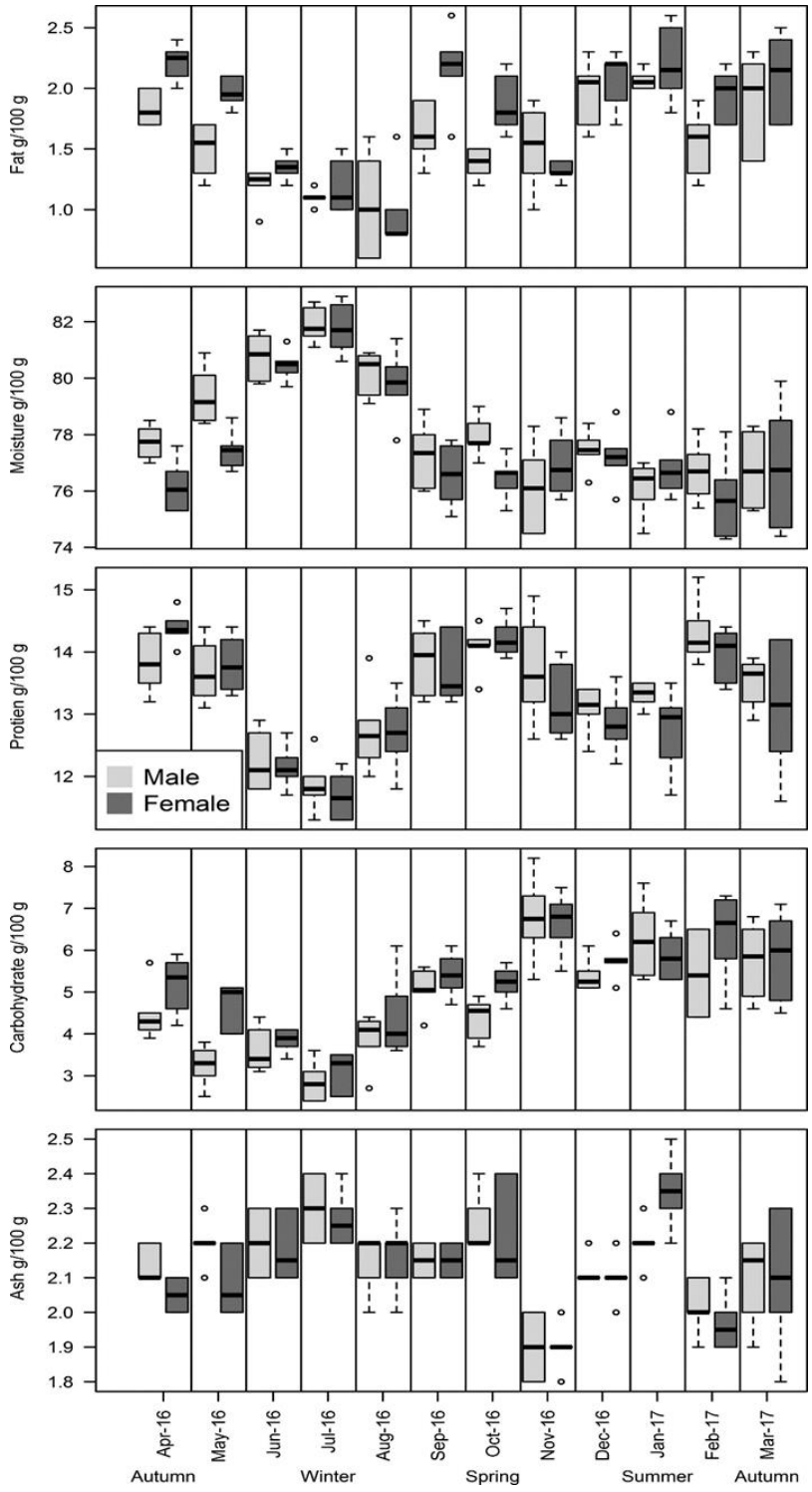


Appendix IV Amino acids before fermentation at Day 0 and after fermentation at Day 4, Day 46, and Day 74 stored in 35°C after fermentation, three replicates averaged over trials of 3 batches



Appendix V	Mean concentration of amino acid (µmol g-1) for three different designated patterns proposed to be affected by season.					
Amino acids	Pattern 1		Pattern 2		Pattern 3	
	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Tryptophan	0.009	0.011	0.009	0.008	0.001	0.004
Tyrosine	0.060	0.061	0.041	0.031	0.001	0.102
Arginine	0.114	1.388	0.976	0.064	0.015	0.060
Phenylalanine	0.015	0.026	0.020	0.012	0.001	0.112
Tryptamine	0.001	0.000	0.001	0.001	0.000	0.001
Histidine	0.108	0.109	0.093	0.123	0.073	0.285
Methionine	0.003	0.002	0.003	0.002	0.001	0.047
Glutamic acid	0.649	0.516	0.400	0.405	0.260	0.538
Glutamine	0.011	0.194	0.097	0.014	0.005	0.013
Tyramine	0.000	0.000	0.000	0.000	0.000	0.000
Aspartic acid	0.128	0.366	0.296	0.243	0.129	0.247
Asn, Orn	0.025	0.333	0.154	0.097	0.024	0.064
Hyp, Leu, Ile	0.012	0.023	0.024	0.006	0.005	0.098
Cystine	0.003	0.028	0.018	0.002	0.003	0.009
Valine	0.011	0.104	0.066	0.007	0.014	0.034
Threonine	0.020	0.050	0.031	0.008	0.010	0.116
Proline	0.149	0.102	0.066	0.057	0.023	0.133
Histamine	0.001	0.003	0.023	0.014	0.001	0.007
Serine	0.028	0.238	0.171	0.019	0.037	0.062
Alanine	0.374	0.344	0.254	0.276	0.151	0.355
Glycine	0.977	0.882	0.727	0.707	0.436	0.752
Lysine	0.003	0.024	0.044	0.014	0.005	0.041

Appendix VI Seasonal profiles of the lipid, moisture, protein, carbohydrate and ash content (g 100 g^{-1} wet weight, w/w) of male and female greenshell mussels collected from April 2016 to March 2017 (Source Miller and Tian, 2018).



Appendix VII Profile of thirty-two fatty acids identified in unfermented and fermented *Perna* stored at three temperatures (4°C, ambient and 35°C) to Day 74. They comprised 13 saturated fatty acids and 19 unsaturated fatty acids

	Day 0	Day 4	Day 11 4°C	Day 11 Ambient	Day 11 35°C	Day 18 4°C	Day 18 Ambient	Day 18 35°C	Day 46 4°C	Day 46 Ambient	Day 46 35°C	Day 74 4°C	Day 74 Ambient	Day 74 35°C
Palmitic	23.00	22.12	21.29	21.29	22.04	22.01	22.80	23.61	24.07	23.76	23.88	23.10	24.73	24.61
cis-4,7,10,13,16,19-Docosahexaenoic	21.42	22.57	22.44	23.03	23.48	22.30	22.42	21.03	24.92	22.37	21.47	22.20	21.81	21.94
cis-5,8,11,14,17-Eicosapentaenoic	15.43	15.58	15.75	16.10	16.99	16.10	16.33	15.20	17.35	16.06	14.94	15.19	15.28	15.46
Palmitoleic	6.68	6.51	6.13	5.99	6.38	6.47	6.27	6.45	6.36	6.97	6.98	7.01	7.13	7.14
Stearic	4.46	4.70	4.03	4.08	2.53	3.98	2.80	1.65	3.18	0.84	0.49	4.26	0.94	0.45
Linoleic	3.95	3.98	4.01	4.15	4.20	3.83	4.20	4.32	4.25	4.20	4.17	4.21	4.32	4.36
Pentadecanoic	3.72	3.71	3.62	3.57	3.76	3.61	3.60	3.78	4.10	4.05	4.01	3.88	4.17	4.12
cis-11,14-Eicosadienoic	2.54	2.58	2.67	2.77	2.61	2.36	2.54	2.56	2.64	1.64	2.46	0.00	0.00	1.33
Henicosanoic	2.34	2.52	2.74	2.82	2.58	2.33	2.57	2.60	2.68	2.56	2.46	2.39	2.65	2.57
Linolelaidic	2.10	2.11	2.08	2.04	2.16	2.10	2.10	2.13	2.27	2.21	2.20	2.08	2.18	2.42
Caproic	1.90	1.64	2.30	1.67	0.82	2.90	0.90	2.03	0.82	1.79	2.75	0.01	0.04	0.25
Alpha-Lenolenic	1.84	1.92	1.78	1.75	1.95	1.84	1.79	1.72	1.96	1.98	1.88	1.82	1.83	1.91
cis-11,14, 17-Eicosatrienoic	1.50	1.45	1.40	1.60	0.94	1.29	0.38	1.08	1.71	1.52	1.75	1.48	1.60	1.62
Undecanoic	1.48	0.76	0.60	0.43	0.37	0.73	1.27	2.41	0.25	1.38	2.23	1.95	1.73	1.57
Caprylic	1.16	0.94	0.98	0.79	0.77	0.87	0.94	0.93	0.79	0.71	0.80	0.93	1.02	0.95
Gamma-Lenolenic	1.14	1.16	1.13	1.22	1.37	1.14	1.19	1.15	1.26	1.23	1.23	1.19	1.25	1.22
Heptadecenoic	1.01	0.99	1.03	1.07	1.16	0.98	1.07	1.11	1.07	1.06	1.08	1.08	1.17	1.22
Erucic	0.78	0.83	1.42	1.25	1.12	0.74	0.82	0.83	0.86	0.80	0.76	0.83	0.79	0.81
Lignoceric	0.72	0.75	0.81	0.84	0.77	0.81	0.98	0.90	0.92	0.87	0.85	0.90	0.97	1.09
cis-10-Heptadecenoic	0.70	0.72	0.64	0.66	0.62	0.66	0.72	0.76	0.68	0.70	0.71	0.74	0.80	0.76
Arachidic	0.40	0.47	0.48	0.49	0.38	0.38	0.41	0.38	0.40	0.40	0.29	0.46	0.39	0.36
cis-8,11,14-Eicosatrienoic	0.38	0.40	0.45	0.41	0.36	0.41	0.57	0.60	0.47	0.49	0.57	0.41	0.56	0.56
cis-11-Eicosenoic	0.32	0.39	0.34	0.35	0.27	0.30	0.31	0.30	0.32	1.09	0.30	2.52	2.56	1.52
Arachidonic	0.27	0.35	0.71	0.49	1.17	0.67	1.76	1.33	0.84	0.51	0.87	0.33	0.85	0.68
cis-10-Pentadecenoic	0.26	0.37	0.26	0.25	0.31	0.27	0.25	0.27	0.27	0.29	0.28	0.33	0.35	0.33
Palmitolic	0.17	0.16	0.72	0.72	0.74	0.71	0.74	0.45	0.80	0.14	0.13	0.16	0.17	0.20
Myristic	0.13	0.10	0.07	0.06	0.05	0.06	0.09	0.18	0.04	0.15	0.21	0.19	0.25	0.20
Lauric	0.06	0.04	0.02	0.01	0.01	0.02	0.04	0.09	0.05	0.07	0.11	0.10	0.13	0.10

Capric	0.06	0.03	0.04	0.03	0.02	0.06	0.08	0.09	0.05	0.07	0.09	0.13	0.16	0.14
Oleic/Elaidic	0.03	0.10	0.06	0.04	0.03	0.04	0.03	0.01	0.05	0.02	0.00	0.03	0.02	0.02
Myristoleic	0.03	0.03	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.05	0.03	0.04	0.06	0.06
Tridecanoic	0.02	0.03	0.00	0.00	0.00	0.01	0.01	0.03	0.01	0.02	0.03	0.05	0.06	0.04

Appendix VIII	Names of volatile compounds identified before and after fermentation

Names of volatile compounds
Sulphur compounds
Dimethyl sulfide
Methyl hydrogen disulfide
Fucose, cyclic ethylene mercaptal
Miscellaneous compounds
Azetidine, 1-nitroso-
Azetidine, 1,3-dimethyl-
1,3,5-Triazine
Furan, 2-ethyl-
Furan, 2-(2-pentenyl)-
Furan, 2-(1-pentenyl)-
Lilac aldehyde D
Aldehydes
2-Pentenal
2-Hexenal
Heptanal-
2-Heptenal
Benzaldehyde
Octanal
2,4-Heptadienal
1H-Pyrazole-4-carboxaldehyde, 1-ethyl-
2-Octenal-
Nonanal
2-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-
2,6-Nonadienal
Benzaldehyde, 4-ethyl-

2,4-Undecadienal

1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-

Alcohols

1-Pentanol

1-Pentyn-3-ol, 3-ethyl-

1-Hexanol

Cyclohexanol, 2-methyl-, propionate, trans-

1-Heptanol

2-Butene-1,4-diol

1-Octen-3-ol

1-Octanol

10-Undecyn-1-ol

(-)-Myrtenol

Alkanes

Octane

Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-

4-Chlorobutanoic anhydride

Butane, 1-chloro-3-methyl-

Heptane, 2,2,4,6,6-pentamethyl-

Decane

Benzene, 1,2,3,5-tetramethyl-

2,2,4,4-Tetramethyloctane

Decane, 2,6,6-trimethyl-

Cyclopropane, 1,1-dimethyl-2-(3-methyl-1,3-butadienyl)-

Cyclopentane, 1-ethenyl-3-methylene-

Dodecane

Cyclohexane, cyclopropyl-

D-Limonene

Esters

6-Fluoro-2-trifluoromethylbenzoic acid, 2-formyl-4,6-dichlorophenyl ester

Glycine, N-phenyl-, ethyl ester

Isophthalic acid, di(2-isopropylphenyl) ester

Ethyl fluoroformate

Fumaric acid, dipropargyl ester

15-Hydroxy-7-oxodehydroabietic acid, methyl ester, TMS derivative

Dibutyl phthalate

Ketones

2H-Pyran-2-one, 4-ethenyltetrahydro-

2-Nonanone

3,5-Octadien-2-one

Cyclohexanone, 2-(2-nitro-2-propenyl)-

9-Decen-2-one, 5-methylene-

2-Undecanone

5,7-Octadien-4-one, 2,6-dimethyl-

2-Dodecylcyclohexanone

3,4-Hexanedione

Amines

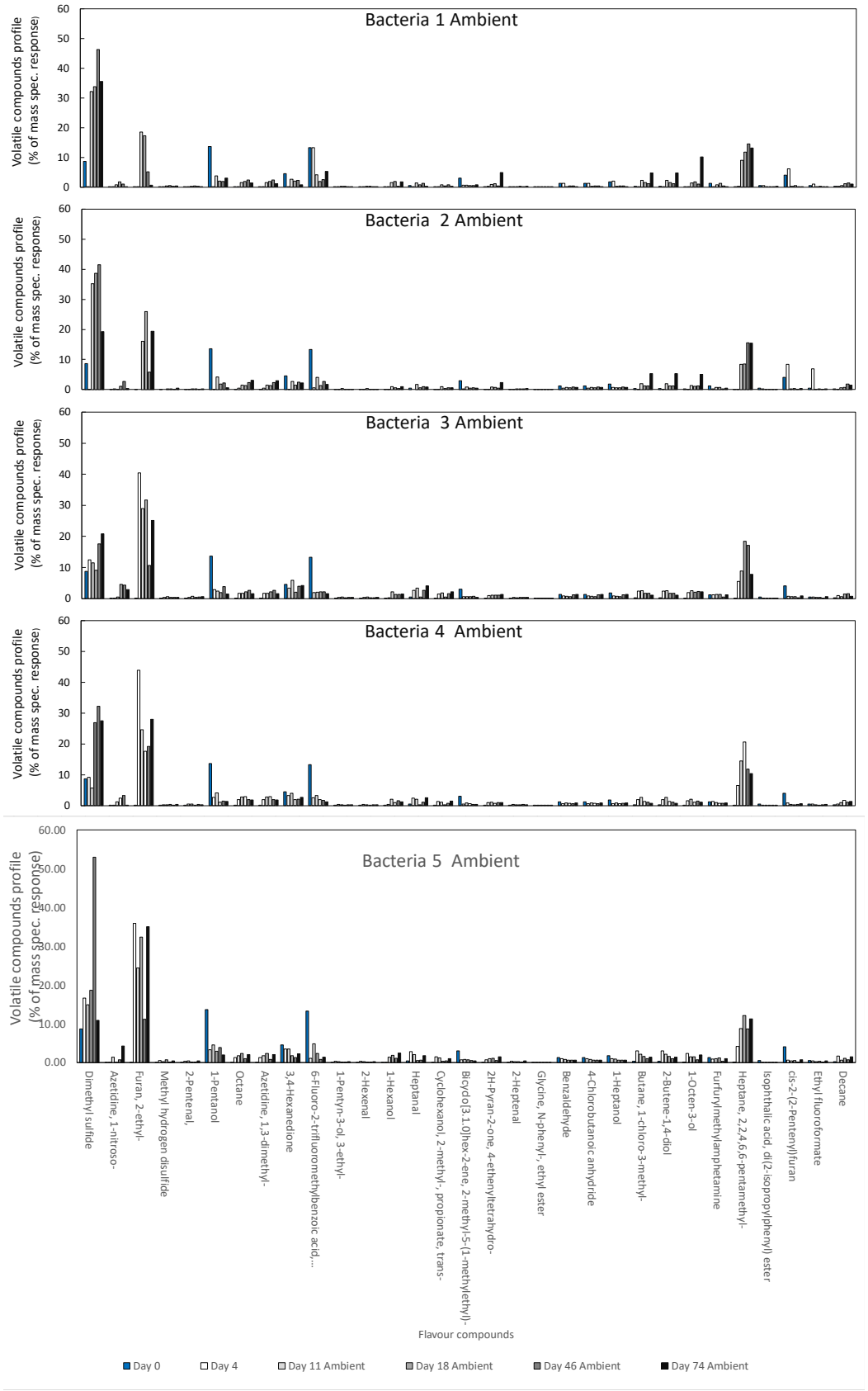
Furfurylmethylamphetamine

Methanamine , N-pentylidene-

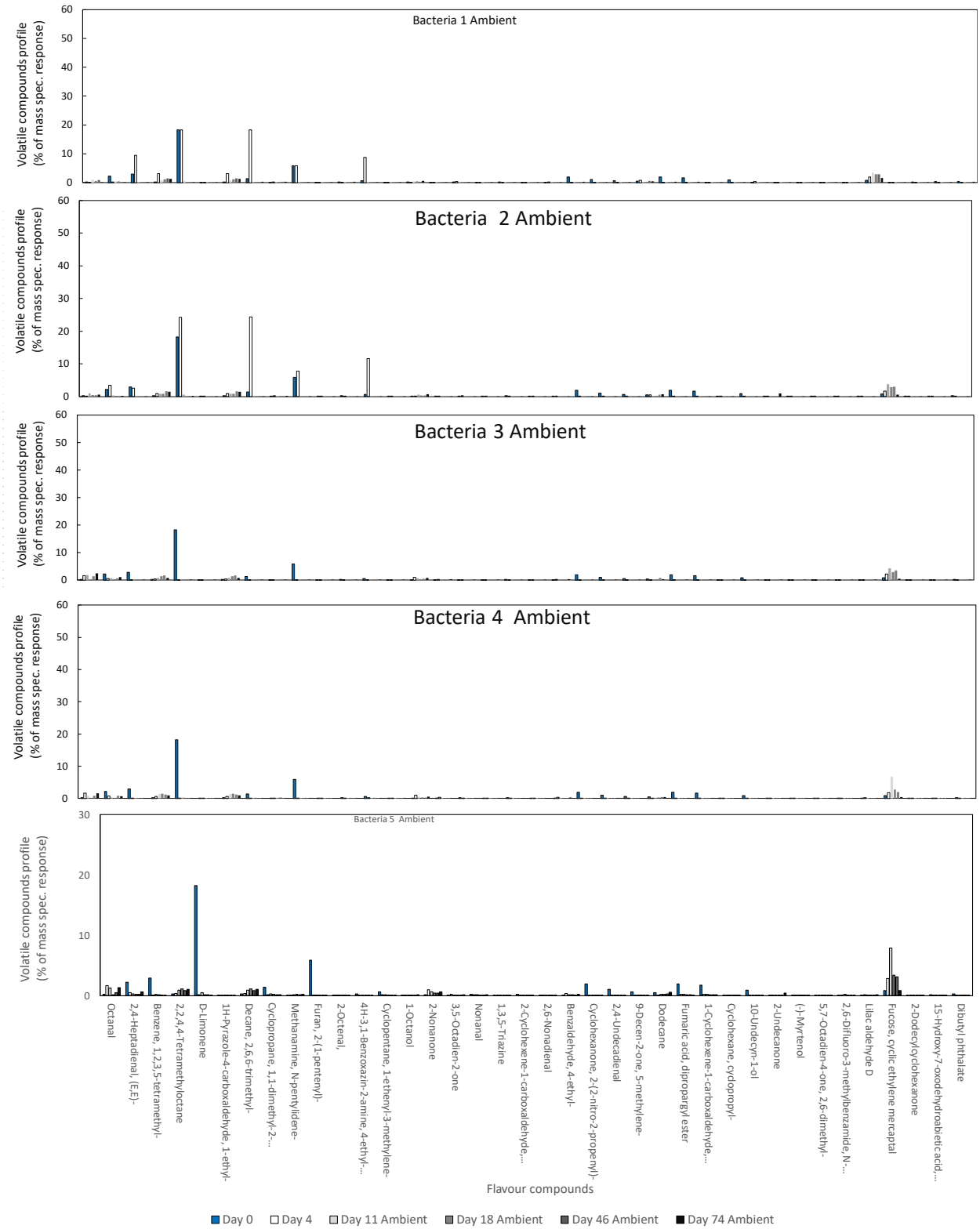
2,6-Difluoro-3-methylbenzamide, N-(3-chloro-4-fluorophenyl)-

4H-3,1-Benzoxazin-2-amine, 4-ethyl-N-[4-(1-methylethyl)phenyl]-

Appendix IX Volatile compound (1 to 31) of Perna at Day 0 and during storage for 74 days at Ambient temperature with five cultures. Compounds are shown in order of elution.



Appendix X Volatile compound (32 to 69) of Perna at Day 0 and during storage for 74 days at ambient temperature with five cultures. Compounds are shown in order of elution.



Appendix XI Identification of the cream colony grown in Oxford modified medium from *Perna* fermented by Culture 4 (A), the alignment hit table showing summary of information for target sequence found by alignment search using the basic local alignment.

Description	Max Score	Total Score	Query Cover	Expectation value	Percent confidence
Staphylococcus sp. SDB 2975 16S ribosomal RNA gene, partial sequence	409	818	100	2.00E-110	100
Staphylococcus sp. SDB 2975 chromosome, complete genome	409	4910	100	2.00E-110	100
Staphylococcus simulans strain NadiaE16 16S ribosomal RNA gene, partial sequence	409	818	100	2.00E-110	100

Appendix XII Identification of the cream colony grown in Oxford modified medium from *Perna* fermented by Culture 4 (B) the alignment hit table showing summary of information for target sequence found by alignment search using the basic local alignment.

Description	Max Score	Total Score	Query Cover	Expectation value	Percent confidence
Staphylococcus sp. SDB 2975 16S ribosomal RNA gene, partial sequence	534	1064	100	4.00E-148	100
Staphylococcus sp. SDB 2975 chromosome, complete genome	534	6384	100	4.00E-148	100
Staphylococcus simulans strain NadiaE16 16S ribosomal RNA gene, partial sequence	534	1064	100	4.00E-148	100

Appendix XIII Identification of the cream colony grown in Oxford modified medium from *Perna* fermented by Culture 3 (B) Alignment Hit Table showing summary of information for target sequence found by alignment search using the basic local alignment sequence tool (BLAST) bioformatics (consensus sequence of pairwise alignment).

Description	Max Score	Total Score	Query Cover	Expectation value	Percent confidence
Staphylococcus sp. SDB 2975 16S ribosomal RNA gene, partial sequence	444	444	100	3.00E-121	100
Staphylococcus sp. SDB 2975 chromosome, complete genome	444	2665	100	3.00E-121	100
Staphylococcus simulans strain NadiaE16 16S ribosomal RNA gene, partial sequence	444	444	100	3.00E-121	100

Appendix XIV Identification of the black colony grown in Oxford modified medium from *Perna* fermented by Culture 5 (B), Alignment Hit Table showing summary of information for target sequence found by alignment search using the basic local alignment sequence tool (BLAST) bioformatics (consensus sequence of pairwise alignment).

Description	Max Score	Total Score	Query Cover	Expectation value	Percent confidence
Listeria monocytogenes strain FDAARGOS_555 chromosome, complete genome	516	3098	100	6.00E-143	99.65
Listeria monocytogenes strain FDAARGOS_554 chromosome, complete genome	516	3098	100	6.00E-143	99.65
Listeria monocytogenes strain 3 16S ribosomal RNA gene, partial sequence5165161006.00E-14399.65	516	516	100	6.00E-143	99.65

Appendix XV Identification of the black colony grown in Oxford modified medium inoculated by the original *Listeria monocytogenes* (B) Alignment Hit Table showing summary of information for target sequence found by alignment search using the basic local alignment sequence tool (BLAST) bioformatics (consensus sequence of pairwise alignments).

Description	Max Score	Total Score	Query Cover (%)	Expectation value	Percent confidence
Listeria monocytogenes strain FDAARGOS_555 chromosome, complete genome	752	4516	100	0	100
Listeria monocytogenes strain FDAARGOS_554 chromosome, complete genome	752	4516	100	0	100
Listeria monocytogenes strain 166 16S ribosomal RNA gene, partial sequence	752	752	100	0	100